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Loss of STAT3 in Mouse Embryonic Fibroblasts Reveals Its Janus-Like Actions on Mitochondrial Function and Cell Viability

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Abstract

STAT3 has been implicated in mitochondrial function; however, the physiological relevance of this action is not established. Here we studied the importance of STAT3 to the cellular response to stimuli, TNF α and serum deprivation, which increase mitochondrial reactive oxygen species (ROS) formation. Experiments were performed using wild type (WT) and STAT3 knockout (KO) mouse embryonic fibroblasts (MEF). Both WT and STAT3 KO MEF expressed similar levels of tumor necrosis factor receptor 1 (TNFR1) and exhibited comparable I κ B α degradation with TNF α . However, in the absence of STAT3 nuclear accumulation of NF κ B p65 with TNF α was attenuated and induction of the survival protein c-FLIP_L was eliminated. Nonetheless, WT MEF were more sensitive to TNF α -induced death which was attributed to necrosis. Deletion of STAT3 decreased ROS formation induced by TNF α and serum deprivation. STAT3 deletion was associated with lower levels of complex I and rates of respiration. Relative to WT cells,

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mitochondria of STAT3 KO cells released significantly more cytochrome c in response to oxidative stress and had greater caspase 3 cleavage due to serum deprivation. Our findings are consistent with STAT3 being important for mitochondrial function and cell viability by ensuring mitochondrial integrity and the expression of pro-survival genes.

Keywords

tumor necrosis factor alpha; reactive oxygen species; cell death; mitochondria; NFκB

1. Introduction

Signal transducer and activator of transcription 3 (STAT3) was originally identified as a transcription factor involved in a diverse number of activities including the acute phase response, inflammation, wound healing, cellular growth, cancer, and embryonic development [1–7]. Recently, STAT3 was reported to have a role in maintaining proper mitochondrial function independent of transcription [8,9]. Mitochondria isolated from pro-B cells or mouse cardiac myocytes in which the STAT3 gene was deleted exhibited reduced ADP-stimulated respiration and activities of complexes I and II of the electron transport chain [10]. Activities were restored in the STAT3 knockout (KO) pro-B cells by the expression of a mitochondria-targeted STAT3 protein with mutations in either the DNA binding domain or the tyrosine site important for dimer formation and subsequent DNA binding. Several studies followed that reported a role for STAT3 in preventing mitochondrial permeability transition pore (mPTP) opening and in mitochondrial reactive oxygen species (ROS) production [11–14]. For instance, calcium-sensitivity of mPTP opening, which can trigger cell death, was found to be enhanced in mitochondria from hearts of STAT3 KO mice [11]. Both positive and negative actions of mitochondrial STAT3 on ROS production have been reported [7]. However, a coherent picture of the significance of STAT3 to mitochondrial function in the context of cellular physiology has not emerged. Here we studied the consequences of STAT3 deletion on cellular survival in mouse embryonic fibroblasts (MEF) in response to stimuli known to increase mitochondrial ROS and induce cell death, tumor necrosis factor alpha (TNFα) and serum deprivation [15,16]. Our findings are consistent with a cellular model in which STAT3 co-ordinates mitochondrial function with mitochondrial integrity and pro-survival gene expression, so as to confront situations that determine cellular survival or death.

2. Materials and Methods

2.1. Materials

Antibodies for STAT3 pS727 (sc-8001-R), IκBα (sc-1643), IκBα pS32/36 (sc-101713), NFκB p65 (sc-372), superoxide dismutase 2 (SOD2; sc-137254), GRIM19 (sc-66195), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; sc-166545 & sc-25778), and cytochrome c (SC-7159) were from Santa Cruz Biotechnology. Cell Signaling Technology was the source for antibodies against STAT3 pY705 (9131), STAT3 (9139), cleaved caspase 3 (9661), lysine-specific demethylase 1 (LSD1; 2184), cellular FLICE-like inhibitory protein long form (c-FLIP_L; 8510), B-cell lymphoma-extra large (Bcl-xL; 21640), cellular inhibitor of apoptosis protein 1 (c-IAP1; 4952). The antibody against tumor necrosis factor receptor 1 (TNFR1; PA1-40282) was from Thermo Scientific. Human recombinant TNFα, xanthine, and protease inhibitor cocktail for use with mammalian cell and tissue extracts (P8340) were from Sigma-Aldrich. Calbiochem was the source for xanthine oxidase from buttermilk. Mouse recombinant leukemia inhibitory factor (LIF) was from Millipore. NE-PER nuclear and cytoplasmic extraction reagents were from Thermo Scientific. NecroX-5

(NCX5) was from Enzo Life Sciences and glutathione monoethyl ester (GME) was from Santa Cruz Biotechnology. Cell culture supplies were from Invitrogen.

2.2. Cells

Mouse Embryonic Fibroblasts (MEF) are a well-established model system for studying basic cell processes. These cells are easy to maintain in culture, proliferate rapidly, and are readily susceptible to genetic manipulations, including gene deletion. Wild type (WT) and STAT3-deleted (KO) mouse embryonic fibroblasts (MEF) were obtained from Valeria Poli (University of Torino, Torino, Italy) and maintained in high glucose DMEM with 10% fetal bovine serum (+FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin [17]. Cultures were determined to be mycoplasma-free using the Universal Mycoplasma Detection kit (ATCC). Cells were plated at a density of 23,800 cells/cm² and were used for experiments 72 h later upon reaching confluence. For some experiments, the medium was changed to DMEM with reduced (0.5%) serum (–FBS) 16 h beforehand so as to attenuate the NFκB p65 pro-survival signaling of TNFα that blocks its pro-apoptotic and pro-necrotic actions [16,18].

2.3. Westerns

Cells were placed on ice and washed 2× with ice-cold Hanks' buffered saline solution (HBSS). Cell lysates were prepared by scraping cells into ice-cold RIPA-based kinase extraction buffer (Boston Bioproducts; BP-116K) containing 1 mM vanadate and 7.5 µL/mL protease inhibitor cocktail and were cleared by centrifugation at 100,000 g for 20 min at 4°C. Protein amounts were determined with the DC Protein Assay (Bio-Rad). Lysate samples containing equal amounts of protein in Laemmli's-SDS reducing buffer were separated by SDS-PAGE and transferred to nitrocellulose membranes, which were blocked for 1 h in Odyssey blocking buffer (LI-COR) diluted 1:1 with phosphate buffered saline (PBS). Co-immunoprecipitations were carried out as described [19] with lysates prepared in Pierce IP lysis buffer and sonicated (3× 5 sec pulses, 5 sec between pulses, 40% duty cycle) with a Fisher Scientific Model 120 Sonic Dismembrator. Lysates were cleared by centrifugation at 100,000 g for 20 min at 4°C. Membranes were incubated overnight with primary antibody in blocking buffer diluted 1:1 with PBS containing 0.1% tween 20 (PBST). Membranes were rinsed 4× 5 min in PBST and incubated 45 min with LI-COR secondary antibody: IRDye 800CW goat-anti-mouse (926–32210) or IRDye 680RD goat-anti-rabbit (926–68071) at 1.5 µL in 20 mL blocking buffer:PBST. Membranes were rinsed 3× 5min with PBST and 1× 5 min with PBS. For the c-IAP1 antibody, PVDF membranes were used and processed as recommended by the manufacturer. Immunoreactive bands were quantified using the Li-COR Odyssey infrared imaging system.

2.4. Immunohistochemistry

Cells were plated onto 22 mm² coverslips (Corning) and grown to 70–80% confluence under standard culture conditions. The cells were switched to reduced serum medium and after 16 h were treated with 1 nM TNFα for 1 h. The cells were fixed with ice-cold methanol for 7–10 min. After a brief wash with PBS, the cells were incubated with blocking solution (1% BSA in PBS) for 1 h followed by primary antibody against NFκB p65 at 1:250 dilution for 1 h. Cells were washed and incubated with Alexa Fluor 488 labeled goat-anti rabbit secondary antibody (Molecular Probes, 1:400) for 1 h. Subsequently, the cells were washed and nuclei were counter-stained with 300 nM DAPI for 1 min. The coverslips were mounted on glass slides using anti-fade reagents and allowed to dry overnight for fluorescent microscopy imaging.

2.5. FACS analysis

Cells were plated in 60 mm dishes. The medium was transferred to a 15 mL tube and each dish was washed with 1 mL HBSS, which was added to the 15 mL tube. One mL TrypLE Express (Invitrogen) was added and the dish incubated 5 min at 37°C. Cells were dislodged by gentle pipetting and added to the 15 mL tube. Cells were collected by centrifugation at 500 g for 5 min at 4°C. Pellets were washed 2× with 3 mL ice-cold PBS and cells gently resuspended with 400 µL binding buffer (FITC Annexin V Apoptosis Detection Kit II; BD Pharmingen). A 100 µL aliquot was transferred to a 5 mL BD Falcon polystyrene round-bottom tube (12 × 75 mm) with strainer cap on ice. To each tube, 5 µL propidium iodide (PI) and 3 µL Annexin-FITC were added. Contents were mixed gently and incubated 15 min on ice in the dark. No-label, PI-only, FITC-only, and dual-label controls were done. To each sample 400 µL binding buffer was added for FACS assay on the Gallios flow cytometer (Beckman Coulter).

2.6. Reactive oxygen species formation

Intracellular generation of ROS was determined following a microplate assay procedure using the oxidant-sensitive 2',7'-dichlorofluorescein-diacetate (DCFDA-Invitrogen, Grand Island, NY, U.S.A) as described elsewhere [20]. Cells were isolated by trypsinization and loaded with 100 µM DCFDA in HBSS for 30 min at 37°C. Afterwards, the dye was washed away with fresh HBSS and cells were placed in a 96-well plate (0.5×10^6 cells/well). Vehicle or TNF α (1 nM) were added and the increase in fluorescence at 530 nm with 485 nm excitation was recorded in 30 min intervals for up to 120 min in a fluorescence plate reader (Fluorocount, Packard). ROS production was calculated as changes in fluorescence/30 min/ 0.5×10^6 cells. The linear phase of the curve (between 60 and 90 min after adding of TNF α) was used for calculation.

2.7. Cellular respiration

WT and STAT3 KO MEF were grown in 75 cm² flasks and harvested immediately before each experiment. Collected cells were centrifuged at 300 g for 3 min at room temperature and resuspended in 2.5 mL of 25 mM HEPES pH 7.2, 118 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, and 20 mM glucose. Cells were counted using a NucleoCounter NC-100 (Chemometec). WT and STAT3 KO cells were harvested for each experiment and the respiration assays of the two cell types were run side-by-side in the two chambers of the Oroboros Oxygraph. Glucose-driven coupled respiration (O₂ consumption) was measured for roughly 12 min in order to obtain a linear rate. The uncoupler FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) was then titrated into the reactions in order to obtain the rate of uncoupled respiration. The final concentration of FCCP varied from 0.2 µM to 0.6 µM, depending upon the amount of cells used in the assay. Antimycin A (2.5 µM) was added to inhibit electron transfer through complex III. Antimycin A inhibited all reactions by at least 98% indicating that the observed O₂ consumption was due to mitochondrial electron transfer. In order to measure activity of cytochrome c oxidase (complex IV), ascorbate (3 mM) was added and the rate of O₂ consumption measured; the one-electron carrier TMPD (N,N,N',N'-tetramethyl-p-phenylenediamine, 0.6 mM) was added and the rate of O₂ consumption was measured again. Ascorbate reduces TMPD which then reduces cytochrome c at its binding site on complex IV. The rate of O₂ consumption in the presence of ascorbate alone was subtracted from the measurement with ascorbate plus TMPD. All of the respiration rates were normalized to the number of cells used in each assay, producing units of nmol O₂ consumed/min/ 10^6 cells.

2.8. Mitochondrial multiprotein complexes

Relative abundances of mitochondrial oxidative phosphorylation multiprotein complexes were determined by a modification of the procedure originally described by Schägger and von Jagow [21]. At least 5×10^6 cells per sample were collected from 60 mm culture dishes as described under FACS analysis. PBS-washed cells were resuspended with 1 ml of $1 \times$ cytosol extraction buffer mix containing DTT and Protease Inhibitors (BioVision Mitochondria/Cytosol Fractionation Kit), and then incubated on ice for 10 min prior to sonication for 3×5 sec pulses at 40% power setting. Cells were inspected microscopically to ensure breakage, and then the homogenates were centrifuged at 700 g for 10 min at 4°C. The supernatant was transferred a fresh 1.5 ml tube, and centrifuged at 10,000 g for 30 min at 4°C. The supernatant was removed. The pellet (intact mitochondria) was resuspended in 0.1 ml KME buffer (100 mM KCl, 0.5 mM EGTA, 50 mM MOPS, pH 7.4) and stored overnight at -80°C before proceeding. Protein content was determined and the samples (containing 30 µg protein) were prepared for blue native gel electrophoresis by adding n-dodecyl-β-D-maltoside and Coomassie G-250 according to the manufacturer's protocol (Novex NativePAGE Sample Prep Kit; Invitrogen). Electrophoresis was performed with NativePAGE 4–16% Bis-Tris gels using the fast Coomassie G-250 staining protocol and Novex NativeMark unstained protein standard. Gels were fixed and destained, and relative intensities of the mitochondrial oxidative phosphorylation multiprotein complexes measured using BIO-RAD Quantity One software after acquisition on a BIO-RAD Molecular Imager Gel Doc XR System.

2.9. Cytochrome c release

Cells in 60 mm culture dishes were either exposed to *in situ*-generated superoxide/hydrogen peroxide by incubation with 0.01 U/mL xanthine oxidase and 500 µM xanthine for 2 h or left untreated as controls. Cells were collected as described under FACS analysis. Each cell pellet was pre-washed by resuspension in 1 mL wash buffer (1mM K₂HPO₄, 1mM DTT, 1mM EGTA; 10mM NaCl, 120mM KCl, 20 mM HEPES, pH 7.0). Cells were then recovered by centrifugation at 500 g for 5 min at 4°C. Cell pellets were permeabilized by resuspension with 200 µL permeabilization buffer (80 µg/mL digitonin, 1 mM K₂HPO₄, 1mM DTT, 1mM EGTA 10mM NaCl, 120mM KCl, 20 mM HEPES, pH 7.0) and protease inhibitor cocktail, incubated on ice for 2 min, then centrifuged at 10,000 g for 10 min at 4°C. Supernatants were collected for analysis of released cytochrome c. For comparison of non-released cytochrome c, the remaining cell pellets were solubilized with an equal volume of RIPA-based extraction buffer and cleared by centrifugation at 100,000 g for 20 min at 4°C. Samples containing equal amounts of protein were heated at 102°C for 5 min in Laemmli's SDS reducing buffer, subjected to SDS-PAGE and Western Blot analysis for cytochrome c content.

2.10. Statistical analysis

Results are expressed as mean $\pm$ SEM for (n) number of independent observations. Statistical significance of differences between two values was determined by Student's t-test. ANOVA followed by Newman-Keuls post-hoc test was used for multiple comparisons.

3. Results

3.1. Impact of STAT3 on TNFα – NFκB signaling

We first assessed whether loss of STAT3 interfered with TNFα intracellular signaling. No differences were seen in levels of TNFR1 between WT and STAT3 KO MEF that were maintained in growth medium or between WT and STAT3 KO MEF that were serum-deprived overnight, although a lower molecular weight band of ~50 kDa was more

prominent after serum-deprivation (Fig. 1A), perhaps due to deglycosylation of the receptor [22].

So as to minimize protective signaling associated with TNF α , studies were performed in cells incubated in reduced serum medium. Under these conditions, TNF α -induced comparable S32/36 phosphorylation of I κ B α in WT and STAT3 KO MEF, which sequesters NF κ B p65 in the cytoplasm [23]; fold-increase: 5.30 ± 0.39 in WT vs. 5.36 ± 0.48 in KO, $n = 3$ (Fig. 1B). Serine phosphorylation decreases I κ B α protein stability and at 15 min comparable decreases in I κ B α protein levels were seen in WT and STAT3 KO MEF following TNF α stimulation, 91.0 ± 2.2 % in WT vs. 95.9 ± 2.7 % in KO, $n = 3$ (Fig. 1B).

We next assessed whether crosstalk occurs between STAT3 and TNF α -induced signaling in MEF. TNF α did not induce canonical STAT3 signaling as indexed by Y705 phosphorylation or interfere with STAT3 activation by LIF (Fig. 2 A and B); however, TNF α induced STAT3 S727 phosphorylation though less so than LIF (Fig. 2 A and C). STAT3 has recently been implicated in nuclear retention of NF κ B p65 [24], and as Fig. 3A shows lower levels of nuclear p65 were detected by immunohistochemistry in STAT3 KO cells compared to WT MEF in response to TNF α stimulation. This observation was confirmed by isolating nuclei from WT and STAT3 KO MEF and probing for NF κ B p65 by Western immunoblotting (Fig. 3B).

3.2. Role of STAT3 in protective protein induction

NF κ B p65 activation by TNF α has been linked to induction of several survival proteins, including c-FLIP_L, Bcl-xL, and c-IAP1. C-FLIP_L at physiological levels of expression appears to inhibit caspase 8 activation and both TNF α -induced apoptosis and necrosis [25,26]. Thus we reasoned that reduced NF κ B p65 uptake in STAT3 KO MEF would result in reduced induction of protective proteins. As seen in Fig. 4 A & B, induction of c-FLIP_L was observed in WT but not STAT3 KO MEF. Levels of the mitochondrial pro-survival protein Bcl-xL were comparable between WT and STAT3 KO MEF and were not affected by TNF α stimulation (Fig. 4 A & C). This observation is consistent with reports suggesting that STAT3 involvement in Bcl-xL expression is cell-type specific [27,28]. Full length c-IAP1, which promotes survival signaling from TNFR1 by ubiquitination of RIP1 [29], was not detected in WT MEF and was barely detectable in either control or TNF α -treated STAT3 KO MEF (Fig. 4A). A smaller-size immunodetectable band (45–50 kDa) was observed with the c-IAP1 antibody in both WT and STAT3 KO MEF that may represent the more stable splice variant of c-IAP1 that has been described in mouse cells, which lacks the CARD and RING domains and was proposed to inhibit Bax-mediated apoptosis [30]. Levels of the splice variant of c-IAP1 were not affected by TNF α and were 46% less in STAT3 KO MEF.

3.3. Effect of TNF α on cell viability

Even though c-FLIP was induced in WT MEF and not STAT3 KO MEF, WT MEF were more sensitive to the cytotoxic effects of TNF α (Fig. 5A), which was attributable to greater necrosis (Fig. 5B). STAT3 KO MEF were less sensitive to TNF α (Fig. 5A). However, viability was less in STAT3 KO vs. WT MEF in the absence of TNF α , indicating increased sensitivity to serum-deprivation (Fig. 5A). For cells maintained in growth medium, there was no difference in viability between untreated WT and STAT3 KO MEF, but the greater cytotoxic effects of TNF α on WT MEF was retained (Fig. 5C). Loss of viability of TNF α -treated STAT3 KO MEF seems to be attributed to necrosis (Fig. 5B), while serum depletion increased the population of cells staining for markers of both apoptosis and necrosis (Fig. 5D).

3.4. Loss of STAT3 abolishes TNF α -induced ROS formation

ROS has been implicated in both TNF α -induced apoptosis and necrosis [16]. As seen from Fig. 6A, TNF α induced ROS formation from serum-deprived WT MEF. There was a trend towards increased ROS formation in STAT3 KO MEF that did not reach significance. Also, basal levels of ROS were much greater in WT MEF than STAT3 KO MEF. In cells maintained in serum (Fig. 6B), basal levels of ROS were significantly lower than in reduced serum and similar between WT and STAT3 KO MEF. Under these conditions, TNF α induced ROS formation in WT but not STAT3 KO MEF.

3.5. Loss of STAT3 decreases mitochondrial respiration

Mitochondria are the primary source of ROS in response to TNF α [16]. Rates of coupled mitochondrial respiration tended to be lower in STAT3 KO MEF compared to WT MEF (Fig. 7A), and uncoupled respiration was statistically less in STAT3 KO MEF (Fig. 7 B & C), indicating less mitochondrial respiratory capacity in STAT3 KO MEF. No significant difference was observed between WT and STAT3 KO in mitochondrial number as assessed by complex IV activity in the absence of a proton gradient (Fig. 7D), a measure of inner mitochondrial membrane content [31]. Consistent with the lower maximum rates of respiration in STAT3 KO MEF is our finding of 33% lower levels of complex I in the electron transport chain relative to complex V, i.e., ATP synthase (Fig. 7E). Co-immunoprecipitation studies provided evidence for the physical association of STAT3 with GRIM19, a critical component of complex I (Fig. 7F).

3.6. Loss of STAT3 increases mitochondrial sensitivity to ROS

Serum-deprivation caused an increase in ROS formation in both WT and STAT3 KO MEF, but to a much lower extent in STAT3 KO MEF (Fig. 6). Nonetheless, serum deprivation induced more cell death in STAT3 KO MEF with features of apoptosis and necrosis (Fig. 5D), which would be consistent with greater mitochondrial sensitivity to ROS due to STAT3 elimination. Consistent with the FACS data, we observed that in cells incubated with serum STAT3 KO attenuated TNF α -induced caspase 3 cleavage to p17, the most catalytically active form of caspase 3 (Fig. 8A). Interestingly, some p17 formation was seen in STAT3 KO MEF in the absence of TNF α stimulation. Serum-deprivation markedly increased p17 levels in STAT3 KO MEF under basal conditions and TNF α further enhanced p17 above levels observed in WT MEF (Fig. 8B). Interestingly, p17 levels with TNF α were less in WT MEF when FBS was reduced (Fig. 8A vs. 8B). This was possibly because conditions for cell growth affected (A) the time courses for caspase 3 cleavage or p17 degradation or (B) the relative contributions of apoptosis and necrosis to cell death.

We next assessed whether increased p17 formation was attributable to mitochondrial ROS generation. Serum deprivation-induced p17 formation was significantly reduced by treatment of STAT3 KO cells with NCX5 (Fig. 8C), a cell permeable antioxidant that localizes mostly in the mitochondria [32]. Treatment of STAT3 KO MEF with GME, which undergoes hydrolysis by intracellular esterases to release glutathione [33], also attenuated p17 formation with serum deprivation (Fig. 8D). No difference was observed between serum-deprived WT and STAT3 KO MEF in levels of SOD2, which detoxifies mitochondrial superoxide into hydrogen peroxide (Fig. 8E). Lastly, cleavage of caspase 3 requires cytochrome c release from mitochondria and we were able to demonstrate that ROS increased cytosolic levels of cytochrome c in STAT3 KO MEF, but not in WT MEF (Fig. 9).

4. Discussion

In this study, we assessed the consequences of deleting STAT3 in MEF under conditions associated with mitochondrial ROS production. In addition to our finding that the induction

of ROS was positively correlated with mitochondrial activity, four novel consequences associated with the loss of STAT3 were observed: (A) nuclear accumulation of NF κ B p65 was attenuated and associated with the loss of induction of the survival protein c-FLIP_L in response to TNF α stimulation; (B) the respiratory capacity of MEF was diminished; (C) levels of complex I were reduced; and (D) mitochondria exhibited enhanced fragility to oxidative stress.

We found lower levels of complex I in STAT3 KO MEF relative to ATP synthase. Others reported reduced activity of complex I of mitochondria from STAT3 deleted cells [10]. The basis for reduced complex I activity is not established, but perhaps related to the interaction of STAT3 with GRIM19, a protein critical for the assembly and function of complex I [34]. We confirmed such a GRIM19-STAT3 interaction in MEF (Fig. 7F). There are reports of deficiencies in other respiratory complexes in the absence of STAT3. Ras-transformed MEF with STAT3 deletion were reported to exhibit reduced complex III and V activities [35], and STAT3 knockdown in HL-1 atrial myocytes was associated with a reduced amount of complex I and activities of complexes I, II, III, and IV [36].

It has been reported that STAT3 deletion enhances mitochondrial ROS [8,9]. Deletion of STAT3 increased mitochondrial ROS in osteoblast/osteocytes [14], astrocytes [12], and certain hematopoietic stem/progenitor cell subpopulations [13]. Reduced rates of flux through the electron transport chain and/or buildup of the mitochondrial membrane potential due to impaired ATP synthesis is associated with higher rates of ROS formation [37]. In STAT3 KO hematopoietic stem/progenitor cells rates of respiration and mitochondrial membrane potential were enhanced, while ATP production was decreased [13]. Thus, these observations are consistent with a model in which impaired ATP synthesis, together with a possible defect in one of the respiratory complexes, leads to electron backup in the electron transport chain and increased ROS formation. STAT3 deletion decreased ATP synthesis in astrocytes; in this case, mitochondrial membrane potential was decreased [12]. Notably, lower cellular levels of glutathione were observed in STAT3-deleted astrocytes [12], raising the possibility that the observed increase in mitochondrial ROS was secondary to a decrease in antioxidant defenses or a consequence of ROS-induced damage to the electron transport chain. The STAT3 inhibitors Stattic [38,39] and phospho-valproic acid [40] also induced mitochondrial ROS generation.

In contrast to these studies positively linking STAT3 deletion or inhibition to ROS formation, we found that STAT3 KO MEF did not show enhanced ROS formation under basal conditions (Fig. 6) and this was associated with lower rates of respiration (Fig. 7 A–D). STAT3 KO MEF also exhibited lower levels of ROS formation than WT MEF following TNF α treatment or with serum deprivation (Fig. 6). Others have reported that STAT3 knockdown in L929 mouse fibrosarcoma cells did not enhance ROS generation [41]. Although respiration was not measured in the L929 study, overall lower mitochondrial respiratory activity offers a straightforward explanation for the lower rates of ROS formation in STAT3 KO MEF and STAT3 depleted L929 cells. TNF α and serum starvation were shown to induce ROS via recruitment of reactive oxygen species modulator 1 (Romo1) to mitochondria [42,43]. Romo1 causes mitochondrial membrane depolarization and enhances ROS from complex III [44]. Complex I and III are the primary sites of ROS production in mitochondria, which is reflective of electron flow [45]. Thus, reduced complex I activity would be expected to decrease ROS formation at the level of complex III. Other factors such as a higher abundance of uncoupling proteins may have contributed as well [46].

We observed lower levels of nuclear p65 in STAT3 KO MEF following TNF α stimulation than in WT MEF (Fig. 3), although levels of TNFR1 and I κ B α degradation were

comparable (Fig. 1). The enhanced nuclear p65 levels frequently observed in cancer cells was recently attributed to the acetylation of p65 brought about by the binding of p65 to STAT3 in association with p300 [24]. The pro-death effects of TNF α are opposed by NF κ B-regulated anti-apoptotic or anti-necrotic gene products [47], and we observed that the protective protein c-FLIP_L was not induced by TNF α in STAT3 KO MEF (Fig. 4). However, TNF α induced greater cell death in WT MEF than STAT3 KO MEF (Fig. 5). Impaired ROS formation in STAT3 KO MEF likely explains this paradox as sustained ROS activation and subsequent JNK activation is responsible for TNF α -induced cell death [16]. These observations highlight the Janus-like nature of STAT3 signaling in MEF in the context of TNF α signaling: on the one hand contributing to the induction of protective proteins, while being necessary for maintaining proper mitochondrial function and ROS generation linked to cell death (Fig. 10).

We also observed that STAT3 KO MEF were more sensitive to serum deprivation (Fig. 5) and oxidative stress, which caused cytochrome c release (Fig. 9) and caspase 3 cleavage (Fig. 8). These actions presumably involve opening of the mPTP. The direct involvement of STAT3 is supported by evidence that STAT3 binds cyclophilin D and inhibits calcium-induced mPTP opening in cardiac myocytes [11] and that a dominant negative GRIM19 sensitizes cells to undergo apoptosis [48]. In addition, there was no difference between WT and STAT3 KO MEF in levels of SOD2, the principal means by which superoxide from the electron transport chain is detoxified. STAT3 was linked to SOD2 expression in mouse neuronal cells [49]. Alternatively and independently, loss of STAT3 results in a lower level of a splice variant of c-IAP1 in MEF cells (Fig. 4), and this splice variant has an enhanced ability to inhibit Bax-induced apoptosis. Bax has been implicated in serum depletion-induced cell death [50–52]. Elschami et al. [36] recently reported that STAT3 knockdown in HL-1 adult atrial myocytes lead to disruption of mitochondrial ultrastructure. This observation complements our study, which provides additional insights into mitochondrial function, ROS production, and sensitivity to oxidative stress.

5. Conclusions

Our study demonstrated that the opposing actions of STAT3 upon mitochondrial function have a direct bearing upon cell fate determination. In cells exposed to TNF α , STAT3 was important for the nuclear accumulation of NF κ B and expression of survival proteins. Paradoxically, STAT3 also affected the relative composition of mitochondrial complexes, mitochondrial respiration, and ROS production, which are linked to TNF α -stimulated cell death. Yet this detrimental effect was accompanied by a STAT3-dependent protective effect, as illustrated by the increased susceptibility of mitochondria from STAT3-deficient cells to release cytochrome c when exposed to ROS. The next challenge will be to delineate how multiple opposing actions of STAT3 are integrated into the appropriate biological response. In summary, our findings illustrate the central importance of STAT3 in determining mitochondrial integrity and function, thereby tipping the balance between cellular survival and death.

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STAT3 has opposing pleiotropic roles in modulating TNF α signaling.

- Loss of STAT3 reduces NF κ B nuclear levels and protective protein induction.
- Its loss decreased TNF α -induced death by lowering respiration and ROS formation.
- STAT3 deletion increased ROS-induced cytochrome c release.
- STAT3 is vital for integrating mitochondrial function and structural integrity.

Figure 1. TNF α signaling in wild type (WT) and STAT3 knockout (KO) MEF

(A) Levels of TNFR1 were assayed by Western blotting in lysates of WT and STAT3 KO MEF kept in growth medium containing 10% FBS (+ FBS) or in medium with 0.5% FBS (– FBS) for 16 h. GAPDH was probed as a loading control. (B) Cells were treated with vehicle (–) or 1 nM TNF α for 5 or 15 min. Cell lysates were prepared and analyzed by Westerns for I κ B α S32/36 phosphorylation (pI κ B α) and I κ B α . Extracts were also probed for STAT3 as a control. Results shown are representative of 3 independent experiments.

Figure 2. Impact of TNF α on STAT3 signaling in MEF

(A) Cells made quiescent by overnight incubation in reduced serum medium were stimulated with 1 nM TNF α and/or 0.45 nM LIF for 5, 15 or 45 min. Cell lysates were probed for STAT3 Y705 phosphorylation (pY STAT3) or S727 phosphorylation (pS STAT3) and total STAT3. (B and C) Data were quantified and normalized to levels of phosphorylation in untreated cells (basal). *P < 0.05, **P < 0.01, and ***P < 0.001 vs. untreated cells. Results are shown as the mean $\pm$ SEM of 3 independent experiments.

Figure 3. Absence of STAT3 reduces nuclear accumulation of NFκB p65 in TNFα-treated MEF
 (A) Cells on coverslips were treated with 1 nM TNFα for 1 h and fixed with methanol. After washing with PBS, the cells were incubated with blocking solution followed by primary antibody against NF-κB p65 for 1 h. Cells were washed and incubated with a secondary antibody with fluorescent probe for 1 h. Subsequently, cells were washed and counter-stained with DAPI for nucleus and observed by fluorescent microscopy. Results shown are representative of 3 independent experiments. (B) MEF were treated with vehicle (-) or 1 nM TNFα (+) for 1 h. Nuclei were isolated using the NE-PER nuclear and cytoplasmic extraction kit from Thermo Scientific. Equal amounts of nuclear proteins were separated by SDS PAGE followed by Western blotting for NFκB p65 and LSD1 as a loading control. **P < 0.01 (n = 3).

Figure 4. Protective proteins in wild type (WT) vs. STAT3 knockout (KO) MEF

Cells were made quiescent by overnight incubation in reduced serum medium and then stimulated with vehicle (–) or 1 nM TNF α (+) for 24 h. Cell extracts were prepared and probed for c-FLIP_L, Bcl-xL, and c-IAP1. GAPDH was probed for as a loading control. (B and C) Data for c-FLIP_L and Bcl-xL were quantified and normalized to levels of GAPDH.

***P < 0.001 vs. other conditions (n = 3).

Figure 5. Differential effect of TNFα on cell viability of wild type (WT) vs. STAT3 knockout (KO) MEF

(A) Serum-deprived WT and STAT3 KO MEF were treated with vehicle or 1 nM TNFα for 24 h. Cells viability (lack of propidium iodide and/or Annexin V staining) was determined by FACS analysis. (B) Necrosis was assessed as propidium iodide staining in the absence of Annexin V staining. (C) Cell viability was determined in MEF maintained throughout in growth medium (10% FBS) and treated for 24 h with vehicle or 1 nM TNFα. (D) Shown is the percentage of serum-deprived WT and STAT3 KO MEF that were doubly labeled by propidium iodide and Annexin V, which represents late apoptosis. On average an insignificant number of cells (< 1.5%) were stained by Annexin V alone and this did not change among conditions. *P < 0.05, **P < 0.01, and ***P < 0.001 (n = 3).

Figure 6. Production of ROS by wild type (WT) and STAT3 knockout (KO) MEF in response to TNF α

Intracellular generation of ROS in response to 1 nM TNF α was measured with DCFDA in MEF that were maintained in (A) reduced serum medium (– FBS) or (B) growth medium (+ FBS). ROS production was calculated as changes in fluorescence/30 min/ 0.5×10^6 cells. *P < 0.05 and **P < 0.01, n = 3.

Figure 7. Mitochondrial respiration and complex I levels in wild type (WT) and STAT3 knockout (KO) MEF

WT and STAT3 KO MEF were grown in medium containing 10% FBS. Cellular respiration of isolated cells was measured with an Oroboros Oxygraph in a solution of HEPES (25 mM) pH 7.2, NaCl (118 mM), KCl (4.8 mM), MgSO₄ (1.2 mM), KH₂PO₄ (1.2 mM), and glucose (20 mM) in the absence (A) or presence (B) of the mitochondrial uncoupler FCCP. The ratio of uncoupled to coupled respiration is shown in (C). (D) The activity of cytochrome c oxidase (complex IV) in the absence of a proton-motive force was assayed as a measure of inner mitochondrial content. Respiration rates were normalized to the number of cells used in each assay and expressed as nmol of O₂ consumed/min/10⁶ cells. *P < 0.05, **P < 0.01, n = 3. (E) Multiprotein complexes were analyzed by blue native gel electrophoresis from mitochondria that were isolated from WT and STAT3 KO MEF and disrupted by free-thawing. Levels of complex I (~1000 kDa) were normalized to levels of complex V (~750 kDa). **P < 0.01, n = 3. (F) STAT3 co-immunoprecipitates with GRIM19. Proteins in cell lysates from 3 separate passages of WT MEF were immunoprecipitated with an antibody to GRIM19 (+). Immunoprecipitated proteins were separated by SDS-PAGE and immunoblotted for STAT3. As a control, a lysate was processed without adding the GRIM19 antibody (-).

Figure 8. Caspase 3 cleavage product p17 in wild type (WT) and STAT3 knockout (KO) MEF

Cells were maintained in (A) growth medium (+ FBS) or (B) reduced serum medium (– FBS) and stimulated with vehicle (–) or 1 nM TNF α (+) for 24 h. (C) STAT3 KO MEF were maintained in reduced serum medium for 24 h either without treatment (–) or in the presence of vehicle, 0.05 % v/v DMSO (–/DMSO) or with 10 μ M necroX-5 (–/NCX5). Cells maintained in growth medium with 10% FBS served as a control (+). (D) STAT3 KO MEF were kept in growth medium (+) or reduced serum medium (–) in the absence or presence of 2 mM GME for 24 h. Cell lysates were analyzed by Western blotting for caspase 3 cleavage product p17 and GAPDH as a loading control. (E) Cell lysates from serum-deprived (16 h) WT and STAT3 KO MEF were analyzed for SOD2 and GAPDH (loading control). **P < 0.01 and ***P < 0.001 (n = 3).

Figure 9. Cytochrome c release with oxidative stress in wild type (WT) and STAT3 knockout (KO) MEF

MEF were incubated for 2h in the absence or presence of a ROS generating system of 500 μ M xanthine and 0.01 U/mL xanthine oxidase. The extent of cytochrome c release was quantified by immunoblotting cytosolic extracts of digitonin-permeabilized MEF and normalizing the results to total cytochrome c levels obtained by full disruption of mitochondria using a RIPA-based extraction buffer. * $P < 0.05$ vs. other conditions ($n = 3$).

Figure 10. Janus-like role of STAT3 in viability of MEF involving mitochondrial function

Binding of TNFα to TNFR1 leads to the formation of Complex I and the activation of NFκB through the serine phosphorylation and subsequent ubiquitin (Ub)-dependent degradation of IκBα. Activation of NFκB p65 and p50 induces expression of survival genes such as c-FLIP and c-IAP1. c-IAP1 maintains complex I through ubiquitination of RIP1. Pro-death Complex II is formed through deubiquitination of RIP1 and is comprised of TRADD, FADD, caspase 8, and RIP1. c-FLIP inhibits caspase 8, thereby inhibiting apoptosis and necrosis. Inhibition of caspase 8 leads to the formation of complex III consisting of RIP1 and RIP3 (not shown), which is linked to programmed necrosis (necroptosis). We observed that nuclear levels of NFκB p65 are enhanced by STAT3. However, the positive effect of STAT3 on survival gene expression is offset by a direct role of STAT3 in maintaining mitochondrial function, which paradoxically contributes to ROS generation in response to TNFα that is linked to necrosis. STAT3 is important as well for maintaining mitochondrial integrity in response to ROS generation. Loss of STAT3 results in enhanced mPTP opening and release of cytochrome c and caspase 3 activation in response to oxidative stress and ROS. FADD, Fas-Associated protein with Death Domain; RIP1, receptor interacting protein 1; TRADD, tumor necrosis factor receptor type 1-associated DEATH domain protein; TRAF2, TNF receptor-associated factor 2. See Mughal W. et al. [53] for additional details on TNFα signaling.