Role of the Ca\textsuperscript{2+}-Calcineurin-Nuclear Factor of Activated T cell Pathway in Mitofusin-2-Mediated Immune Function of Jurkat Cells

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Abstract

Background: Mitofusin-2 (MFN2), a well-known mitochondrial fusion protein, has been shown to participate in innate immunity, but its role in mediating adaptive immunity remains poorly characterized. In this study, we explored the potential role of MFN2 in mediating the immune function of T lymphocytes.

Methods: We manipulated MFN2 gene expression in Jurkat cells via lentiviral transduction of MFN2 small interfering RNA (siRNA) or full-length MFN2. After transduction, the immune response and its underlying mechanism were determined in Jurkat cells. One-way analysis of variance and Student’s t-test were performed to determine the statistical significance between the groups.

Results: Overexpression of MFN2 enhanced the immune response of T lymphocytes by upregulating Ca\textsuperscript{2+} (359.280 ± 10.130 vs. 266.940 ± 10.170, \(P = 0.000\)), calcineurin (0.513 ± 0.014 vs. 0.403 ± 0.020 nmol/L, \(P = 0.024\)), and nuclear factor of activated T cells (NFATs) activation (1.040 ± 0.086 vs. 0.700 ± 0.115, \(P = 0.005\)), whereas depletion of MFN2 impaired the immune function of T lymphocytes by downregulating Ca\textsuperscript{2+} (141.140 ± 14.670 vs. 267.060 ± 9.230, \(P = 0.000\)), calcineurin (0.054 ± 0.030 nmol/L vs. 0.404 ± 0.063 nmol/L, \(P = 0.000\)), and NFAT activation (0.500 ± 0.025 vs. 0.720 ± 0.061, \(P = 0.012\)). Furthermore, upregulated calcineurin partially reversed the negative effects of MFN2 siRNA on T cell-mediated immunity evidenced by elevations in T cell proliferation (1.120 ± 0.048 vs. 0.580 ± 0.078, \(P = 0.040\)), interleukin-2 (IL-2) production (473.300 ± 24.100 vs. 175.330 ± 12.900 pg/ml, \(P = 0.000\)), and the interferon-\(\gamma\)/IL-4 ratio (3.080 ± 0.156 vs. 0.953 ± 0.093, \(P = 0.000\)). Meanwhile, calcineurin activity inhibitor depleted the positive effects of overexpressed MFN2 on T cells function.

Conclusions: Our findings suggest that MFN2 may regulate T cell immune functions primarily through the Ca\textsuperscript{2+}-calcineurin-NFAT pathway. MFN2 may represent a potential therapeutic target for T cell immune dysfunction-related diseases.

Key words: Calcineurin; Calcium; Jurkat Cells; Mitofusin-2 Protein; Nuclear Factor of Activated T cells

INTRODUCTION

Mitochondria are dynamic organelles that play major roles in energy metabolism and the regulation of cellular proliferation.\textsuperscript{[1]} Optimal mitochondrial function is ensured by an intricate control system that tightly couples fusion and fission.\textsuperscript{[2]} Mitofusin-2 (MFN2), the key member in this control system, mediates outer mitochondrial membrane fusion to maintain steady-state mitochondrial morphology and participates in numerous life processes, including cell proliferation and apoptosis, oxidative metabolism, and autophagy.\textsuperscript{[3-7]} Recently, abundant evidence has demonstrated that MFN2 also plays a key role in innate immunity against viral infection, not by affecting mitochondrial fusion, but instead by regulating mitochondria-endoplasmic reticulum (ER) contact sites.\textsuperscript{[7-9]} Since the mitochondria and ER are major intracellular calcium pools, MFN2-mediated ER–mitochondria tethering allows efficient calcium transfer...
between the ER and mitochondria,[8,10] which is required for intracellular calcium homeostasis.[9] This suggests the possibility that MFN2 functions as a regulator of the intracellular calcium concentration. Since intracellular Ca\(^{2+}\) concentrations and signals are crucial for the activation of T cells,[11] it is possible that MFN2 has a role in regulating T cell function by modulating ER-mitochondria tethering and, consequently, intracellular Ca\(^{2+}\) signals.

The Ca\(^{2+}\)-calcineurin-nuclear factor of activated T cells (NFATs) pathway plays a central role in T cell activation, proliferation, and Th1/Th2 polarization.[12,13] It has been demonstrated that T cell activation is initiated by an increase in the cytoplasmic level of calcium, which activates calcineurin through the binding of a regulatory subunit and activation of calmodulin binding.[14] Calcineurin induces NFATs that induce the transcription of the interleukin-2 (IL-2) gene.[15] IL-2 activates T-helper lymphocytes and induces the production of other cytokines, which are believed to influence the extent of the immune response significantly. Our previous study suggested that upregulation of MFN2 reversed high-mobility group box-1 protein (HMGB1)-induced inhibition of T cell proliferation through the Ca\(^{2+}\)-NFAT pathway.[16] In addition, the protective effect of MFN2 on T cell immune dysfunction was found to be dependent on other signaling pathways associated with NFAT but not mitogen-activated protein kinase.[17] Therefore, in the present study, we investigated the role of MFN2 in mediating the immune function of T lymphocytes in vitro and determined whether MFN2-mediated regulation of T cells was associated with the Ca\(^{2+}\)-calcineurin-NFAT pathway.

## Methods

### Ethical approval

This study was exempted from the ethical approval.

### Media and reagents

RPMI-1640, fetal bovine serum (FBS), glutamine, penicillin, streptomycin, and 4-(2-hydroxyethyl)-1-piperazineneethanesulfonic acid were purchased from Gibco (Grand Island, NY, USA). Phorbol myristate acetate (PMA) and ionomycin were purchased from the Beyotime Institute (Nanjing, China). FK506, MFN2, and β-actin primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Methyl-thiazolyl-tetrazolium (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits for IL-2, IL-4 and interferon (IFN)-γ were obtained from Biosource (Worcester, MA, USA). Fluoro-3/AM and pluronic F-127 were obtained from Molecular Probes (Eugene, OR, USA). TRIzol reagent was obtained from Invitrogen (Carlsbad, CA, USA). Total RNA isolation and reverse transcription systems were purchased from Promega (Madison, WI, USA). The Biomol Green Calcineurin Assay kit was purchased from Biomol (Plymouth Meeting, PA, USA). Nuclear extract and TransAM NFAT kits were obtained from Active Motif (Carlsbad, CA, USA). Nondenaturing lysis buffer and protease inhibitor cocktail were purchased from Applygen Technologies Inc., (Beijing, China). An Amersham enhanced chemiluminescence (ECL) Advance Western Blotting Detection kit was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden).

### Cell culture and stimulation

Jurkat E6-1 human T-lymphocyte leukemia cells (purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) were cultured in RPMI-1640 medium containing 10% FBS and 1% antibiotics (penicillin and streptomycin) and incubated at 37°C in humidified air with 5% CO\(_2\). Cell viability was measured by Trypan blue exclusion before each experiment.

After transfection with lentiviral vectors (LVs) with or without target genes, T cells (1 × 10\(^6\)/ml) were continuously cultured for 6, 12, 24, or 48 h in the presence or absence of PMA (50 ng/ml)/ionomycin (1 µmol/L). Cells were then collected for Western blot analysis, real-time polymerase chain reaction (RT-PCR), or flow cytometric analysis, and the culture supernatants were collected for cytokine analysis by ELISA.

### Lentiviral vector transduction and green fluorescent protein reporter gene detection

Small interfering RNAs (siRNAs) containing the target sequence 5'-GTCAGGTTACCTATCCAAA-3' were designed to bind to MFN2 mRNA. Full-length human MFN2 cDNA was obtained from GenScript Corporation (Piscataway, NJ, USA). LV expressing DNA fragments encoding red fluorescent protein (RFP)-tagged MRN2 siRNAs (MRN2-siRNA) and green fluorescent protein (GFP)-tagged full-length MFN2 (LV-MFN2) were constructed, packed, and purified using reagents from GeneChem Co., Ltd., (Shanghai, China). As a control, LVs expressing GFP alone (LV-GFP) or RFP with a nonsense sequence (TTCTCCGAACGTGTCACGT; control-siRNA) were also generated. LVs expressing DNA fragments encoding a GFP-tagged constitutively active calcineurin (LV-calcineurin) lacking the regulatory domain of calcineurin A by introducing a stop codon at nucleotide 1259 were also constructed, packed, and purified by GeneChem Co., Ltd.[18] For this experiment, a LV expressing GFP alone (LV-GFP2) was also generated. Transduction was performed according to the manufacturer’s protocol, as previously described.[19] The transduction efficiencies of the LVs in Jurkat cells were monitored by fluorescence microscopy 3 days after transduction. The results showed that the transduction efficiency was >80% at a multiplicity of infection of 50.

### T cell proliferation assay

The T cell proliferation rate was determined using an MTT assay. Briefly, after transduction with LVs for 48 h, cells (1 × 10\(^6\)/ml) were inoculated into 96-well plates at a volume of 0.2 ml per well and cultured at 37°C in 5% CO\(_2\). Then, the cells were treated with or without PMA (50 ng/ml) and ionomycin (1 µmol/L) for 6, 12, 24, and 48 h...
before 100 µl supernatant was procured. Next, 20 µl of MTT (5 mg/ml) was added to each well, and the plates were incubated in a CO2 incubator for 4 h. The resulting formazan was solubilized in 100 µl Triton-ISOP solution. After all the crystals had been dissolved overnight, the optical density was measured using a microplate reader (Spectra MR, Dynex, Richfield, MN, USA) at a wavelength of 540 nm.

**Enzyme-linked immunosorbent assay**

Levels of cytokines, including IL-2, IL-4, and IFN-γ, in the cell culture supernatants were measured with commercially available human ELISA kits by strictly following the manufacturer’s protocols. The plates were read using a microplate reader (Spectra MR, Dynex).

**Western immunoblot analysis**

Cell lysis, protein separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis, protein transfer to polyvinylidene difluoride membranes, and blot development using an ECL system (Santa Cruz Biotechnology) were performed as previously described. Membranes were probed with primary antibodies recognizing MFN2 and β-actin. Immunoreactive bands were detected with ECL reagents. Protein levels were quantified by densitometry using NIH image software and normalized to β-actin levels.

**RNA isolation and real-time polymerase chain reaction**

After the cells were treated as indicated, total RNA was extracted from cells using TRIzol Isolation Reagent (Invitrogen), and the concentration of purified total RNA was spectrophotometrically determined at 260 nm. Reverse transcription was completed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). The primers used for RT-PCR were as follows: human MFN2 (forward: 5’-TGGCTCAAGACTATAAAGCTGC-3’ and reverse: 5’-GGAGGACACAAGGGTGGA-3’) and human β-actin (forward: 5’-TGACGTGGACATCCGAAAG-3’ and reverse: 5’-CTGGAAGGTGACAGCGAGG-3’). cDNA was amplified with an initial incubation at 94°C for 4 min followed by 30 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C and an additional extension step of 5 min at 72°C after the last cycle. The resultant PCR products were detected with a Bio-Rad GelDoc XR system (Hercules, CA, USA), and levels were expressed as the ratio of MFN2 to β-actin.

**Intracellular calcium measurement**

Jurkat cells from all treatment groups were labeled with 5 mmol/L Fluo-3/AM (Molecular Probes) in Hanks’ balanced salt solution (HBSS) containing 0.06% Pluronic F-127 (Molecular Probes) for 30 min at 37°C. Following incubation, the cells were added to five times the probe volume and incubated for another 30–40 min at 37°C in the dark. Labeled cells were washed twice, resuspended with prewarmed HBSS, and analyzed by flow cytometry using a FACScan (BD Bioscience, Mountain View, CA, USA) after 10 min of rest at room temperature.

**Calcineurin phosphatase activity assay**

Calcineurin enzyme activity was analyzed with the Biomol Green Calcineurin Assay kit according to the manufacturer’s instructions. Soluble protein extracts were prepared on ice in lysis buffer containing proteinase inhibitors. To measure the calcineurin phosphatase activity, protein extract concentrations were quantified, and equal amounts of protein were incubated for 30 min according to the instructions of the assay kit. Liberated phosphate was colorimetrically measured at 620 nm.

**Nuclear factor of activated T cell activity assay**

A nuclear extract kit (Active Motif) was used to extract the nuclear protein by strictly following the manufacturer’s protocols, as previously reported. Protein concentrations were measured using the Bradford protein assay kit (Applygen Technologies Inc.). The ELISA-based electrophoretic mobility shift assay (TransAM NFAT kit) was used to quantify the amount of active NFAT in the nuclei (6 µg). Briefly, active NFAT was purified from a nuclear lysate upon binding to an immobilized oligonucleotide containing a 5’-AGGAAA-3’ motif and was then measured by ELISA.

**Statistical analysis**

SPSS software (version 17.0, Chicago, IL, USA) was used for the analysis of all data, which were presented as the mean ± standard deviation (SD). We performed one-way analysis of variance (ANOVA) to determine the statistical significance of differences among the groups and Student’s t-test to verify the statistical significance between two arbitrary groups. Differences at *P* < 0.05 were considered statistically significant.

**Results**

**Lentiviral vector transduction efficiency in Jurkat cells**

The GFP/RFP-positive cell ratio was used to assess the transduction efficiency of the target gene in Jurkat cells in our study. The efficiencies of the LV transduction of MFN2-siRNA (for depletion of MFN2) and LV-MFN2 (for overexpression of MFN2) were determined by fluorescence microscopy to be over 80% [Figure 1a]. MFN2 mRNA expression levels in Jurkat cells were determined by RT-PCR. The results showed that MFN2 mRNA expression was significantly lower in MFN2-siRNA cells than that in control-siRNA cells (0.570 ± 0.130 vs. 1.930 ± 0.220, *P* = 0.002) and significantly higher in LV-MFN2 cells than that in LV-GFP cells (3.130 ± 0.480 vs. 2.330 ± 0.510, *P* = 0.030). There were no significant differences in MFN2 mRNA expression between the normal, LV-GFP, and control-siRNA groups (1.870 ± 0.440, 2.330 ± 0.510, vs. 1.930 ± 0.220, respectively, *P* = 1.000 and 0.872) [Figure 1b and 1d]. Similar results were obtained by Western blotting [Figure 1c and 1e].

The efficiency of the LV transduction of LV-calcineurin (for overexpression of calcineurin) was above 80% [Figure 1a]. Calcineurin phosphatase activity as measured
by the calcineurin assay kit was significantly higher in LV-calcineurin cells than that in LV-GFP2 cells (0.587 ± 0.040 vs. 0.260 ± 0.020 nmol/L, *P* = 0.000), while there was no significant difference between the normal and LV-GFP2 groups [Figure 1f]. These data suggest that these lentivirus-mediated transductions were efficient and stable.

**Effect of mitofusin-2 on the immune function of T-lymphocytes**

To investigate the effect of MFN2 on the immune function of T-lymphocytes, Jurkat cells were transfected with LV-MFN2 or MFN2-siRNA and were then stimulated with or without PMA (50 ng/ml)/ionomycin (1 µmol/L) for various lengths of time (6, 12, 24, or 48 h). Then, representative indices of the immune function of T lymphocytes – including T cell proliferation; IL-2 production, which is a hallmark of T cell activation; and the IFN-γ (Th1 cytokine)/IL-4 (Th2 cytokine) ratio, which reflects the polarization of T lymphocytes – were assessed by MTT assay and ELISA. As shown in Figure 2, the immune response of T-lymphocytes in the MFN2-siRNA group was significantly attenuated, while that in the LV-MFN2 group was markedly elevated after PMA/ionomycin treatment. Specifically, T cells transfected with MFN2-siRNA exhibited reduced proliferation (0.718 ± 0.110 vs. 1.200 ± 0.114, *P* = 0.000 or 0.808 ± 0.093 vs. 1.120 ± 0.125, *P* = 0.015; Figure 2a) as well as reduced IL-2 production (221.650 ± 12.560 vs. 414.090 ± 26.810 pg/ml, *P* = 0.000; Figure 2b) after treatment with PMA/ionomycin for 24 or 48 h. MFN2-siRNA cells also showed a decrease in the IFN-γ/IL-4 ratio (1.120 ± 0.117 vs. 2.100 ± 0.165, *P* = 0.000 or 1.320 ± 0.136 vs. 2.550 ± 0.216, *P* = 0.000; Figure 2c) after treatment with PMA/ionomycin for 12 or 24 h. In contrast, T cells transfected with LV-MFN2 exhibited enhanced proliferation (1.250 ± 0.058 vs. 1.010 ± 0.134, *P* = 0.001 or 1.540 ± 0.124 vs. 1.210 ± 0.158, *P* = 0.003; Figure 2a) and IL-2 production (461.050 ± 23.050 vs. 355.170 ± 17.900 pg/ml, *P* = 0.000 or 633.830 ± 11.790 vs. 413.130 ± 18.220 pg/ml, *P* = 0.000; Figure 2b) after being exposed to PMA/ionomycin for 24 or 48 h. In contrast, T cells transfected with LV-MFN2 exhibited enhanced proliferation (1.250 ± 0.058 vs. 1.010 ± 0.134, *P* = 0.001 or 1.540 ± 0.124 vs. 1.210 ± 0.158, *P* = 0.003; Figure 2a) and IL-2 production (461.050 ± 23.050 vs. 355.170 ± 17.900 pg/ml, *P* = 0.000 or 633.830 ± 11.790 vs. 413.130 ± 18.220 pg/ml, *P* = 0.000; Figure 2b) after being exposed to PMA/ionomycin for 24 or 48 h. Based on these results, cultured cells transfected with lentivirus were stimulated with PMA/ionomycin for 24 h in subsequent experiments.
Changes in $\text{Ca}^{2+}$, calcineurin, and nuclear factor of activated T cell in T-lymphocytes following changes in mitofusin-2 expression

In an initial attempt to investigate the potential role of the $\text{Ca}^{2+}$-calcineurin-NFAT pathway in the MFN2-mediated immune response of T-lymphocytes, we determined the levels of cytoplasmic $\text{Ca}^{2+}$, calcineurin, and NFAT activation after stimulation with PMA/ionomycin for 24 h using flow cytometry, a calcineurin assay kit, and a fast-activated cell-based ELISA, respectively. Treatment of the LV-MFN2 group with PMA/ionomycin for 24 h resulted in the most significant elevation in $\text{Ca}^{2+}$ levels (359.280 ± 10.130 vs. 266.940 ± 10.170, $P = 0.000$, Figure 3a and 3c), calcineurin phosphatase activity (0.513 ± 0.014 vs. 0.403 ± 0.020 nmol/L, $P = 0.024$, Figure 3b), and NFAT activation (1.040 ± 0.086 vs. 0.700 ± 0.115, $P = 0.005$, Figure 3d) in T-lymphocytes. However, a marked decrease in cytoplasmic $\text{Ca}^{2+}$ levels (141.140 ± 14.670 vs. 267.060 ± 9.230, $P = 0.000$), calcineurin phosphatase activity (0.054 ± 0.030 vs. 0.404 ± 0.063 nmol/L, $P = 0.000$), and NFAT activation (0.500 ± 0.025 vs. 0.720 ± 0.061, $P = 0.012$) was noted in the MFN2-siRNA group [Figure 3].

These results indicate that the $\text{Ca}^{2+}$-calcineurin-NFAT pathway may be critically involved in the MFN2-mediated immune response of T lymphocytes.

Role of calcineurin in nuclear factor of activated T cell activation following changes in mitofusin-2 expression

It was previously reported that MFN2 expression was reduced following stimulation with a high dose of HMGB1 in mouse CD4+ T-lymphocytes. It was also found that the HMGB1-mediated inhibition of T cell proliferation, accompanied by IL-2 production and a decrease in IFN-γ/IL-4, partially occurred through the Ca²⁺-NFAT signaling pathway. To clearly elucidate the potential role of calcineurin in the regulation of NFAT and the immune function of T-lymphocytes, we used LV-calcineurin to upregulate FK506 to suppress the expression of active calcineurin. As shown in Figure 4a and 4c, calcineurin levels and NFAT activation were attenuated in the MFN2-siRNA group after treatment with PMA/ionomycin for 24 h (0.053 ± 0.021 vs. 0.397 ± 0.065 nmol/L, $P = 0.000$ and 0.478 ± 0.038 vs. 0.718 ± 0.062, $P = 0.007$). However, when MFN2-siRNA cells were further transfected with LV-calcineurin (with an incubation time of 48 h for each vector), we observed significant elevations in calcineurin (1.157 ± 0.100 vs. 0.067 ± 0.015 nmol/L, $P = 0.000$; Figure 4a) and NFAT activation (0.940 ± 0.046 vs. 0.447 ± 0.059, $P = 0.000$; Figure 4c). Similarly, elevations in calcineurin and NFAT activation in the LV-MFN2 group were reversed by pretreating the cells for 48 h with 20 nmol/L FK506 (0.519 ± 0.044 vs. 0.277 ± 0.045 nmol/L, $P = 0.000$; Figure 5a) and NFAT activation (1.072 ± 0.111 vs. 0.618 ± 0.0697, $P = 0.000$; Figure 5b and 5d). Thus, the MFN2-mediated changes in NFAT activation were partially reversed by regulating active calcineurin expression, indicating that calcineurin plays an essential role in the regulation of MFN2-mediated T-lymphocyte immune function.

Effect of calcineurin expression on the immune function of T-lymphocytes

To elucidate the role of calcineurin in modulating MFN2 and its effect on the immune function of T-lymphocytes, we investigated whether the effects of MFN2 on the immune response of T-lymphocytes could be reversed by changing the expression of active calcineurin. T cells transfected with MFN2-siRNA exhibited reductions in proliferative activity (0.580 ± 0.078 vs. 1.260 ± 0.071, $P = 0.040$; Figure 5a), IL-2 release (175.330 ± 12.900 vs. 413.250 ± 15.900 pg/ml, $P = 0.000$; Figure 5c), and the IFN-γ/IL-4 ratio (1.030 ± 0.091 vs. 2.570 ± 0.097, $P = 0.000$; Figure 5e) after treatment with PMA/ionomycin for 24 h. However, when the MFN2-siRNA group was transfected with LV-calcineurin to simultaneously increase the active calcineurin level, T cell proliferation (1.120 ± 0.048 vs. 0.580 ± 0.078, $P = 0.040$) IL-2 production (473.300 ± 24.100 vs. 175.330 ± 12.900 pg/ml, $P = 0.000$; Figure 5a and 5e), and the IFN-γ/IL-4 ratio (3.080 ± 0.156 vs. 0.953 ± 0.093, $P = 0.000$; Figure 5e) were significantly elevated. Similarly, elevations in

![Figure 2: Effect of MFN2 on the immune function of Jurkat cells in response to PMA/ionomycin. Jurkat cells were transfected with LV-MFN2 and MFN2-RNAi and then stimulated with PMA (50 ng/ml) plus ionomycin (1 mmol/L) for various lengths of time (6, 12, 24, and 48 h). (a) A methyl-thiazolyl-tetrazolium cell proliferation assay was used to assess Jurkat cells activity. Levels of IL-2 (b) and the IFN-γ/IL-4 ratio (c) were measured by ELISA. Results are shown as mean ± standard deviation. *$P < 0.05$ versus the controls; †$P < 0.01$ versus the controls; ‡$P < 0.01$ versus the control-RNAi or LV-GFP group. IFN: Interferon; IL: Interleukin; LV: Lentiviral vector; MFN2: Mitofusin-2; GFP: Green fluorescent protein; PMA: Phorbol myristate acetate.
Figure 3: Effect of MFN2 on intracellular calcium, calcineurin expression, and NFAT activity in Jurkat cells. Jurkat cells were transfected with LV-MFN2 and MFN2-RNAi and then stimulated with PMA (50 ng/ml) plus ionomycin (1 mmol/L) for various lengths of time. (a and c) Intracellular calcium was measured by FACS with the fluorescent probe Fluo-3/AM. (b) Calcineurin activity was measured using a calcineurin assay kit. (d) NFAT activity in Jurkat cells was measured by ELISA. *P < 0.05, †P < 0.01 versus the control group; ‡P < 0.05, §P < 0.01 versus the control-RNAi or LV-GFP group. LV: Lentiviral vector; MFN2: Mitofusin-2; GFP: Green fluorescent protein; NFAT: Nuclear factor of activated T cells; PMA: Phorbol myristate acetate.

Figure 4: Regulation of calcineurin expression reverses MFN2-induced NFAT activation in Jurkat cells. After various pretreatments, Jurkat cells were stimulated with PMA plus ionomycin for 24 h. Jurkat cells were pretreated with MFN2-RNAi and then LV-calcineurin, and calcineurin activity was measured by commercial assay kit (a) and NFAT activity was determined by ELISA (c). Jurkat cells were pretreated with LV-MFN2 and then FK-506, and calcineurin activity (b) and NFAT activity (d) were measured. *P < 0.05 vs. the control group; †P < 0.05 vs. the control-RNAi or LV-GFP group; ‡P < 0.05 vs. the LV-MFN2 or MFN2-RNAi group. LV: Lentiviral vector; MFN2: Mitofusin-2; GFP: Green fluorescent protein; NFAT: Nuclear factor of activated T-cell. P/I: PMA (phorbol myristate acetate)/ionomycin; ELISA: Enzyme-linked immunosorbent assay.
T cell proliferation (1.520 ± 0.082 vs. 1.270 ± 0.082, \( P = 0.029 \); Figure 5b), IL-2 production (642.780 ± 8.010 vs. 410.710 ± 23.900 pg/ml, \( P = 0.000 \); Figure 5d), and the IFN-\( \gamma \)/IL-4 ratio (4.570 ± 0.071 vs. 2.650 ± 0.055, \( P = 0.000 \); Figure 5f) observed in the LV-MFN2 group were reversed by the administration of FK506. Therefore, calcineurin appeared to play an important role in the process by which MFN2 affects T lymphocyte-mediated immunity.

**Discussion**

Mitochondria have long been considered the power houses of eukaryotic cells. However, it is becoming increasingly apparent that mitochondria are intimately involved in a broad range of immune-related pathways, functioning as signaling platforms and contributing to effector responses.\[21-24\] Thus, in addition to their well-appreciated roles in cellular metabolism and programmed cell death, mitochondria appear to function as a centrally positioned hub in the innate immune system.\[25\] In general, mitochondria regulate cell signaling in two ways: by serving as physical platforms on which protein-protein signaling interactions occur and by regulating levels of intracellular signaling molecules, including Ca\(^{2+}\) and reactive oxygen species.\[26\] MFN2, located in the outer mitochondrial membrane, controls mitochondrial dynamics,\[27-29\] thereby exerting an effect on antiviral signaling, mainly by interacting with the mitochondrial antiviral signaling protein to inhibit antiviral signaling pathways.\[7,30\] Nevertheless, limited information is available concerning the function of MFN2 in the adaptive immune response. In order to provide such information, we manipulated the expression of MFN2 in Jurkat cells and investigated the related signaling pathways in the cell-mediated immunity of T-lymphocytes.

The major finding of the present study is that the immune function of T-lymphocytes is regulated by MFN2 *in vitro*. Upon the upregulation of MFN2 in Jurkat cells, the immune response of T cells was enhanced; in contrast, when MFN2 was depleted, T-lymphocyte immune capacity was inhibited, as evidenced by a reduction in proliferation activity and
a shift in polarization from Th1 to Th2. Our results thus demonstrate that MFN2 plays an important role in adaptive immunity in vitro.

T cell activation is largely initiated by T cell receptor ligation, leading to the activation of phosphoinositide phospholipase C, which then catalyzes the generation of inositol-1,4,5-trisphosphate (IP3). This interacts with its receptor (IP3R) on the ER surface, thereby inducing calcium efflux from the ER and subsequent depletion of the calcium store. This calcium store depletion activates calcium release-activated channels, thereby inducing a sustained influx of calcium to activate the cell signaling pathways of T cells.[31] Thus, Ca\(^{2+}\) plays a key role in initiating T cell activation and differentiation.[32-34] In our experiments, we assessed levels of cytoplasmic Ca\(^{2+}\) after altering MFN2 expression, and the results showed that MFN2 did indeed influence Ca\(^{2+}\) levels in T cells. In line with these findings, de Brito and Scorrano found that MFN2 ablation or silencing in mouse embryonic fibroblasts (MEFs) and HeLa cells disrupted ER morphology and ER-mitochondria interactions, thereby reducing the efficiency of mitochondrial Ca\(^{2+}\) uptake in response to stimuli that generate IP3.[38]

The calcineurin-NFAT pathway plays an essential role in the regulation of the immune system by modulating the T cell response to stimuli and regulating the development and function of T and B cells, as well as other hematopoietic cells.[35] The present study revealed that MFN2 could influence cytoplasmic Ca\(^{2+}\) levels, activating calcineurin to dephosphorylate NFAT in the nucleus. Calcineurin has been identified as a key signaling enzyme in T-lymphocyte activation.[18] To confirm the role of calcineurin in MFN2-mediated T cell immune function, we further altered the expression of active calcineurin after manipulating the expression of MFN2. Our data suggested that MFN2-mediated changes in NFAT activation and T cell function were partially reversed by regulating active calcineurin expression; therefore, calcineurin contributes to the role of MFN2 in regulating T cell function. Further studies are necessary to determine whether other Ca\(^{2+}\) signaling molecules, such as protein kinase C, calmodulin kinases, and cytoskeletal proteins, also participate in the MFN2-mediated immune function of T cells.[36]

MFN2 acts as a suppressor of proliferation and may inhibit the proliferation of vascular smooth muscle cells and MEFs.[37] Dasgupta et al.[38] reported that MFN2 exhibited antiproliferative properties in activated peripheral blood T cells. However, in the present study, MFN2 overexpression markedly enhanced T cell proliferation in Jurkat cells. Several factors could explain this discrepancy. First, MFN2 may exert different functions in different cell types. Mounting evidence has shown that MFN2 is involved beyond fusion, acting to regulate mitochondrial metabolism, apoptosis, the shape of other organelles, and even progression through the cell cycle.[39] Thus, MFN2 may be involved in different pathophysiological processes in different systems.[40-42] Further studies are necessary to determine if this effect is present in other types of immune cells. Second, MFN2 may mediate different effects in T cells under different stimuli. In a previous study, the overexpression of MFN2 inhibited cellular proliferation in CD4\(^{+}\) T cells activated by concanavalin A (ConA).[19] However, surprisingly, in ConA and HMGB1 costimulated CD4\(^{+}\) T cells, the overexpression of MFN2 promoted the proliferation of T cells.

The NFAT gene family was first identified in 1988 as a family of transcription factors that activate IL2 gene expression in response to T cell activation.[43] This family contains four classic members (NFAT\(_{c}\)) as well as NFAT\(_{b}\).[44] However, in this study, we did not assess the different types of NFAT and so could not confirm the types of NFAT that are definitively activated in T cell immune function; therefore, further study is required to answer this question.

In summary, MFN2 plays a key role in regulating T cell function, mainly through the Ca\(^{2+}\)-calcineurin-NFAT pathway. Our results indicate that the targeting of MFN2 may represent a novel interventional strategy for T cell dysfunction, which occurs in autoimmune diseases, sepsis, and cancer. Exploring the potential significance and unraveling the precise regulatory mechanism underlying the regulation of Ca\(^{2+}\) by MFN2 are warranted in further studies.

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### Conflicts of interest

There are no conflicts of interest.

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