

Original Article

Targeting pathogenic Th17.1 cells via JAK-STAT3 pathway: A novel approach with tofacitinib for refractory myasthenia gravis

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ABSTRACT

There remains a critical unmet need for effective, accessible, and well-tolerated therapies for myasthenia gravis (MG) who are refractory to current immunotherapies. The janus kinase and signal transducer and activator of transcription (JAK-STAT) signaling pathway plays a pivotal role in maintaining immune homeostasis by regulating cytokine-mediated responses. However, insights into the involvement of the JAK-STAT signaling in MG pathogenesis are still preliminary. In this study, we observed elevated levels of *JAK2* in MG and further assessed the clinical efficacy of the pan-JAK inhibitor, tofacitinib, administered over 24 weeks in a cohort of 19 patients with refractory MG (NCT04431895). Tofacitinib significantly reduces the glucocorticoid dose and improves MG-relevant clinical scores and quality of life. The immunomodulatory effects of tofacitinib were mediated through downregulation of p-STAT3, IL-6, and IL-23, resulting in suppression of pathogenic Th17.1 cells in MG. Collectively, our results suggest that a novel approach to suppress pathogenic Th17.1 cells via the JAK-STAT3 signalling pathway with tofacitinib is effective and well-tolerated for treating patients with refractory MG.

Introduction

Myasthenia gravis (MG) is a B-cell-mediated, T-cell-dependent, autoantibody-mediated disease, which targets the postsynaptic membrane of the neuromuscular junction, leading to hallmark muscle weakness and fatigability [1]. Conventional management of MG has primarily involved acetylcholinesterase inhibitors, corticosteroids, immunosuppressants, and thymectomy [2]. Over the past few decades, emerging immunotherapies for MG mainly target the immune cascade, including key B-cell-associated molecules, distinct elements of complement cascade activation, and fragment crystallizable neonatal receptors participating in the IgG recycling [3]. Although current treatments have markedly improved the quality of life for MG, limitations remain regarding the therapeutic efficacy, treatment duration, cost-effectiveness, and medication adherence associated with administration modalities [4,5]. Among patients with MG, up to 15 % are refractory to the standard immunotherapies [6,7]. Furthermore, a

recent analysis of insurance claims data revealed escalating individual and societal costs associated with neurological disorders, highlighting the substantial financial burden imposed by new-to-market medications for conditions such as MG [8]. There are unmet needs for the development of therapeutic strategies with high cost-effectiveness for these patients with refractory MG.

In current knowledge on the pathogenesis of MG, autoantigen-reactive T cells facilitate B cell activation by inducing robust proliferative responses and cytokines, thereby promoting the production of pathogenic autoantibodies and class switch recombination [9]. Autoreactive T cells from MG patients mainly consist of the Th17.1 phenotype, which serves as a biomarker of disease severity in MG [10,11]. With the help of IL-23 and IL-12, Th17 cells display considerable plasticity that shift toward a Th17.1 phenotype, thus secreting the pathogenic cytokines, including IFN- γ , IL-17, and granulocyte-macrophage colony-stimulating factor (GM-CSF). Moreover, emerging evidence demonstrates

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that Th17.1 cells exhibit selective glucocorticoid resistance, suggesting their potential pathogenic role in treatment-refractory MG [12,13]. In the conversion of Th17.1, cytokines, such as IL-6 and IL-23, drive the differentiation from Th17 toward a Th1 phenotype. These highlight the essential roles of IL-6, IL-23, and Th17.1 cells as targets for the modulation of autoimmune responses in MG [14,15].

The Janus kinase (JAK) and signal transducer and activator of transcription (STAT) signaling pathway is an important immune response pathway that regulates the transcription of immune cytokines to hold immune homeostasis. The JAK2/STAT3 signaling pathway mediates signal transduction downstream of IL-23 and IL-6. JAK2 inhibitors have been demonstrated to alleviate experimental autoimmune myasthenia gravis (EAMG) by restoring the Th17/Treg balance [16]. Despite the pathophysiological importance of the JAK/STAT signaling pathway in the pathogenesis of MG, current knowledge has been primarily characterized in EAMG models, with limited evidence in patients with MG. Tofacitinib is a non-selective JAK inhibitor that inhibits all JAK kinases to regulate a broad range of immune responses and has been shown to ameliorate EAMG severity significantly [17]. Clinical trials have established the efficacy and tolerability of tofacitinib in several autoimmune diseases, including ulcerative colitis, rheumatoid arthritis, and psoriatic arthritis, though its therapeutic benefits for MG remain unclear [18,19].

In this 24-week prospective pilot study, we evaluated the therapeutic potential of tofacitinib for patients with refractory generalized MG (gMG). Using transcriptome sequencing, we investigated whether and how tofacitinib modulates the immune subsets in MG patients. In addition to inhibiting STAT3 phosphorylation, tofacitinib significantly attenuated both the differentiation and pathogenic function of Th17.1 cells.

Methods

Participants

We conducted a prospective pilot study of 19 patients aged 18–70 years with refractory MG treated at Huashan Hospital (Shanghai, China) between June 2020 and July 2023 (ClinicalTrials.gov Identifier: NCT04431895). All patients were categorized as Myasthenia Gravis Foundation of America class II to IV and showed abnormal repetitive nerve stimulation results. All patients were documented inadequate response to standard immunotherapy, including at least 3 months of combined glucocorticoid (0.75–1 mg/kg/day) and either azathioprine (≥ 100 mg/day) for over 6 months, or other immunosuppressive therapies (i.e. mycophenolate mofetil, cyclosporine, tacrolimus) for at least 3 months. Patients were excluded if they had received rituximab within 6 months before screening, had intravenous immunoglobulin or plasma exchange within 1 month of screening, had active hepatitis B, concurrent autoimmune diseases, active infections, or malignancies other than thymoma. All participants provided written informed consent. The full details of the inclusion and exclusion criteria are provided in the supplementary data.

Patients received tofacitinib at a dose of 5 mg twice daily during a 24-week treatment period while receiving a prednisolone regimen (10–40 mg/day at baseline). Efficacy was assessed with the MG-Activities of Daily Living (MG-ADL) scale, Quantitative Myasthenia Gravis (QMG) score, Myasthenia Gravis Composite (MGC) scale, and the 15-item revised version of the Myasthenia Gravis Quality of Life (MG-QOL15r) questionnaire at baseline and weeks 4, 8, 12, 16, and 24. The primary outcome of this study was the change from baseline in QMG Scores at week 24. Minimal symptom expression (MSE) is denoted by an MG-ADL score of 0 or 1. For acetylcholine receptor antibody-positive patients, the levels of serum antibody were detected by radioimmunoassay at baseline and Week 16. Safety was assessed through the incidence of adverse events throughout the study.

Human samples

Blood samples were obtained from MG patients and healthy controls (HCs) at Huashan Hospital with informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood samples using Ficoll-based density gradient separation (Cytica). For cytokine determination, blood was centrifuged directly after withdrawal, and serum was stored at -80°C until analysis. The use of all samples in this study was approved by the Ethics Committees at the Huashan Hospital, Fudan University.

scRNA-seq sample preparation and sequencing

For single-cell RNA sequencing (scRNA-seq), mononuclear cells (MNCs) were isolated from peripheral blood using red blood cell lysis buffer (Miltenyi Biotec). Single cell suspension from blood samples was stained for viability assessment using Calcein-AM (Thermo Fisher Scientific) and Draq7 (BD Biosciences). The BD Rhapsody system was used to capture the transcriptomic information of the single cells. Single-cell capture was achieved by random distribution of a single-cell suspension across $>200,000$ microwells through a limited dilution approach. Beads with oligonucleotide barcodes were added to saturation so that a bead was paired with a cell in a microwell. The cells were lysed in the microwell to hybridize mRNA molecules to barcoded capture oligos on the beads. Beads were collected into a single tube for reverse transcription and ExoI digestion. Upon cDNA synthesis, each cDNA molecule was tagged on the 5' end (that is, the 3' end of an mRNA transcript) with a unique molecular identifier (UMI) and cell barcode indicating its cell of origin. Whole transcriptome libraries were prepared using the BD Rhapsody single-cell whole-transcriptome amplification (WTA) workflow, including random priming and extension (RPE), RPE amplification PCR, and WTA index PCR. The libraries were quantified using a High Sensitivity DNA chip (Agilent) on a Bioanalyzer 2200 and the Qubit High Sensitivity DNA assay (Thermo Fisher Scientific). All libraries were sequenced by DNBSEQ-T7 Sequencer (MGI) on a 150 bp paired-end run.

ScRNA-seq data analysis

We applied fastp with default parameter, filtering the adaptor sequence, and removed the low-quality reads to achieve clean data. To quantify the gene expression of the single-cell data, we used STARsolo (version 2.7.10a) along with the human GRCh38 reference genome. Cells contained over 500 expressed genes, and the mitochondria UMI rate below 5 % passed the cell quality filtering and mitochondria genes were removed in the expression table. The Seurat package (version 4.4.0) was used for cell normalization and regression based on the expression table according to the UMI counts of each sample and the percent of mitochondria rate to obtain the scaled data. Principal component analysis (PCA) was constructed based on the scaled data with the top 2000 highly variable genes, and the top 10 principal components were used for tSNE construction and UMAP construction. Utilizing a graph-based cluster method (resolution = 0.5), we acquired the unsupervised cell cluster result based on the PCA top 10 principal and we calculated the marker genes by the FindAllMarkers function with the Wilcoxon rank sum test algorithm under criteria as $\log_2\text{FC} > 0.25$, P value < 0.05 , and $\text{min.pct} > 0.1$. The cell type clusters were annotated based on cluster markers and well-known cell-type markers. In the CD4 (+) T cell cluster, genes with $\log_2\text{FC} > 0.5$ and adjusted P value < 0.05 found by DESeq2 were assigned as differentially expressed. Gene function enrichment analysis was done with clusterProfiler. Pathway analyses were predominantly performed on the hallmark pathways described in the ontology gene sets C5 of the molecular signature database [20]. To assign pathway activity, gene set variation analysis was performed using standard settings in the GSVA package (version 1.22.4).

Bulk RNA-seq library preparation and sequencing

Total RNA was extracted from MNCs of MG patients treated with tofacitinib with TRIzol reagent (Invitrogen) following the standard protocol. In brief, the NEBNext Ultra RNA Library Prep Kit (Illumina) was used to generate the sequencing library, the TruSeq PE Cluster Kit v3-cBot-HS (Illumina) was used to perform sample clustering, and the library preparations were sequenced on an Illumina Novaseq platform.

Bulk RNA-seq data analysis

Raw bulk RNA sequencing (RNAseq) reads were aligned to the hg38 reference genome, and gene expression profiles were quantified using the STAR aligner. Differential expression analysis of two groups in RNA-seq was performed using the DESeq2 R package (version 1.20.0). DESeq2 provides statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting *P* value was adjusted using the Benjamini and Hochberg approach for controlling the false discovery rate. Genes with fold-change >2 and adjusted *P* value < 0.05 found by DESeq2 were assigned as differentially expressed. Gene function enrichment analysis was done with clusterProfiler. We analyzed Gene Set Enrichment Analysis (GSEA) using the local version of the GSEA analysis tool.

Real-time quantitative polymerase chain reaction (qPCR)

Total RNA was extracted using RNAiso Plus (TaKaRa) and reverse transcribed into cDNA using PrimeScript RT reagent Kit with gDNA

Eraser (TaKaRa) according to the protocol provided. RT-qPCR was performed using TB Green Premix Ex Taq (TaKaRa) in 96-well plates. Plates were read with the QuantStudio 5 Real-Time PCR System (Applied Biosystems). The primer sequences are shown in [Supplementary Table 1](#). Amplifications were carried out with the primers described in the Supplementary Table.

Th17.1 Cell subset differentiation

Naïve CD4(+) T cells were isolated from PBMCs of immunotherapy-naïve MG patients by immunomagnetic negative selection according to the manufacturer's instructions (Stemcell). Purified naïve CD4(+) T cells were seeded in a 96-well round-bottom microplate (Corning) at a density of 10^6 cells per mL and cultured with X-VIVO 15 (Lonza) in a 37 °C 5 % CO₂ incubator. To induce Th17.1 cell differentiation, the medium was supplemented with human CD3/CD28 T cell activator (Stemcell), 50 U/mL IL-2 (BD), 2.5 µg/mL anti-human IL-4 antibody (Invitrogen), 20 ng/mL IL-1β (Biolegend), 30 ng/mL IL-6 (Biolegend), and 30 ng/mL IL-23 (Biolegend). Tofacitinib (10 nM; Selleckchem) or equal dimethyl sulfoxide (Sigma-Aldrich) were added to the culture media, which was replaced on day 3, followed by cell splitting. On day 7, cells were harvested for intracellular staining, while supernatants were collected for ELISA analysis.

Enzyme-linked immunosorbent assay (ELISA)

Serum IL-10 levels were assayed by a commercial ELISA kit (R&D) following the manufacturer's protocol. Similarly, GM-CSF and IL-17A

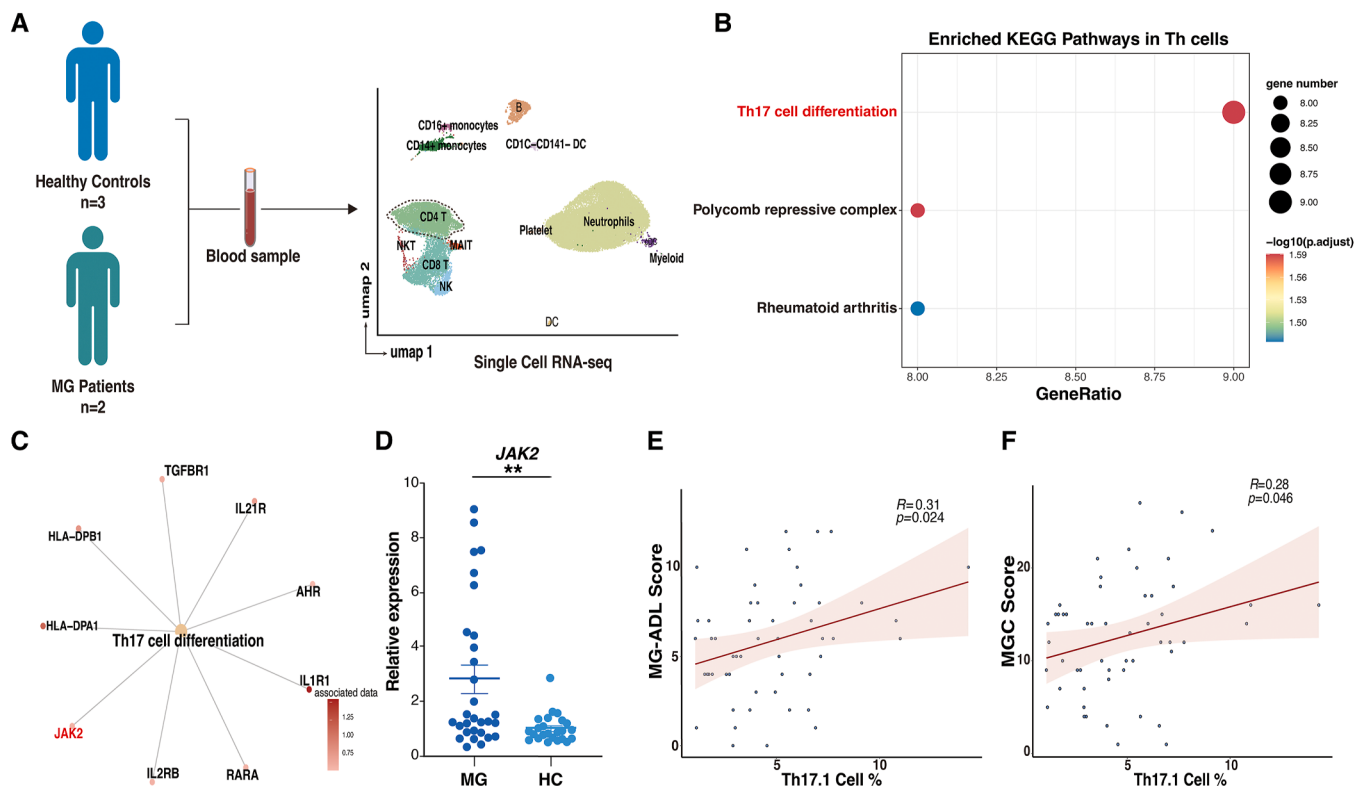


Fig. 1. Enhanced Th17 cell differentiation pathways and elevated JAK2 expression in myasthenia gravis (MG) patients. **A** Single-cell RNA sequencing (scRNA-seq) was performed on peripheral blood (PB) samples from treatment-naïve MG patients ($n = 2$) and matched healthy controls ($n = 3$). **B** Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of upregulated genes of CD4(+) T cells in MG patients. **C** Key upregulated genes associated with Th17 cell differentiation pathways. **D** JAK2 expression in peripheral blood mononuclear cells (PBMCs) from immunotherapy-naïve MG patients and matched healthy controls was measured using RT-qPCR (MG patients $n = 30$, healthy controls $n = 24$). **E**, **F** Correlation between MG-ADL score (**E**) or MGC score (**F**) and paired circulating Th17.1 cell frequencies in refractory MG patients ($n = 53$). Data are presented as mean $\pm$ standard error of the mean (SEM). Statistical analysis was conducted using a two-sided Wilcoxon rank-sum test (**D**) or Pearson Correlation test (**E**, **F**). Statistical significance is indicated by $**p < 0.01$.

concentrations in cell supernatants were measured with commercially available ELISA kits (R&D) according to the manufacturer's instructions.

Flow cytometry

For the blood sample, 200 μ L whole blood was stained with a cocktail of surface markers (BD Biosciences). Immune cell populations were defined as follows: CD4(+) T cells (CD3⁺ CD4⁺), Tregs (CD3⁺ CD4⁺ CD25^{hi} CD127^{dim}). For the frequency and the levels of p-STAT3 in CD4(+)T subsets, 200 μ L whole blood was stained with surface antibodies including CD4, CD45RA (eBioscience), CD27, CXCR3, CCR4, and CCR6 (Biolegend). To induce the phosphorylation of STAT3, 50 ng/mL IL-6 was added to the blood sample and incubated at 37 °C for 15 min. The samples were fixed in Lyse/Fix Buffer (BD Biosciences) at 37 °C for 10 min, and washed once with 1 % bovine serum albumin (BSA). The cells were permeabilized with cold Perm Buffer III (BD Biosciences) for 30 min on ice, washed twice with 1 % BSA, then stained with p-STAT3 (BD Biosciences) for 30 min, and washed once with 1 % BSA before analysis.

For intracellular cytokine staining, cells were incubated for 5 h with the cell activation cocktail (Biolegend). Surface staining was performed by incubation with CD4 and fixable viability dye (eBioscience) for 20 min at room temperature. Intracellular staining was performed using the Fixation/Permeabilization Kit (BD Biosciences). Briefly, cells were fixed and permeabilized for 20 min on ice and stained with IL-17A and IFN- γ in permeabilization buffer for 30 min at room temperature.

The samples were analyzed on an Attune NxT Flow Cytometer (Thermo Fisher Scientific), and the data was analyzed on FlowJo v10 software (LLC).

Statistical analysis

The normality of data was assessed individually, and the F test was conducted to determine the similarity in the variances. For two independent samples, unpaired two-sided student t-test was used to calculate significance, while a paired samples t-test is applied for paired samples. Mann-Whitney U test for non-normally distributed data. Significance was assumed to be reached at $P < 0.05$. In all graphs, exact P values are shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. NS, not significant. All data are presented as mean $\pm$ s.e.m. Each dot in the main figures represents one sample. All statistical analyses were performed using GraphPad Prism (v.9.5.0; <https://www.graphpad.com/>), R (v4.4.0), and SPSS Statistics (26.0).

Results

JAK2 and Th17 cell differentiation is elevated in CD4(+) T cells of patients with MG

To elucidate the pathogenesis of MG and identify potential therapeutic targets, we applied scRNA-seq from peripheral blood of patients with immunotherapy-naïve MG and age-/sex-matched HCs (Fig. 1A). To define the major cell population, we identified the majority of known immune cell types, including neutrophils, CD4(+) T cells, CD8(+) T cells, monocytes, and B cells (Fig. 1A, Fig. S1). Compared with HCs, Th17 cell differentiation-related pathway, particularly the JAK2 expression was upregulated in CD4(+) T cells from MG patients (Fig. 1B–D). We have previously reported a significant increase in Th17.1 cell levels in MG patients compared to HCs [21]. In the present study, we further demonstrate that the frequency of Th17.1 cells is significantly correlated with disease severity, as assessed by both MG-ADL and MGC scores in refractory MG patients (Fig. 1E and F). Taken together, these findings suggest that JAK2-mediated Th17.1 cell differentiation plays a critical role in the pathogenesis of refractory MG.

Demographic and baseline clinical characteristics of tofacitinib-treated refractory MG

A total of 780 patients with MG were screened between June 2020 and July 2023, of whom 19 refractory gMG patients (mean [SD] age, 44.3 [11.3] years) were finally enrolled in this study (Fig. 2). The baseline characteristics are shown in Table 1. Patient characteristics were representative of the refractory MG population, among which 4 patients had thymoma and had undergone thymoma resection before enrollment, while one early-onset MG (EOMG) received thymectomy. The majority of patients (89 %) were positive for anti-acetylcholine receptor (AChR) antibodies, while two individuals were negative for both anti-AChR and anti-muscle-specific receptor tyrosine kinase (MuSK) antibodies, namely seronegative myasthenia gravis (SNMG). Most refractory MG patients (14 [74 %] of 19) had been treated with steroids and more than one immunosuppressive treatment before enrollment, as shown in Table 2.

Tofacitinib improves clinical outcomes and glucocorticoid response in refractory MG

Most patients (17 [89 %] of 19) received tofacitinib combined with oral glucocorticoids during the study, while two patients received intravenous immunoglobulin (IVIg) (400 mg/kg/day, 5 days) at week 8 due to the new-onset COVID-19 infection. Significant clinical improvements were observed in MG patients from week 4 and sustained through the end of the study, as measured by QMG (mean reduction of 6.2 points, SEM of 1.51), ADL (mean reduction of 3.6 points, SEM of 0.78), and MGC (mean reduction of 6.6 points, SEM of 1.70) at week 24 (Fig. 3A–C, E). Patients treated with tofacitinib showed improvement in MG-QOL15r scores as early as week 8, which was sustained through week 24 (mean reduction of 11.0 points, SEM of 2.44) (Fig. 3D). By the end of

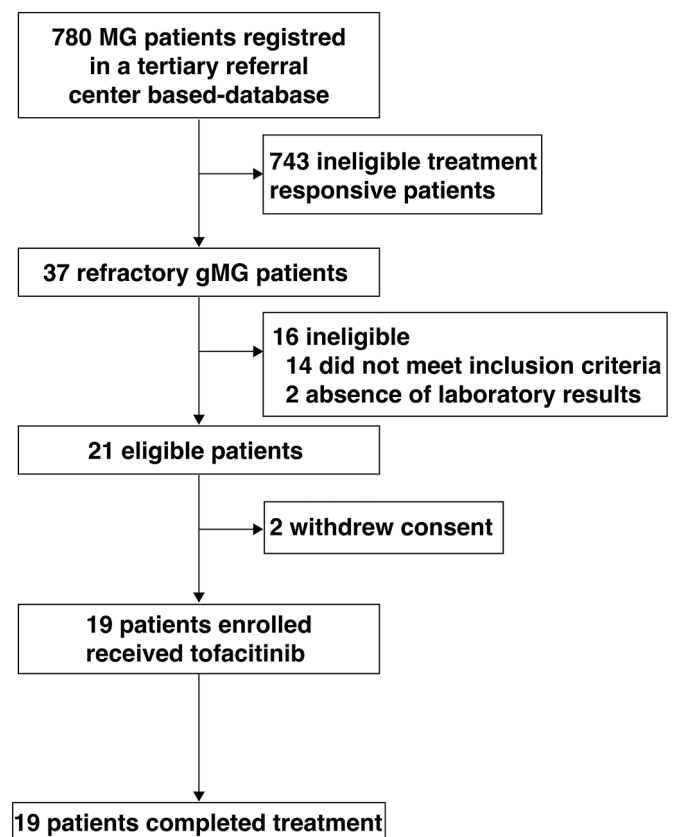


Fig. 2. Flow diagram of the enrollment of study participants.

Table 1

Demographic and baseline clinical characteristics of tofacitinib-treated refractory MG.

	All patients (n = 19)
Age, years	44.3 (11.3)
Sex	
Female	14 (74 %)
Male	5 (26 %)
MG subtypes	
EOMG	10 (52.6 %)
LOMG	3 (15.8 %)
TAMG	4 (21.1 %)
SNMG	2 (10.5 %)
MGFA class at screening	
IIa/b	13 (68 %)
IIIa/b	5 (26 %)
IVa/b	1 (5 %)
Previous thymectomy	
B1	1 (5.3 %)
B2	3 (15.8 %)
Thymic hyperplasia	1 (5.3 %)
Acetylcholine receptor antibody-positive	17 (89 %)
Acetylcholine receptor and MuSK antibody-negative	2 (11 %)
Baseline scores	
Total MG-ADL score	6.0 (2.9)
Total quantitative myasthenia gravis score	15.0 (4.4)
Total myasthenia gravis composite score	13.0 (6.0)
Total MG-QOL15r score	21.2 (13.0)
Myasthenia gravis therapy before baseline	
Steroid	19 (100.0 %)
Tacrolimus	16 (84 %)
Azathioprine	8 (42 %)
Mycophenolate mofetil	5 (26 %)
PE or IVIG	10 (53 %)
Rituximab	4 (22 %)

Data are mean (SD) or n (%). EOMG = Early-onset Myasthenia Gravis. IVIG = Intravenous Immunoglobulin. LOMG = Late-onset Myasthenia Gravis. TAMG = Thymoma-associated Myasthenia Gravis. SNMG = Double-Seronegative Myasthenia Gravis. MG-ADL = Myasthenia Gravis Activities of Daily Living. MGFA = Myasthenia Gravis Foundation of America. MG-QOL15r = 15-item revised version of the Myasthenia Gravis Quality of Life. MuSK = Muscle-specific Receptor Tyrosine Kinase. PE = Plasma Exchange.

the study, MSE was achieved by 10 patients (52.6 %), including two SNMG. Notably, tofacitinib significantly led to the dose reduction of prednisolone from week 16 and declined to 13.68 mg (SEM 1.81) at week 24 (Fig. 3F). The levels of anti-AChR antibody in MG decreased at

week 16, though not statistically significant ($P = 0.093$; Fig. 3G). No severe treatment-emergent adverse events were reported during the study. Given these therapeutic effects, we next explored the potential mechanisms underlying the benefit of tofacitinib in MG.

Tofacitinib suppresses IL-6 and IL-23 expression in patient-derived peripheral blood mononuclear cells

As a pan-JAK inhibitor, tofacitinib can modulate multiple signalling pathways associated with cytokines [22]. To comprehensively assess how tofacitinib altered the immune landscape of MG, we performed Bulk RNAseq on PBMCs of MG patients at pre- and post-treatment stage (Fig. 4A). As a result, IL-17-associated pathways was significantly downregulated after tofacitinib treatment (Fig. 4B). We observed that tofacitinib downregulated cytokines associated with Th17.1 cells including IL-6 and IL-23, rather than broadly inhibiting the entire Th17-related pathway (Fig. 4C–E). In addition, *IFN- γ* mRNA expression levels were significantly reduced, while *JAK2* and *T-bet* mRNA expression showed a non-significant downward trend (Fig. 4F, Fig. S2). Together, these results indicate that tofacitinib alleviates MG by inhibiting cytokines associated with Th17.1 cell differentiation.

Tofacitinib suppresses p-STAT3 expression in pathogenic Th17.1 cells

Based on RNA sequencing results and the crucial involvement of the JAK-STAT signalling pathway in CD4(+) T cells, we next measured the expression of CD4(+) T cell subsets in pre- and post-treatment MG patients (Fig. 5A and B, Fig. S3). Compared with baseline, the frequencies of total CD4(+) T cells, Th1, and Th17.1 cells declined marginally but not significantly post-tofacitinib treatment (Fig. 5C, D, F). Meanwhile, the proportions of classical Th17 and Th2 cells remained stable (Fig. 5E–G). In contrast, both immunosuppressive Tregs and serum levels of the immunoregulatory cytokine IL-10 were significantly upregulated following tofacitinib treatment in MG patients (Fig. 5H, Fig. S3).

Given that the cell frequency alone does not fully reflect their pathogenic potential and the pivotal role of p-STAT3 in T cell differentiation and glucocorticoid resistance, we determined p-STAT3 levels in CD4(+) T cell subsets of pre- and post-treatment MG patients. We found a reduction in p-STAT3 levels across all Th cell subsets, with the most pronounced inhibition seen in Th17.1 and Th1 cells following tofacitinib treatment in MG patients (Fig. 6A and B). To further verify the inhibitory effect of tofacitinib on Th17.1 cells, naïve CD4(+) T cells isolated from

Table 2

Baseline characteristics and medical history of refractory MG patients.

No.	Age	Sex	AChR Positive	MGFA Class	Thymoma	Thymectomy	MG Duration, y	Previous treatments (duration)
1	32	F	No	IIIa	No	No	3	MMF (1w), TK (5 m)
2	34	F	Yes	IIa	No	No	3	AZA (7 m)
3	66	F	Yes	IIIa	No	No	8	TK (3 m)
4	29	M	Yes	IVa	No	No	15	AZA (12 m), PE (4c), RTX (4c)
5	48	F	Yes	IIb	B1	Yes	1	TK (9 m)
6	35	F	Yes	IIIb	No	No	9	AZA (7 m), IVIG (2c), RTX (4c), TK (2y)
7	34	F	Yes	IIa	No	No	9	TK (8 m)
8	61	F	Yes	IIIa	B2	Yes	2	PE (1c), TK (5 m)
9	35	M	Yes	IIIa	No	Yes	33	AZA (15 m), IVIG (1c), PE (2c), RTX (2c), TK (5y)
10	47	F	No	IIa	No	No	11	TK (16 m)
11	39	F	Yes	IIb	B2	Yes	1	AZA (6 m), TK (13 m)
12	40	F	Yes	IIb	No	No	16	AZA (1y), IVIG (5c), RTX (4c), TK (5y)
13	51	F	Yes	IIb	No	No	6	AZA (1y), TK (2y)
14	39	M	Yes	IIb	No	No	10	IVIG (2c), MMF (14 m), TK (3 m)
15	57	M	Yes	IIa	No	No	3	IVIG (6c), MMF (3 m)
16	62	F	Yes	IIa	No	No	2	MMF (3 m), TK (3 m)
17	34	F	Yes	IIb	No	No	6	IVIG (1c), TK (5 m)
18	53	M	Yes	IIb	B2	Yes	3	IVIG (1c), TK (2y)
19	45	F	Yes	IIb	No	No	3	AZA (1w), IVIG (1c), MMF (21 m), TK (8 m)

AChR = Acetylcholine receptor. AZA = azathioprine. IVIG = Intravenous Immunoglobulin. MGFA = Myasthenia Gravis Foundation of America. PE = plasma exchange. MMF = mycophenolate mofetil. RTX = rituximab. TK = tacrolimus. c = cycles. m = months. w = weeks. y = years.

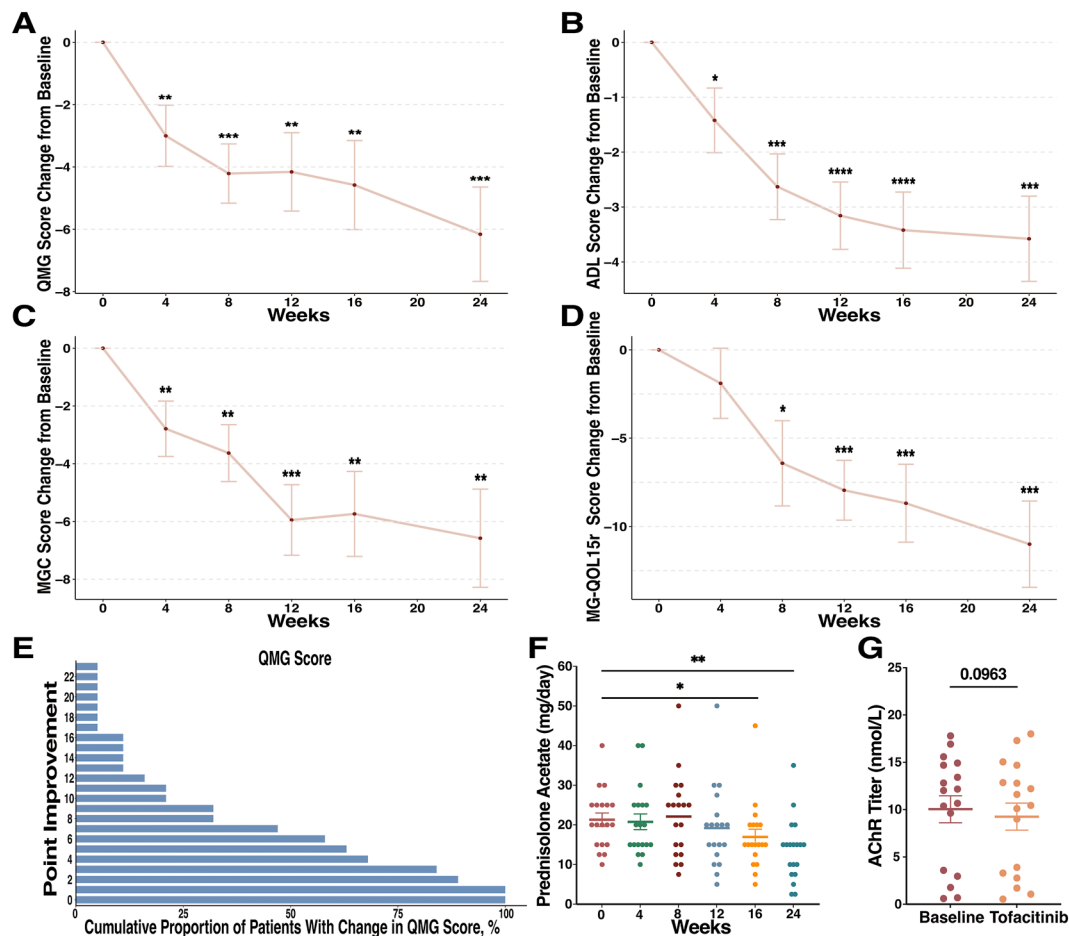


Fig. 3. Tofacitinib improves clinical outcomes and restores glucocorticoid response in refractory MG patients. **A–D** Temporal Profile of Quantitative Myasthenia Gravis (QMG) scores (A), Myasthenia Gravis Activities of Daily Living (MG-ADL) scores (B), Myasthenia Gravis Composite (MGC) scores (C), and MG Quality of Life 15–Revised (QOL15r) scores (D) in refractory MG patients ($n = 19$) with tofacitinib treatment through the 24-week follow-up. **E** Minimum point improvement in QMG score in MG patients **F** Longitudinal assessment of prednisolone acetate dosage in refractory MG Patients ($n = 19$) over 24 weeks with tofacitinib treatment. **G** The level of serum anti-acetylcholine receptor (AChR) antibody in the MG patients ($n = 17$) at baseline and week 16 post-treatment as determined by radioimmunoassay (RSR). Data are presented as mean $\pm$ standard error of the mean (SEM). Statistical analysis was conducted using Student's two-sided paired t -test (A–F) or two-sided Wilcoxon rank-sum test (G). Statistical significance is indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

treatment-naïve MG patients were cultured in vitro under Th17.1-polarizing conditions in the presence of tofacitinib (Fig. 6C). As expected, tofacitinib treatment led to the downregulation of Th17.1 (IL-17+ IFN- γ +) and ex-Th17 (IL-17- IFN- γ -) cell differentiation, but did not significantly affect Th17 (IL-17+ IFN- γ -) cells (Fig. 6D–G). We then ruled out that the inhibition of cell differentiation was caused by the cytotoxicity of tofacitinib (Fig. 6H). In addition, the expression of pathogenic inflammatory cytokines IL-17A and GM-CSF secreted by Th17.1 cells in the supernatants was reduced (Fig. 6I and J). Collectively, these findings demonstrated that tofacitinib effectively suppressed both the differentiation and pro-inflammatory effector functions of Th17.1 cells in MG.

Discussion

While recent therapeutic advances in MG have significantly improved clinical outcomes for treatment-refractory cases, the prohibitive cost creates significant accessibility challenges, driving the need for developing alternative treatment options for refractory MG [23,24]. Here, we demonstrate that the JAK kinase inhibitor tofacitinib rapidly improves clinical symptoms and restores glucocorticoid response in refractory MG. The therapeutic efficacy potentially arises from modulating immune homeostasis in CD4(+) T cells, particularly by suppressing the p-STAT3 level in pathogenic Th17.1 cells, thereby

reducing the secretion of pro-inflammatory cytokines, including IL-6, IL-23, IL-17A, and GM-CSF, while simultaneously upregulating the frequency of Tregs and the production of IL-10. Our study provided preliminary data that tofacitinib ameliorated refractory gMG, probably via the inhibition of p-STAT3 in Th17.1 cells and the modulation of the immune homeostasis. These results may provide a promising treatment strategy for refractory MG.

Understanding the immunopathogenic mechanism of MG is crucial for the development of novel immunotherapies. Here, the scRNA-seq revealed that the JAK2-mediated Th17 cell differentiation-related signaling pathway was enriched in MG. The pathogenic Th17.1 cells that mediate both autoimmune pathogenesis and glucocorticoid resistance have been showed involved in various autoimmune diseases, including MG [25]. Studies have shown CD4(+) T cells exhibit increased Th17.1 cell populations and elevated IL-17 production in MG [26]. JAK2 mediates the differentiation of naïve T cells into Th17.1 cells via the IL-6/JAK2/STAT3 signaling axis [27]. Moreover, it has been shown that both JAK2 inhibitors and pan-JAK kinase inhibitors-tofacitinib can ameliorate EAMG, while the efficacy of JAK kinase inhibitors in MG patients has not been reported [16]. Taken together, these findings suggest that therapeutic targeting of the JAK-STAT pathway may offer a potential treatment for refractory MG by inhibiting the differentiation and function of pathogenic Th17.1 cells [28].

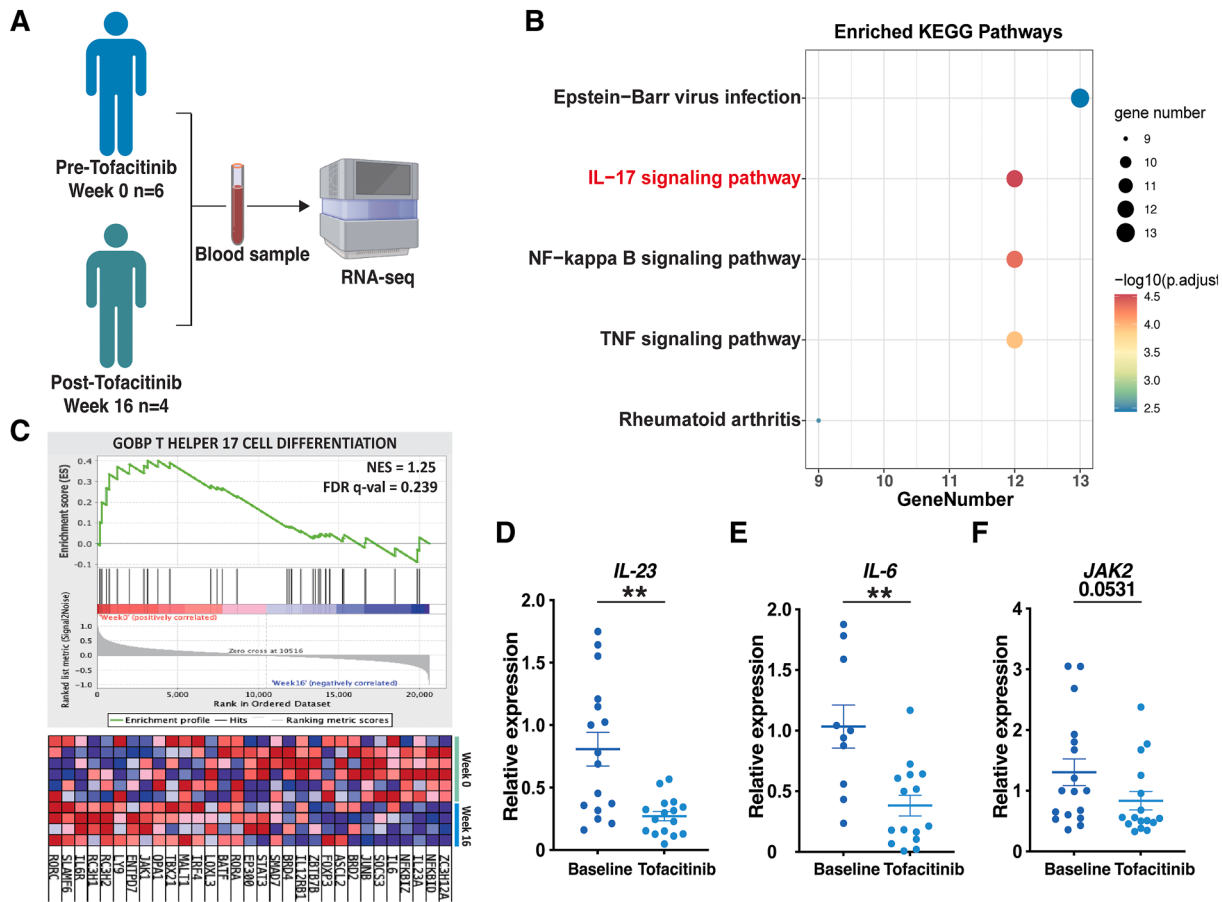


Fig. 4. Tofacitinib suppresses Th17 cell differentiation pathways in MG patients. **A** Bulk RNA sequencing (RNA-seq) was performed on peripheral blood mononuclear cells (PBMCs) from MG patients at baseline ($n = 6$) and week 16 post-tofacitinib treatment ($n = 4$). **B** KEGG pathway enrichment analysis of downregulated genes in PBMCs of MG patients with tofacitinib treatment. **C** Gene Set Enrichment Analysis (GSEA) showing suppression of Th17 cell differentiation pathways in MG patients. **D-F** Key Th17 cell differentiation pathways related genes expression, including IL-23 (D), IL-6 (E), and JAK2 (D) in PBMCs from MG patients ($n = 10-17$ for each group) at baseline and week 12 post tofacitinib treatment were measured using RT-qPCR. Data are presented as mean $\pm$ standard error of the mean (SEM). Statistical analysis was conducted using Student's two-sided unpaired *t*-test (E) or two-sided Wilcoxon rank-sum test (D, F). Statistical significance is indicated by ** $p < 0.01$.

Refractory MG represents a subgroup of patients with insensitivity to the immunosuppressive therapies, uncontrolled symptoms, recurrent and frequent crises, and frequent hospitalizations [29]. These patients exhibit a higher prevalence of female sex, younger at disease onset, and thymoma comorbidity compared to treatment-responsive MG patients [30]. The study enrolled a total of 19 refractory gMG patients who were representative of the broader refractory population, including 4 thymoma-associated MG patients. Refractory status was defined as the lack of treatment response despite adequate therapies with glucocorticoid in addition to either azathioprine for over 6 months or other immunosuppressants for at least 3 months. The results demonstrated that refractory gMG achieved clinical benefit from tofacitinib, as evidenced by reduced prednisolone acetate dose, improved muscle strength, and the quality of life. MSE was achieved in 52.6 % of tofacitinib-treated patients, including two cases with SNMG. The clinical improvements were detectable as early as 4 weeks post-treatment and sustainable through the 24-week follow-up. Compared with conventional immunosuppressants such as azathioprine and mycophenolate mofetil, tofacitinib demonstrated a more rapid improvement in clinical outcomes for MG patients [31]. Given the limited therapeutic options for SNMG patients, we propose that future large-scale clinical studies should evaluate the potential of JAK inhibitors as a viable treatment strategy for these patients [32].

Despite the pathophysiological importance of JAK-STAT signaling in the differentiation of Th17 cells, current knowledge on the mechanism of

JAK inhibitors for MG is primarily based on EAMG studies, with limited clinical validation from studies in MG patients [17]. Notably, these studies have reported that JAK inhibitors ameliorated EAMG by regulating the balance of Th17 and Treg. Although Th17 cells have long been recognized as key drivers of autoimmunity, clinical trial data indicate that IL-17 inhibition alone is insufficient to suppress chronic inflammation or improve clinical outcomes in autoimmune diseases [33]. Under proinflammatory conditions, Th17 cells exhibit phenotypic plasticity by acquiring the ability to produce IFN- γ , IL-17, and GM-CSF, with the Th17.1 subset representing the predominant autoantigen-reactive T cell population in MG [10,34]. Our results indicate that tofacitinib suppresses the IL-17 signaling pathway by downregulating both IL-6 and IL-23. Notably, IL-6 promotes the classical differentiation of Th17 cells and the expression of IL-23 receptor, meanwhile IL-23 promotes Th17.1 cells development through T-bet induction, resulting in characteristic IFN- γ production [35]. Although EAMG studies have been indicated that JAK inhibition improved EAMG mainly by suppressing Th17 cell responses [16,17]. In the present study, tofacitinib had no significant effect on classical Th17 cells, whereas it markedly downregulated IL-6 and IL-23, both critical cytokines for inducing a Th17.1 phenotype. Emerging evidence reveals that IL-23p19 blockade attenuates EAMG progression by suppressing pathogenic Th17 cell expansion [36]. These findings suggest that tofacitinib may ameliorate MG by suppressing the development of pathogenic Th17.1 cells other than classical Th17 cells. Moreover, although tofacitinib demonstrates established inhibitory activity against JAK2, our data revealed only a non-significant reduction in

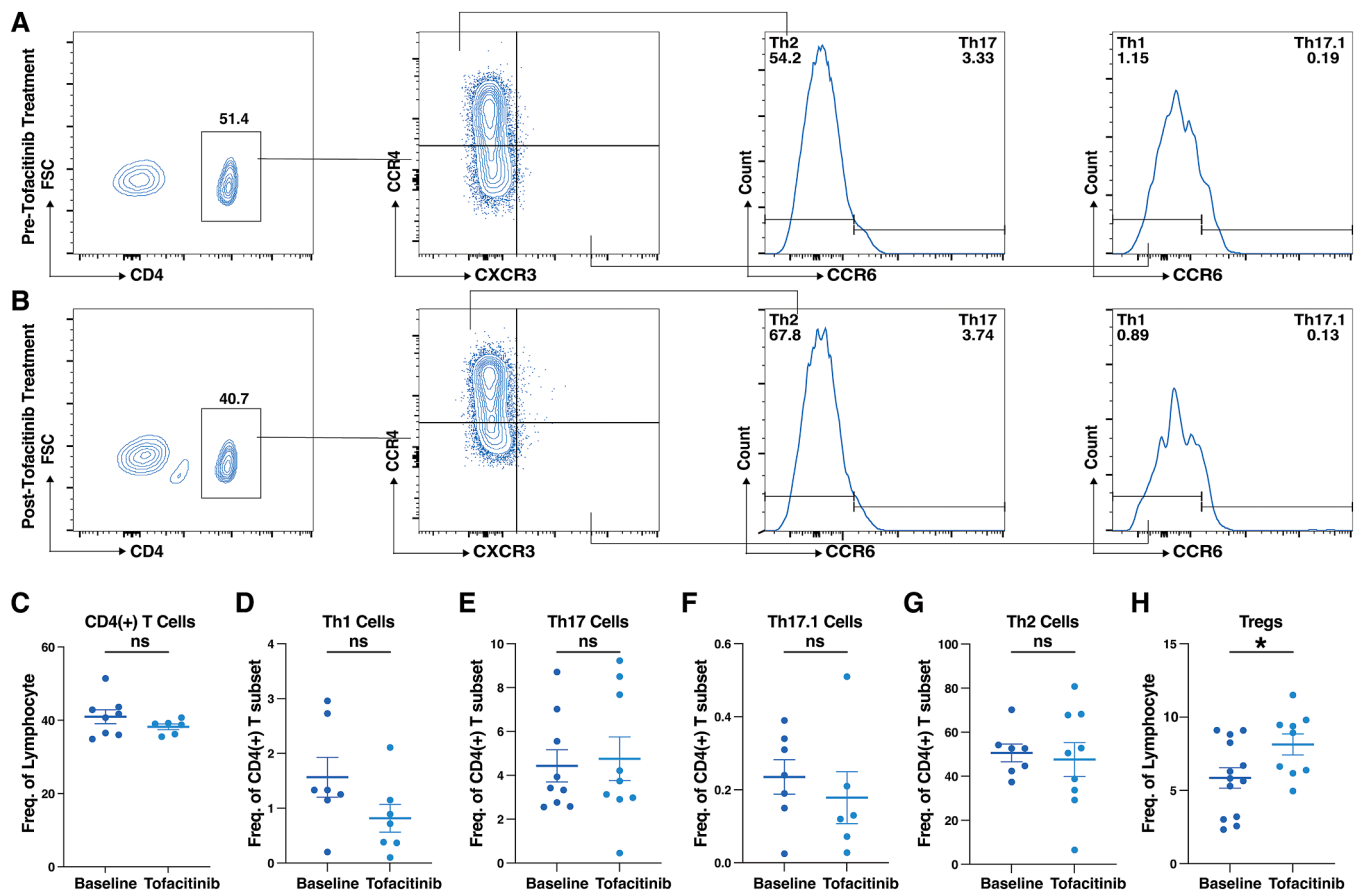


Fig. 5. Tofacitinib alters CD4(+) T helper cell frequencies in MG patients. **A-B** Flow cytometry gating strategy and representative contour plots showing Th cell frequencies in the MG patients at baseline (A) and post-tofacitinib treatment (B). **C-G** Quantitative analysis of T helper cell subset frequencies including CD4(+) T cells (C), Th1 cells (D), Th17 cells (E), Th17.1 cells (F), Th2 cells (G) and Tregs cells (H) in MG patients ($n = 6-13$ for each group) at baseline or 12 weeks after treatment. Data are presented as mean $\pm$ standard error of the mean (SEM). Statistical analysis was conducted using Student's two-sided unpaired t -test (C-H). Statistical significance is indicated by * $p < 0.05$.

JAK2 mRNA expression ($P = 0.0531$) with tofacitinib treatment, which may attributable to the limited statistical power of our small cohort [37].

Tofacitinib has been shown to promote Tregs expansion in EAMG, suggesting its potential immunomodulatory effects [17]. In this study, we demonstrate that tofacitinib modulates the immune homeostasis of MG patients by promoting the expansion of Tregs, while concurrently elevating IL-10 levels. We further investigated the effects of tofacitinib on CD4(+) T cell subsets. The data showed that tofacitinib induced only a marginal, statistically non-significant reduction in total CD4(+) T cells, Th1 cells, and Th17.1 cells. However, cell frequency alone does not fully reflect their pathogenic potential. The transcription factor STAT3 mediates pathogenicity of Th17.1 cells through dual control of IL-23R expression and cellular differentiation, resulting in escape from Treg-mediated immunoregulatory control [38]. Moreover, emerging evidence suggests STAT3 signaling may mediate Th17.1 cell glucocorticoid resistance through upregulation of MDR1/P-glycoprotein expression [39,40]. Our results demonstrate that tofacitinib treatment significantly reduces the phosphorylation levels of STAT3 in bulk CD4⁺ T cells, with the most pronounced suppression observed in Th17.1 cells.

Pathogenic Th17.1 cells produce IL-17A and GM-CSF upon IL-23R engagement, with GM-CSF being particularly critical for the initiation and sustenance of tissue inflammation [41]. GM-CSF is crucial in the progression of MG, as evidenced by our previous findings demonstrating significantly elevated GM-CSF levels in MG patients experiencing crisis

compared to non-crisis cases [21]. The present study demonstrates that tofacitinib attenuates the pathogenic potential of Th17.1 cells by reducing the secretion of IL-17A and GM-CSF. Meanwhile, tofacitinib treatment exhibited a consistent upward trend in Th17 cell frequency, observed both in MG patients and in vitro differentiation assays. Collectively, our findings demonstrate that tofacitinib preferentially downregulates the pathogenic IL-17+IFN- γ + Th17.1 subset, rather than conventional IL-17+IFN- γ - Th17 cells in MG.

This study has several limitations, including its single-center design and relatively small sample size. To further characterize the response to tofacitinib in refractory MG patients, larger, multi-institutional randomized clinical trials with well-defined MG cohorts are required. Despite tofacitinib demonstrated an excellent safety profile in our study, some studies have reported adverse effects of JAK inhibitors, emphasizing the need for long-term follow-up studies to fully characterize tofacitinib's risk-benefit profile in MG management.

Our findings demonstrate that blocking of JAK-STAT3 signaling pathway with tofacitinib improves clinical outcomes and glucocorticoid response in refractory MG, providing a promising, effective, and well-tolerated treatment for patients with refractory gMG. This study elucidates a key mechanism by which tofacitinib restores immune homeostasis in MG, suppressing pathogenic Th17.1 cells via inhibition of p-STAT3, IL-6, and IL-23, while simultaneously promoting the expansion of Tregs and enhancing IL-10 production. Collectively, the data in this

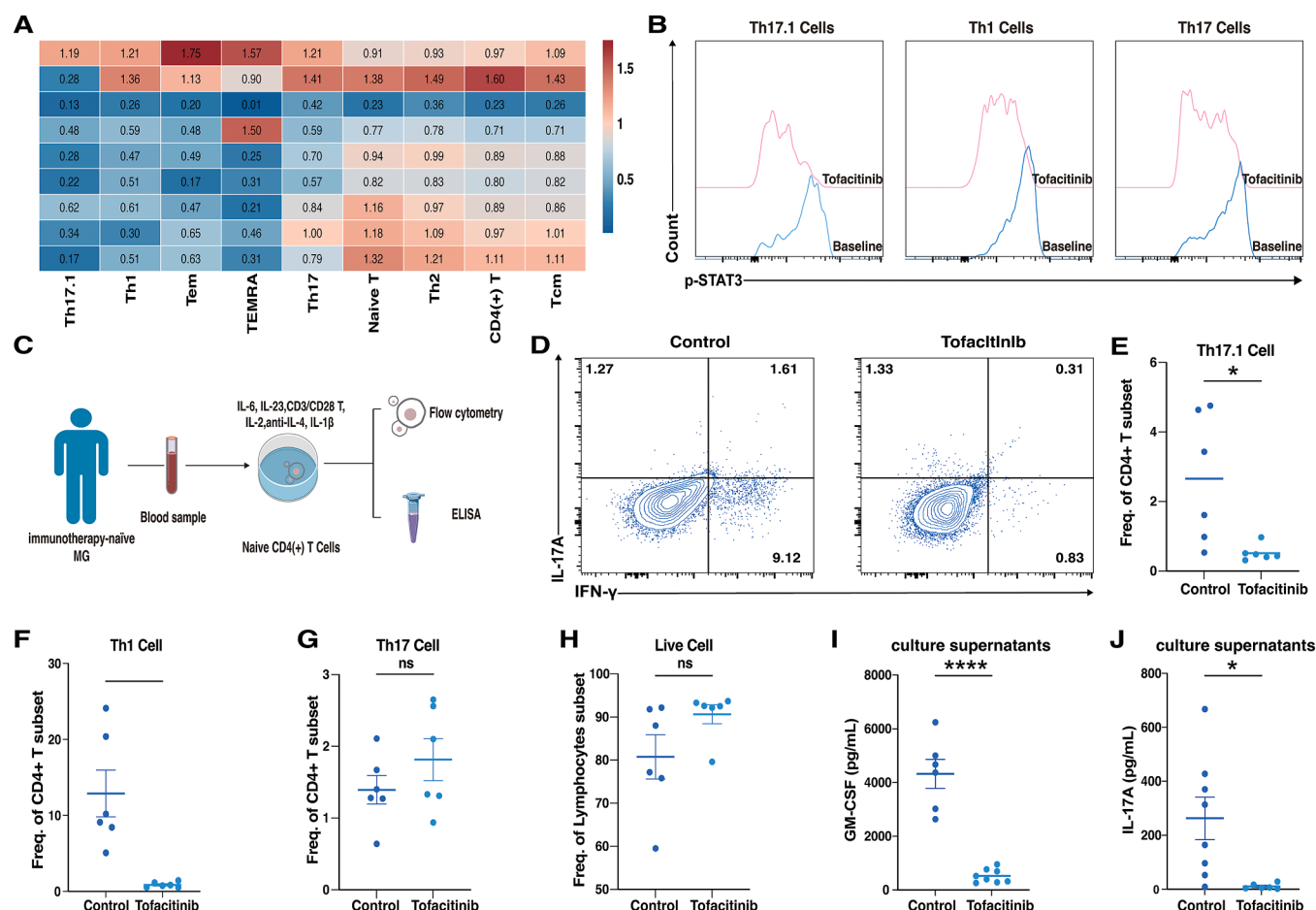


Fig. 6. Tofacitinib inhibits p-STAT3 signaling and pro-inflammatory functions in pathogenic Th17.1 cells. **A** Heatmap depicting tofacitinib suppressing p-STAT3 across CD4⁺ T helper subsets in MG patients ($n = 9$) as determined by phospho-flow cytometry. To quantify relative p-STAT3 activation, we calculated: 1) the stimulation ratio (IL-6-stimulated p-STAT3/unstimulated p-STAT3) at week 12, 2) the corresponding baseline ratio using identical methodology, and 3) the fold-change by dividing the week 12 ratio by the baseline ratio, thereby normalizing all p-STAT3 expression to the pretreatment level. **B** Representative flow cytometry histograms showing the levels of p-STAT3 with IL-6 stimulation in Th17.1, Th1, and Th17 cells at baseline or 12 weeks after treatment. **C** In vitro differentiation of Th17.1 cells was induced using naïve CD4⁺ T cells isolated from the PBMCs of immunotherapy-naïve MG patients. **D** Representative contour plots showing the frequencies of Th17.1, Th1, and Th17 cells during in vitro Th17.1 differentiation, naïve CD4⁺ T cells of treatment-naïve MG patients were treated with tofacitinib or control dimethyl sulfoxide. **E-H** Quantitative analysis of T helper cell subset frequencies, including Th17.1 (E), Th1 (F), Th17 (G), and live cells (H) in vitro Th17.1 differentiation ($n = 6$ for each group). **I-J** The level of supernatants GM-CSF (I) and IL-17A (J) in control and tofacitinib treatment ($n = 6-8$ for each group) in vitro as determined by ELISA. Data are presented as mean $\pm$ standard error of the mean (SEM). Statistical analysis was conducted using Student's two-sided paired *t*-test (D-J). Statistical significance is indicated by * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

study clearly show that position Th17.1 cell-targeted therapy is a promising treatment approach for MG, particularly in glucocorticoid-resistant refractory cases.

Author contributions

R.Z., C.Y., C.B.Z., J.S., and S.S.L. were involved in conception and design of the study. H.H.Z., L.J., and D.X.H. were involved in acquisition of data. J.Y.X., B.G.X., and F.F.L. developed methodology. R.Z., X.H., and Y.R.L. performed the experiments. R.Z., and S.S.L. wrote the manuscript.

Study approval

The Ethics Committee of the Huashan Hospital of Fudan University granted approval for this study (KY2020-019), and all participants provided written informed consent.

Data availability

The data are available from the corresponding author on reasonable request.

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Declaration of Competing Interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.neurot.2026.e00831>.

Abbreviations

AChR	Acetylcholine receptor
EAMG	Experimental autoimmune myasthenia gravis
gMG	generalized MG
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HCS	Healthy controls
IVIG	Intravenous Immunoglobulin
JAK	Janus kinase
MG	Myasthenia gravis
MG-ADL	Myasthenia Gravis Activities of Daily Living
MG-QOL15r	15-item revised version of the Myasthenia Gravis Quality of Life
MSE	Minimal symptom expression
PBMCs	Peripheral blood mononuclear cells
QMG	Quantitative Myasthenia Gravis
RNAseq	Bulk RNA sequencing
scRNA-seq	Single-cell RNA sequencing
SNMG	Seronegative myasthenia gravis
STAT	Signal transducer and activator of transcription

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