Multilevel defects in the hematopoietic niche in essential thrombocythemia

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Supplementary Materials

Supplementary Tables

Table S1. Demographics of patients and healthy donors

	ET (n = 91)	HD (n = 50)
Age (years)	53 (23–79)	34 (14–52)
Female/male	73/18	12/38
Cytogenetics	*JAK2V617F*-positive	-
Treatment	untreated or discontinued	-

ET = essential thrombocythemia; HD = healthy donor

Table S2. Gene-specific primers used in this study

GAPDH	Forward Primer	GGAGCGAGATCCCTCCAAAAT
	Reverse Primer	GGCTGTTGTCATACTTCTCATGG
NES	Forward Primer	CTGCTACCCTTGAGACACCTG
	Reverse Primer	GGGCTCTGATCTCTGCATCTAC
IL-6	Forward primer	ACTCACCTCTTCAGAACGAATTG
	Reverse primer	CCATCTTTGGAAGGTTCAGGTTG
BTG2	Forward primer	CCTGTGGGTGGACCCCTAT
	Reverse primer	GGCCTCCTCGTACAAGACG

FOS	Forward Primer	GGGGCAAGGTGGAACAGTTAT
	Reverse Primer	CCGCTTGGAGTGTATCAGTCA
T-bet	Forward Primer	GTCCAACAATGTGACCCAGAT
	Reverse Primer	ACCTCAACGATATGCAGCCG
GATA3	Forward Primer	GCCCCTCATTAAGCCCAAG
	Reverse Primer	TTGTGGTGGTCTGACAGTTCG
ROR-γt	Forward Primer	GTGGGGACAAGTCGTCTGG
	Reverse Primer	AGTGCTGGCATCGGTTTCG
FOXP3	Forward Primer	GTGGCCCGGATGTGAGAAG
	Reverse Primer	GGAGCCCTTGTCGGATGATG
WDR4	Forward Primer	TAACCGATGACAGTAAGCGTCT
	Reverse Primer	TCTCCTCCGAGGCTATGAAAG
CREB	Forward Primer	ATTCACAGGAGTCAGTGGATAGT
	Reverse Primer	CACCGTTACAGTGGTGATGG
CCND1	Forward Primer	GCTGCGAAGTGGAAACCATC
	Reverse Primer	CCTCCTTCTGCACACATTTGAA
CDKN1C	Forward Primer	CATCCACGATGGAGCGTCTTGTC
	Reverse Primer	TCTGGTCCTCGGCGTTCAGC
WNT9A	Forward Primer	GCATCTGAAGCACAAGTATGAG
	Reverse Primer	CAGAAGCTAGGCGAGTCATC

DAPK1	Forward Primer	ACGTGGATGATTACTACGACACC
	Reverse Primer	TGCTTTTCTCACGGCATTTCT
CEBPA	Forward Primer	GACAAGAACAGCAACGAGTAC
	Reverse Primer	TCATTGTCACTGGTCAGCTC
LEP	Forward Primer	TGCCTTCCAGAAACGTGATCC
	Reverse Primer	CTCTGTGGAGTAGCCTGAAGC
MMP1	Forward Primer	AAAATTACACGCCAGATTTGCC
	Reverse Primer	GGTGTGACATTACTCCAGAGTTG
TLR3	Forward Primer	AATCTGTCTCTGAGTAACAGCC
	Reverse Primer	GGAAAGATCGAGCATAGTGAGA
TLR4	Forward Primer	GACTGGGTAAGGAATGAGCTAG
	Reverse Primer	ACCTTTCGGCTTTTATGGAAAC

Table S3. ELISA kits used in this study

Human IL-6 Quantikine ELISA Kit	D6050 (R&D Systems, Minnneapolis, MN, USA)
Human CXCL12/SDF-1 alpha Quantikine ELISA Kit	DSA00 (R&D Systems, Minnneapolis, MN, USA)
Human SCF Quantikine ELISA Kit	DCK00 (R&D Systems, Minnneapolis, MN, USA)
Norepinephrine ELISA Kit	KA1891 (Abnova, Taiwan, China)

Human IL-1 beta/IL-1F2 Quantikine ELISA Kit	DLB50 (R&D Systems, Minnneapolis, MN, USA)
Human IL-4 Quantikine ELISA Kit	D4050 (R&D Systems, Minnneapolis, MN, USA)
Human CD40 Ligand/TNFSF5 Quantikine ELISA Kit	DCDL40 (R&D Systems, Minnneapolis, MN, USA)
Human Leptin Quantikine ELISA Kit	DLP00 (R&D Systems, Minnneapolis, MN, USA)
Human Thrombopoietin Quantikine ELISA Kit	DTP00B (R&D Systems, Minnneapolis, MN, USA)
Human VEGF Quantikine ELISA Kit	DVE00 (R&D Systems, Minnneapolis, MN, USA)
Human G-CSF Quantikine ELISA Kit	DCS50 (R&D Systems, Minnneapolis, MN, USA)
Human M-CSF Quantikine ELISA Kit	DMC00B (R&D Systems, Minnneapolis, MN, USA)
Human GM-CSF Quantikine ELISA Kit	DGM00 (R&D Systems, Minnneapolis, MN, USA)

Table S4. Antibodies and inhibitors used in this study

Anti-Nestin antibody [10C2]	ab22035 (Abcam, Cambridge, UK)

Anti-CD34 antibody [EP373Y]	(ab81289) (Abcam, Cambridge, UK)
Goat Anti-Mouse IgG H&L (Alexa Fluor® 594)	ab150116 (Abcam, Cambridge, UK)
GAPDH (D4C6R) Mouse mAb	97166 (Cell Signaling Technology, Boston, MA, USA)
Anti-WDR4 antibody [EPR11052]	ab169526 (Abcam, Cambridge, UK)
Goat Anti-Rabbit IgG H&L (HRP)	ab6721 (Abcam, Cambridge, UK)
Rabbit Anti-Mouse IgG H&L (HRP)	ab6728 (Abcam, Cambridge, UK)
Goat Anti-Mouse IgG H&L (Alkaline Phosphatase)	ab97020 (Abcam, Cambridge, UK)
Anti-IL-6 antibody	ab6672 (Abcam, Cambridge, UK)
Anti-AKT3 + AKT2 + AKT1 antibody [Y89]	ab32505 (Abcam, Cambridge, UK)
Anti-AKT3 (phospho S472) + AKT2 (phospho S474) + AKT1 (phospho S473) antibody [EPR18853]	ab192623 (Abcam, Cambridge, UK)
Anti-ERK1 + ERK2 antibody [EPR17526]	ab184699 (Abcam, Cambridge, UK)
Anti-Erk1 (pT202/pY204) + Erk2 (pT185/pY187) antibody [EP197Y]	ab76299 (Abcam, Cambridge, UK)
Anti-GSK3 beta antibody [Y174]	ab32391 (Abcam, Cambridge, UK)

Anti-GSK3 beta (phospho S9) antibody [EPR2286Y]	ab75814 (Abcam, Cambridge, UK)
Anti-CREB antibody [E306]	ab32515 (Abcam, Cambridge, UK)
Anti-CREB (phospho S133) antibody [E113]	ab32096 (Abcam, Cambridge, UK)
Anti-Adrenergic Receptor Antibody, β 3	AB5122 (Merck Millipore, Darmstadt, Germany)
Anti-Tyrosine Hydroxylase antibody	ab112 (Abcam, Cambridge, UK)
Anti-GFAP antibody [2A5]	ab4648 (Abcam, Cambridge, UK)
pan-Akt inhibitor (GDC-0068)	CAS No. : 1001264-89-6 (MedChemExpress, NJ, USA)
ERK1 and ERK2 inhibitor (SCH772984)	CAS No. : 942183-80-4 (MedChemExpress, NJ, USA)
Selective GSK-3 inhibitor (SB216763)	CAS No. : 280744-09-4 (MedChemExpress, NJ, USA)
Ultra-LEAF™ Purified anti-human CD3 Antibody	317326 (BioLegend, San Diego, CA, USA)
Ultra-LEAF™ Purified anti-human CD28 Antibody	302934 (BioLegend, San Diego, CA, USA)
APC anti-human CD69 Antibody	310910 (BioLegend, San Diego, CA, USA)

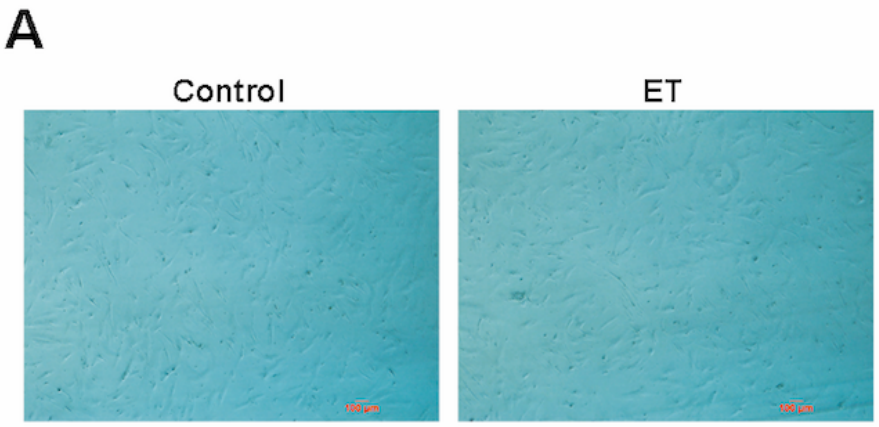
PE anti-human CD25 Antibody	302606 (BioLegend, San Diego, CA, USA)
PE anti-human CD73 (Ecto-5'-nucleotidase) Antibody	344004 (BioLegend, San Diego, CA, USA)
PE anti-human CD105 Antibody	323206 (BioLegend, San Diego, CA, USA)
PE anti-human CD11a Antibody	301208 (BioLegend, San Diego, CA, USA)
PE anti-human CD14 Antibody	367104 (BioLegend, San Diego, CA, USA)
PE anti-human CD19 Antibody	392506 (BioLegend, San Diego, CA, USA)
PE anti-human CD34 Antibody	343606 (BioLegend, San Diego, CA, USA)
PE anti-human IgG Fc Antibody	409304 (BioLegend, San Diego, CA, USA)
FITC anti-human CD90 (Thy1) Antibody	328108 (BioLegend, San Diego, CA, USA)
FITC anti-human CD45 Antibody	368508 (BioLegend, San Diego, CA, USA)

FITC anti-human HLA-DR Antibody	307604 (BioLegend, San Diego, CA, USA)

Supplementary Figures

Figure S1

A



B

Items	*JAK2V617F*$^{+}$ ET (%)	Healthy donors (%)	p
CD73	95.6 ± 0.41	97.19 ± 0.92	> 0.05
CD90	98.00 ± 0.21	98.26 ± 0.33	> 0.05
CD105	85.21 ± 0.37	84.39 ± 0.71	> 0.05
CD11a	-	-	-
CD14	-	-	-
CD19	-	-	-
CD34	-	-	-
CD45	-	-	-
HLA-DR	-	-	-

Figure S1

Immunophenotypes and morphology of mesenchymal stromal cells (MSCs) from healthy donors and patients with *JAK2V617F*-positive ET.

A. Representative morphology of MSCs derived from HDs and patients with *JAK2V617F*-positive ET. There were no obvious differences between the HD-MSCs and ET-MSCs. **B.** Immunophenotypic analysis showed that MSCs from both HDs and *JAK2V617F*-positive ET patients expressed high levels of MSC surface markers (CD73, CD90, and CD105), but did not express hematopoietic surface markers, such as CD11a, CD14, CD19, CD34, CD45, and HLA- DR.

Figure S2

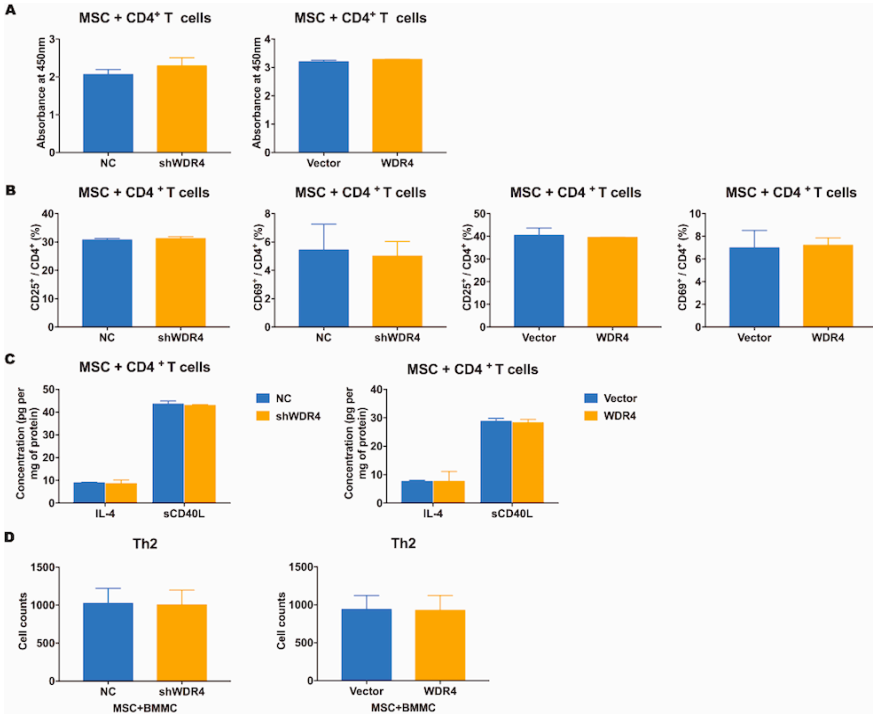


Figure S2

WDR4 in MSCs does not bias the inflammation state of CD4-positive T cells.

A-C. WDR4 in MSCs does not bias the proliferation **(A)**, activation **(B),** secretory phenotype **(C)** of CD4-positive T cells and show no influence on the number of Th2 cells **(D)**.

Figure S3

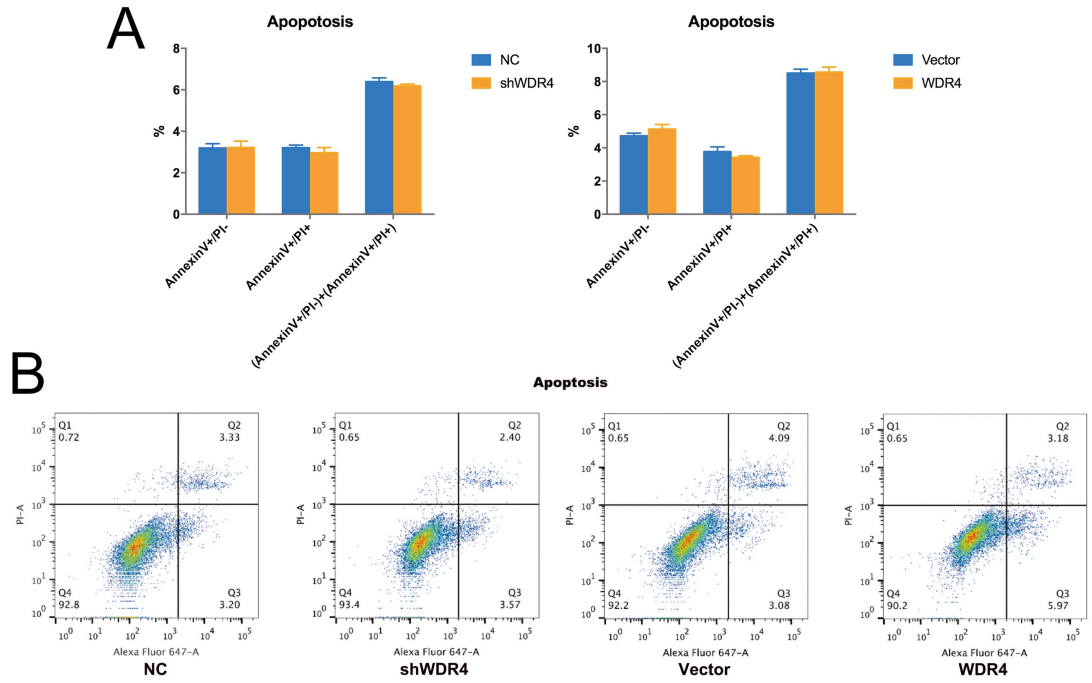


Figure S3

Apoptosis was not influenced by WDR4 in MSCs

A. The level of WDR4 expression had no effect on the apoptosis of MSCs. **B.** Representative micrographs of MSCs transfected with WDR4 complementary DNA (cDNA) or short hairpin RNA (shRNA) and their respective controls.

Supplementary Methods

Patients and samples

Eighty-seven never treated or currently untreated patients with ET and forty-eight healthy donors (HDs) were included in this study with maximum number of specimens in each experiment was forty-two. The patients that had been diagnosed with ET according to the 2016 version of the World Health Organization (WHO) diagnostic criteria for myeloid neoplasms were *JAK2V617F*-positive.[1] The clinical specimens used in the present study were sourced primarily from the pathological cell bank of our hospital. The study was conducted according to the Declaration of Helsinki and approved by our local institutional review board.

Isolation, expansion, and characterization of MSCs

Following isolation by density centrifugation, 2–4 × 10^7 mononuclear cells were cultured in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (Ham) containing 12% fetal bovine serum and 1% penicillin/streptomycin/L-glutamine (Sigma-Aldrich, St Louis, MO, USA) at 37°C and 5% CO_2 in a humidified atmosphere. The medium was changed after 48 h and every 3–4 days thereafter. When the cultures reached 80% confluence, the cells were passaged and frozen for study. To fulfill the criteria of the international society for cellular therapy, and to exclude contamination of MSC cultures by hematopoietic cells, the MSCs were analyzed for the expression of CD73, CD90, CD105, CD11a, CD14, CD19, CD34, CD45, and HLA-DR, by flow cytometry.[2] In addition to the analysis of surface markers, differentiation assays were performed in passage four. We deposited 5 × 10^4

MSCs in 24-well plates, and performed adipogenic, osteogenic, and chondrogenic differentiation using a StemPro™ Adipogenesis Differentiation Kit, a StemPro™ Osteogenesis Differentiation Kit, and a StemPro™ Chondrogenesis Differentiation Kit, respectively (all Invitrogen Gibco, Carlsbad, CA, USA). All images were captured using an Axiovert 25 microscope (Zeiss, Jena, Germany). For the purpose of quantification, the differentiation was graded according to microscopic analysis of the stain index determined by multiplying the score for staining intensity with the score for positive areas. Two members of our experimental team conducted double-blind readings. We used semi-quantitative results to determine the percentage of positive cells and the staining intensity under the microscope. Five high-power fields were observed in each plate, and the staining intensity and percentage of positive cells was counted. The staining intensity was scored as follows: 0 = no dyeing; 1 = weak dyeing; 2 = moderate dyeing; 3 = intense dyeing. The frequency of positive dyeing cells was defined as follows: 0, less than 5%; 1, 5–25%; 2, 26–50%; 3, 51–75%; 4, greater than 75%.[3, 4]

MSC proliferation assay

We deposited 1×10^4 MSCs in 96-well plates. The MSC proliferation assay was performed using Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Rockville, MD, USA), according to the manufacturer's protocol.

Cell cycle analysis of MSCs

For cell cycle analysis, we collected 5×10^4 of the cultured cells and fixed them in 70% ethanol for 30 min. After washing the cells with phosphate-buffered saline (PBS) and treating them with RNase A (Sigma-Aldrich, St. Louis, MO, USA) for 30 min, we incubated the cells with propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) for 15 min, and investigated them using flow cytometry.

Investigation of apoptosis in the MSCs

Apoptosis was determined by detecting phosphatidylserine exposure on the cell plasma membrane. We harvested 5×10^4 cells and washed them in ice-cold PBS, resuspended them in 300ul of binding buffer, and incubated them with 5ul of Annexin V-647 solution for 30 min at 4°C in the dark. This step was followed by further incubation with 5ul of propidium iodide for 5 min, after which the samples were immediately analyzed by flow cytometry.

Investigation of MSC senescence

We deposited 5×10^4 MSCs in 24-well plates, and used a Senescence β-Galactosidase Staining Kit (Cell Signaling Technology, Boston, MA, USA) according to the manufacturer's instructions to evaluate the senescence in the MSCs. The cells were counted using a light microscope, and the fraction of senescent cells (β-galactosidase-positive) was determined.

Transcriptomic and quantitative real-time polymerase chain reaction (qPCR) analysis of the MSCs

Total RNA was isolated from MSCs at passage four using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Following extraction of the total RNA, the eukaryotic mRNA was enriched using Oligo(dT) beads, and the prokaryotic mRNA was enriched by removing rRNA using a Ribo-Zero^{TM} Magnetic Kit (Epicentre, Madison, WI, USA). The enriched mRNA was then separated into short fragments using a fragmentation buffer, and reverse-transcribed into complementary DNA (cDNA) with random primers. Second-strand cDNA molecules were synthesized by DNA polymerase I and RNase H using dNTPs and a buffer. We then purified the cDNA fragments with a QIAquick PCR extraction kit, repaired the ends, added a poly(A) tail, and ligated the molecules to Illumina sequencing adapters. The ligation products were size-selected by agarose gel electrophoresis, amplified by PCR, and sequenced using Illumina HiSeq™ 2500 (Gene Denovo Biotechnology, Guangzhou, China). We carried out gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and gene set enrichment analysis (GSEA) computational assays. To verify the authenticity of the sequencing results, we carried out qPCR with sequence-specific oligonucleotide primers, as described in a previous study.[5]

Isolation of CD34-positive cells

We obtained mononuclear cells by the density gradient method. Subsequently, CD34-positive cells were sorted using magnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany).

Colony-forming unit assay

We co-cultured the MSCs and healthy donor-derived CD34-positive cells for 7 or 14 days. The suspended cells were seeded in methylcellulose medium H4534 (Stem Cell Technologies, Vancouver, Canada), and cultured for 2 weeks. Following co-culture in H3000 medium (StemCell Technologies, Vancouver, Canada), we cultured a suspension of cells on semi-solid medium 04971 (StemCell Technologies, Vancouver) for CFU-MK analysis. Two weeks later, we dehydrated and fixed the cells and sequentially stained the cells according to the manufacturer's instructions. We then counted the clones to evaluate the *in vitro* effect of MSCs on CFU growth.

Isolation of CD4-positive cells

We obtained mononuclear cells from the bone marrow aspirate by the density gradient method, and subsequently sorted the CD4-positive cells using magnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany).

CD4-positive cell proliferation assay

We deposited 1×10^5 CD4-positive cells in 96-well plates. The proliferation assay

was performed using Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Rockville, MD), according to the manufacturer's instructions.

CD4-positive T cell activation

We deposited 1×10^5 CD4-positive cells and stimulated them with soluble anti-CD3 (5 μg/mL) and anti-CD28 (5 μg/mL) in the presence of IL-2 (300 U/mL) in 96-well plates. The CD4-positive T cells were analyzed by flow cytometry using antibodies to CD25 and CD69.

Detection of Th1, Th2, and Th17 cells and regulatory T cells (Tregs)

Bone marrow mononuclear cells (BMMCs) were obtained from HDs and stimulated by standard procedures.[6] Th1, Th2, and Th17 cells were detected using the Human Th1/Th2/Th17 Phenotyping Kit (BD Biosciences, San Jose, CA, USA), and Tregs were detected with the True-Nuclear™ One Step Staining Human Treg Flow™ Kit (Biolegend, San Diego, CA, USA) according to the manufacturer's instructions.

Gene silencing and overexpression

Lentiviruses carrying *WDR4* shRNA or cDNA were purchased from Genechem Co., Ltd. (Shanghai, China). MSC transduction was performed according to the manufacturer's protocols. Briefly, an 80–100% infection rate was achieved.

Phospho-kinase antibody array

Total protein samples were extracted from MSCs and assessed for the phospho-kinases with the Human Phospho-Kinase Array (R&D Systems, Catalog # ARY003B) according to the manufacturer's instructions.

Western blotting

Western blotting was performed using standard procedures. A list of antibodies is provided in *Supplementary Table S4*.

Immunohistochemical and immunofluorescent analyses

Sections (10μm for nerve fibers and 5um for others) were prepared from paraffin-embedded tissue samples and then incubated with antibodies using standard procedures. ImageJ software (NIH, Bethesda, MD, USA) was used to calculate the number of positively stained cells per unit area. A list of antibodies is provided in *Supplementary Table S4*.

Statistical analysis

We analyzed data that conformed to Gaussian distribution using the Student's t-test. Data that did not conform to Gaussian distribution were analyzed using the Mann–Whitney U rank sum test. All data are reported as mean values and SDs or SEMs. We considered data with p-values < 0.05 to be statistically significant. The analysis described above was performed using GraphPad Prism software (version 7.0a).

References

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