RIP1–driven autoinflammation targets IL–1α independently of inflammasomes and RIP3

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The protein-tyrosine phosphatase SHP-1 has critical roles in immune signalling, but how mutations in SHP-1 cause inflammatory disease in humans remains poorly defined. Mice homozygous for the Tyr208Asn amino acid substitution in the carboxy terminus of SHP-1 (referred to as Ptpn6spin mice) spontaneously develop a severe inflammatory syndrome that resembles neutrophilic dermatosis in humans and is characterized by persistent footpad swelling and supplicative inflammation⁶–⁸. Here we report that receptor-interacting protein 1 (RIP1)-regulated interleukin (IL)-1α production by haematopoietic cells critically mediates chronic inflammatory disease in Ptpn6spin mice, whereas inflammasome signalling and IL-1β-mediated events are dispensable. IL-1α was also crucial for exacerbated inflammatory responses and unremitting tissue damage upon footpad microabrasion of Ptpn6spin mice. Notably, pharmacological and genetic blockade of the kinase RIP1 protected against wound-induced inflammation and tissue damage in Ptpn6spin mice, whereas RIP3 deletion failed to do so. Moreover, RIP1-mediated inflammatory cytokine production was attenuated by NF-κB and ERK inhibition. Together, our results indicate that wound-induced tissue damage and chronic inflammation in Ptpn6spin mice are critically dependent on RIP1-mediated IL-1α production, whereas inflammasome signalling and RIP3-mediated necroptosis are dispensable. Thus, we have unravelled a novel inflammatory circuit in which RIP1-mediated IL-1α secretion in response to deregulated SHP-1 activity triggers an inflammatory destructive disease that proceeds independently of inflammasomes and programmed necrosis.

Figure 1 | Ptpn6spin mice develop spontaneous footpad inflammation.

a, b, Spontaneous induction of footpad swelling (a) and lymphomegaly (b) in the popliteal lymph nodes (popLN) of Ptpn6spin mice at 10–16 weeks of age. c–f, Wild-type (WT) and diseased Ptpn6spin mice were harvested at 10–12 weeks of age. c, Numbers (mean ± s.e.m.) of popliteal lymph node cells. d, Immunohistochemistry staining of neutrophils in the popLN of spin mice is characterized by (Fig. 1b). In contrast, lymph nodes that drain non-inflamed areas in Ptpn6WT mice do not display lymphomegaly (Supplementary Fig. 1). Differently Ptpn6spin mice show raised levels of circulating cytokines and chemokines that are associated with granulopoiesis and neutrophil recruitment (Fig. 1c). Complete with augmented production of granulopoietic factors, the inflammatory lesions in the footpads of mutant mice are dominated by neutrophils (Fig. 1d), and neutrophilia ensues in the periphery (Fig. 1e and Supplementary Fig. 2). Moreover, spontaneous disease progression in Ptpn6spin mice is characterized by

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enhanced frequencies of inflammatory T cells that produce high levels of IL-17 and IFN-γ (Fig. 1f and Supplementary Fig. 3) and the accumulation of T cells that exhibit an effector/memory phenotype (CD44hiCD62Llo) (Supplementary Fig. 4). Analysis of mice before the onset of overt disease (4–8 weeks of age) reveals that Ptpn6spin mice possess normal numbers of lymphoid and myeloid cells (Supplementary Fig. 5), and do not display perturbations in T-cell development, regulatory T-cell numbers, or T-cell activation status before disease progression (Supplementary Fig. 6). Furthermore, the Ptpn6spin mutation does not affect inflammatory cytokine production by peripheral T cells and other immune cells in young mutant mice (Supplementary Fig. 7).

Previous work established that IL-1 receptor (IL-1R) signalling is required for Ptpn6spin-mediated inflammatory disease. However, the molecular mechanisms operating upstream of IL-1R engagement that are responsible for spontaneous induction of inflammatory disease are not known. Inflammasome-driven activation of caspase 1 is increasingly recognized as a central instigator of inflammation and disease pathology through its critical role in the production of bioactive IL-1β11. In this context, the NLRP3 inflammasome responds to a multitude of damage-associated danger signals that are associated with autoinflammation2. To test whether aberrant inflammasome activation is responsible for inducing inflammatory disease in response to defective SHP-1 signalling, Ptpn6spin mice were bred to animals that are deficient in the key inflammasome proteins NLRP3 and caspase 1. However, homozygous disruption of neither NLRP3 nor caspase 1 rescued Ptpn6spin mice from footpad inflammation (Fig. 2a and Supplementary Fig. 8) and neutrophil infiltration (Fig. 2b). In full agreement, homozygous deletion of the gene encoding IL-1β also failed to prevent footpad inflammation and granulocyte recruitment in Ptpn6spin mice (Fig. 2a, b and Supplementary Fig. 8), nor was excessive inflammatory cytokine production and cutaneous inflammatory disease rescued by genetic deletion of Tlr4 (Supplementary Fig. 9). In marked contrast, genetic ablation of Il1a provided significant protection from the development of footpad inflammatory disease in Ptpn6 mutant mice (Fig. 2a, b and Supplementary Fig. 8), which was associated with a return to normal neutrophil numbers (Fig. 2b–d), and reduced generation of IL-17-producing CD4+ helper T (Tfh) cells (Fig. 2e). These findings demonstrate that IL-1β has a central role in SHP-1-mediated disease progression, which proceeds independently of inflammasome activation and IL-1β secretion.

Given that IL-1β acts as an alarmin that orchestrates wound-healing responses13–15, we next tested whether defective wound healing might contribute to disease pathogenesis in Ptpn6spin mice. To this end, mice were subjected to microabrasion injury on the plantar surfaces of the hind feet, and monitored for incidence of inflammatory responses. Microabrasion-provoked tissue damage induced similar erythema and oedema in wild-type and Ptpn6spin mice during the first 48 h. However, inflammation at the wound site was fully resolved in wild-type mice by day 14, whereas Ptpn6spin mice developed exacerbated inflammation that was characterized by intense redness and swelling of the affected area (Fig. 3a, b and Supplementary Fig. 10). At day 21, the inability of Ptpn6spin mice to curtail wound inflammation ultimately resulted in the development of a persistent and aggravated state of footpad inflammation characterized by severe cutaneous dermatitis and oedema (Fig. 3a, b and Supplementary Fig. 10). Notably, genetic ablation of IL-1β production in Ptpn6spin mice provided full protection from microabrasion-induced footpad inflammation (Fig. 3a, b and Supplementary Fig. 10). The microabrasion procedure triggered a rapid (4–5 h after wound induction) and potent production of inflammatory cytokines and chemokines in wild-type mice that was further limited Ptpn6spin-mediated disease. a–c, Microabrasion injuries were induced on the plantar surfaces of the footpads of wild-type, Ptpn6spin and Ptpn6spin×Il1a–/– mice. a, Clinical scores based on erythema and oedema as described in detail in the Methods section were recorded daily. b, Percentage of disease-free mice over time. c, Serum levels of granulopoiesis-associated factors 5 h after microabrasion stimulation. d, e, Wild-type (n = 4) and disease-free PTPN6 mutant mice (5–7 weeks old) (n = 5) were immunized with MOG/CFA and pertussis toxin. d, Mean clinical paralysis scores. e, Splenocytes were collected on day 20 and re-stimulated with MOG peptide for 48 h to measure cytokine secretion. f, Wild-type (n = 7), disease-free (4–7 weeks of age) Ptpn6spin (n = 9) and Ptpn6spin×Il1a–/– (n = 5) mice received 250 mg kg–1 of acetaminophen by intraperitoneal injection. The level of serum alanine aminotransferase (sALT) were measured 18–20 h later by ELISA. All bar graphs show mean ± s.e.m. *P<0.05, **P<0.01, ***P<0.001.
exacerbated in Ptpn6<sup>spin</sup> mice (Fig. 3c). Notably, the enhanced secretion of neutrophilic factors in Ptpn6<sup>spin</sup> mice was fully rescued in Ptpn6<sup>spin</sup> mice lacking IL-1α (Fig. 3c). Augmented wound-healing responses in Ptpn6<sup>spin</sup> mice were not the result of global aberrations in inflammation because young Ptpn6<sup>spin</sup> mice did not display abnormalities in immune cell composition or inflammatory cytokine production (Supplementary Figs 5–7). Furthermore, the Ptpn6<sup>spin</sup> mutation did not affect the generation of MOG-specific T cells or neuroinflammation during experimental autoimmune encephalomyelitis (EAE) in young mice (Fig. 3d, e and Supplementary Fig. 11). Commensal bacteria are increasingly recognized for their role in the pathogenesis of autoimmune diseases<sup>16</sup>, and defects in innate immune responses to normal commensal bacteria rather than modifying the bacterial ecology of inflammatory skin lesions. Notably, Ptpn6<sup>spin</sup> mice also were hypersensitive in the caudemphenin (APAP)-induced liver injury model that is considered a model for sterile autoinflammation and wound-healing responses of Ptpn6<sup>spin</sup> mice.<sup>19</sup>

Because inflammatory disease was suppressed when Ptpn6<sup>spin</sup> mice were derived under germ-free conditions<sup>3</sup>, we explored the possibility of footpad-associated dysbiosis in Ptpn6<sup>spin</sup> mice. However, total bacterial counts and composition of the footpad-associated microbiome in microabrasion-induced inflammatory skin lesions were comparable in separately housed wild-type and Ptpn6<sup>spin</sup> mice, respectively (Supplementary Fig. 12). Furthermore, we failed to observe enhanced microabrasion-induced granulopoietic cytokine production in wild-type mice that were co-housed with Ptpn6<sup>spin</sup> mice (Supplementary Fig. 13), indicating that the Ptpn6<sup>spin</sup> mutation alters immune responses to normal commensal bacteria rather than modifying the bacterial ecology of inflammatory skin lesions. Notably, Ptpn6<sup>spin</sup> mice also were hypersensitive in the caudemphenin (APAP)-induced liver injury model that is considered a model for sterile autoinflammation and wound-healing responses of Ptpn6<sup>spin</sup> mice.

To determine whether SHP-1 regulates inflammatory responses in haematopoietic or radioreistant cells, bone marrow chimaera mice were generated. Expression of the hypomorphic Ptpn6<sup>spin</sup> allele in the haematopoietic compartment alone promoted spontaneous footpad inflammation (Fig. 4a) concomitant with augmented cytokine production (Fig. 4b) and neutrophilia (Supplementary Fig. 14). In contrast, chimaeric mice bearing the Ptpn6<sup>spin</sup> mutation only in radioreistant cells failed to develop footpad inflammation (data not shown), suggesting that SHP-1 expression in bone-marrow-derived immune cells rather than non-haematopoietic cells (such as keratinocytes) is critical for induction of the autoinflammatory syndrome. Collectively, these findings suggest that unwarnted IL-1α release in response to dysregulated SHP-1 activity in haematopoietic cells has a pivotal role in the induction of inflammatory disease. To identify the bone-marrow-derived cell populations that are responsible for Ptpn6<sup>spin</sup>-induced inflammation, we investigated inflammatory responses in isolated macrophages and neutrophils as these cell types have been shown to centrally regulate inflammatory and wound-healing responses<sup>19</sup>. The Ptpn6<sup>spin</sup> mutation did not influence inflammatory cytokine production in macrophages (Supplementary Fig. 15). Although Ptpn6<sup>spin</sup> neutrophils produced slightly higher levels of the proinflammatory cytokines granulocyte colony-stimulating factor (G-CSF) and tumour-necrosis factor-α (TNF-α) in response to lipopolysaccharide (LPS) stimulation, production of other pro-inflammatory mediators (KC, IL-6 and IL-1β) was normal in these cells (Supplementary Fig. 16).

We therefore concluded that modest differences in neutrophil-associated cytokine production may contribute to, but are unlikely to account fully for, the marked inflammatory phenotype observed in vivo. The kinase RIP1 is emerging as a key regulator of inflammatory cytokine production and cellular stress<sup>13,21</sup>. To address the in vivo role of RIP1 in exacerbated inflammatory cytokine production, Ptpn6<sup>spin</sup> mice were pre-treated with either vehicle control (PBS), the RIP1 kinase inhibitor necrostatin 1 (Nec1) or the structurally related inactive Nec1 analogue (iNec)<sup>22</sup> before being subjected to microabrasion injury. Unlike iNec, Nec1-mediated in vivo inhibition of RIP1 kinase activity markedly attenuated secretion of inflammatory mediators in Ptpn6<sup>spin</sup> mice to levels comparable to those of wild-type mice (Fig. 4c), suggesting a critical role for RIP1 signalling in Ptpn6<sup>spin</sup>-induced inflammatory disease. RIP1-deficient mice suffer from perinatal lethality<sup>21</sup>, hampering genetic analysis of the role of RIP1 in Ptpn6<sup>spin</sup>-induced autoinflammation. However, the observation that Ptpn6<sup>spin</sup>-mediated autoinflammation stems from the haematopoietic compartment (Fig. 4a, b) provided a rationale to explore the role of RIP1 by means of fetal liver transplantation experiments. To this end, fetal liver cells...
from Ptpn6<sup>Wt</sup>Rip1<sup>+/+</sup>, Ptpn6<sup>Spin</sup>Rip1<sup>+/+</sup> and Ptpn6<sup>Spin</sup>Rip1<sup>−/−</sup> embryos collected at embryonic (E) day E14.5 were transferred into irradiated CD45.1 congenic mice. Reconstitution of recipient mice with control Ptpn6<sup>Spin</sup>Rip1<sup>+/+</sup> fetal liver cells resulted in unremitting footpad swelling, whereas genetic deletion of Rip1 in the haematopoietic compartment provided protection against Ptpn6<sup>Spin</sup>-associated inflammatory disease progression (Fig. 4d and Supplementary Fig. 17) and neutrophilia (Fig. 4e), a hallmark of this inflammatory syndrome. We proposed that targeted MAP kinase and NF-κB signalling drives Ptpn6<sup>Spin</sup>-associated inflammation. In agreement, we found that in vivo RIP1 inhibition markedly dampened local activation of ERK and NF-κB signalling (Fig. 4f). Moreover, pharmacological blockade of NF-κB activation with the IKK-β inhibitor SC-514 and inhibition of ERK signalling with U0126 treatment both abrogated hyperinflammatory cytokine production in Ptpn6<sup>Spin</sup> mice (Supplementary Fig. 18). Importantly, the RIP1 kinase inhibitor Nec1 also inhibited the synthesis of Il1α transcripts (Fig. 4g), further highlighting the role of RIP1 as a critical regulator of NF-κB-induced and IL-1β-driven autoinflammation in Ptpn6<sup>Spin</sup> mice. Notably, IL-1β deletion also attenuated exacerbated ERK and NF-κB signalling in the footpads of Ptpn6<sup>Spin</sup> mice (Fig. 4f), suggesting that RIP1-mediated IL-1β production triggers an inflammatory feedback loop that contributes to disease progression. In addition to driving MAP kinase and NF-κB activation, RIP1 controls induction of necroptosis in conjunction with RIP3 (ref. 20). To verify a potential role for unwarranted necroptosis induction in Ptpn6<sup>Spin</sup>-associated inflammatory disease, Rip3-deficient mice were bred to Ptpn6<sup>Spin</sup> mice. However, unlike deletion of Rip1 and Il1α, genetic ablation of Rip3 expression failed to protect Ptpn6<sup>Spin</sup> mice from exacerbated inflammation in response to microabrasion-induced tissue injury (Fig. 4h and Supplementary Fig. 19). These results indicate that RIP3-mediated necroptosis is dispensable, and suggest a critical role for RIP1-mediated regulation of MAP kinase and NF-κB signalling in driving the inflammatory phenotype of Ptpn6<sup>Spin</sup> mice.

Defective neutrophil homeostasis is associated with numerous devastating human diseases<sup>21</sup>. For instance, neutropenia can cause severe susceptibility to infection, whereas neutrophilia is linked to autoinflammatory disorders. Our results in the Ptpn6<sup>Spin</sup> mice further support an important role for RIP1 in inflammatory and autoinflammatory diseases. We have previously reported that RIP1 is required for neutrophil development, splenocytes and popliteal lymph node cells were re-stimulated with PMA/ionomycin followed by intracellular flow cytometry staining. Formalin-preserved cells and neutrophils was assessed in a blinded manner by a pathologist using discrimination between necrotic and apoptotic cell death by the ability to induce sterile inflammation and any associated references are available in the online version of the paper.

**METHODS SUMMARY**

**Ptpn6<sup>Spin</sup>** mice homozygous for the Tyr208Asn amino acid substitution in the C-terminal Src homology 2 domain of SHP-1 have been described previously<sup>1</sup>. Ptpn6<sup>Spin</sup> mice spontaneously develop a persistent footpad disease that is characterized by paw swelling and cutaneous inflammation at 8–16 weeks of age. Blood was collected by submandibular venipuncture to measure the levels of circulating neutrophils and inflammatory cytokines. To assess T-cell-mediated cytokine production, splenocytes and popliteal lymph node cells were re-stimulated with PMA/Ionomycin followed by intracellular flow cytometry staining. Formalin-preserved cells and neutrophils was assessed in a blinded manner by a pathologist using discrimination between necrotic and apoptotic cell death by the ability to induce sterile inflammation.

**MATERIALS AND METHODS**

**Ptpn6<sup>Spin</sup>** mice (wild-type, Rip1<sup>−/−</sup> and Ptpn6<sup>Spin</sup>Rip1<sup>−/−</sup>) were generated by crossing Ptpn6<sup>Spin</sup> with Rip1<sup>−/−</sup> mice. Fetal liver cells collected at embryonic day E14.5 were transplanted into lethally irradiated wild-type mice to generate Ptpn6<sup>Spin</sup>Rip1<sup>−/−</sup> wild-type chimaeric mice.

**Full Methods** and any associated references are available in the online version of the paper.

**Received 16 June 2012; accepted 9 April 2013.**

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**Acknowledgements** We thank B. A. Buetler, V. M. Dixit and D. R. Green for supplying mice.

**Author Contributions** J.R.L., M.L. and T.-D.K. designed the study; J.R.L. performed the experiments and G.R.L. provided technical assistance; J.R.L., M.L. and T.-D.K. analysed data and wrote the manuscript; M.A.K. and Y.I. provided genetic tools; P.V. performed the histopathology analysis; T.-D.K. oversaw the project.

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METHODS

Mice. Ptpn6<sup>−/−</sup> (ref. 3), Nlrp3<sup>−/−</sup> (ref. 24), Casp1<sup>−/−</sup> (ref. 24), Il1b<sup>−/−</sup> (ref. 25), Il1a<sup>−/−</sup> (ref. 26), Rip1<sup>−/−</sup> (ref. 27) and Rip1<sup>−/−</sup> (ref. 21) mice were previously described. All mice were housed under specific pathogen-free conditions within the Animal Resource Center at St Jude Children’s Research Hospital. Animal studies were conducted under protocols approved by the Institutional Animal Care and Use Committee of St Jude Children’s Research Hospital.

Histopathology. Formalin-preserved feet were processed and embedded in paraffin according to standard procedures. Sections (5 μm) were stained with haematoxylin and eosin (H&E) and examined by a pathologist blinded to the experimental groups. For immunohistochemistry, formalin-fixed paraffin-embedded tissues were cut into 4 μm sections and slides were stained with anti-Gr-1 to stain neutrophils in the footpads.

Microabrasion injury model. A novel microabrasion wound model was developed to evaluate the inflammatory response in a synchronized and controlled fashion. In this model, wild-type and disease-free Ptpn6<sup>−/−</sup> mice (4–8 weeks old) were anaesthetized and the plantar surfaces of the hind paws were irritated by gently rubbing with sterile sandpaper to induce physical trauma and microinjuries. Clinical scores were assigned daily based on the following scale: 0, no disease; 1, erythema; 2, erythema and mild swelling; 3, erythema, swelling and crusty wound formation; 4, weepy wound formation and severe swelling. The development of persistent footpad swelling was used to evaluate disease incidence over time. The levels of proinflammatory mediators that are produced in response to microabrasion injury were measured in the serum 4–5 h after wound induction. For the in vivo necrotostin 1 experiments, mice were given either 50 μg necrotostin 1 (Nec1) (Sigma-Aldrich) or 50 μg of an inactive control analogue (iNec, Calbiochem) by the intraperitoneal route 1 h before microabrasion irritation of the footpads. Circulating cytokine levels were measured in the serum 4–5 h later.

Experimental autoimmune encephalomyelitis (EAE). Age- (5–8 weeks) and sex-matched mice were immunized subcutaneously with 100 μg MOG<sub>35–55</sub> peptide (MEGVWYRSPFSRVVHLYRNGK) emulsified in CFA (Difco Laboratories) to develop disease-free EAE.

Bone marrow chimaeras. Bone marrow was flushed from the femurs and filtered through a 40 μm filter. 3–5 × 10<sup>6</sup> cells in 200 μl PBS were transferred by tail vein injection into lethally irradiated (1,000 rad) mice. Congenic CD45 markers were used to verify chimaerism.

In vitro macrophage stimulation. Bone-marrow-derived macrophages (BMDMs) were generated by culturing bone marrow cells in L-cell-conditioned IMDM medium supplemented with 10% FBS, 1% non-essential amino acid, and 1% penicillin-streptomycin for 5 days. BMDMs were seeded in 12-well cell culture plates and cultured overnight. To evaluate cytokine production, BMDMs were primed with 2 μM <sup>1</sup>ultrapure Escherichia coli-derived LPS (Invivogen) for 4 h followed by 5 mM ATP (Sigma–Aldrich) for an additional 30 min. BMDMs were also separately stimulated with Salmonella enterica serovar Typhimurium (5 MOI) for 4 h and supernatants were collected to evaluate cytokine secretion by ELISA.

Neutrophil culture and in vitro stimulation. Bone marrow cells were isolated from the femurs of mice and neutrophils (CD11b<sup>+</sup> Gr-1<sup>+</sup>) were purified by flow cytometry sorting. Neutrophils (1 × 10<sup>6</sup> cells ml<sup>−1</sup>) were stimulated with 100 ng ml<sup>−1</sup> <sup>1</sup>ultrapure Escherichia coli-derived LPS (Invivogen). Supernatants were collected after 48 h of stimulation and cytokine levels were measured by ELISA.

Western blotting. Footpad protein lysates were collected in RIPA lysis buffer supplemented with complete protease inhibitor cocktail (Roche) and PhosSTOP (Roche) using a tissue homogenizer. Samples were resolved by SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes via electroblotting. Membranes were blocked in 5% non-fat milk and incubated overnight at 4 °C with primary antibodies. The membranes were then probed with horseradish peroxidase (HRP)-tagged secondary antibodies at room temperature for 1 h. Immunoreactive proteins were visualized using the ECL method (Pierce).

Real-time RT–PCR. Total RNA was isolated from the hind paws with Trizol (Invitrogen) according to the manufacturer’s instructions. 1 μg of RNA was reverse-transcribed to cDNA with random RNA-specific primers using the high-capacity cDNA reverse transcription kit (Applied Biosystems). Transcript levels of Il1a and Gulp1 were analysed using SYBR-Green (Applied Biosystems) on an ABI7500 real-time PCR machine according to the manufacturer’s recommendations. Relative expression was calculated using the ΔΔCt standardization method.

Footpad pathology scoring. Footpad haematoxylin and eosin sections were scored based on the extent and severity of inflammation, ulceration and hyperplasia of the mucosa in a blinded fashion by a veterinary pathologist. Severity scores for inflammation were as follows: 0, normal (within normal limits); 1, minimal (small, focal, or widely separated); 2, mild; 3, moderate (moderate multifocal inflammation with dermititis, suppurative, coalescing with intraepithelial and follicular abscesses); 4, marked (marked inflammation, with intraepidermal pustules, epidermal hyperplasia, acantholysis, dermatitis, perifolliculitis); 5, severe (severe inflammation, with intraepidermal pustules, epidermal hyperplasia, acantholysis, dermatitis, perifolliculitis, lesions covering >50% of the section).

APAP-induced hepatotoxicity model. Acetaminophen (Sigma–Aldrich) was dissolved in sterile PBS by heating the solution to 55 °C. Mice that fasted overnight for 18–20 h received 250 mg kg<sup>−1</sup> of acetaminophen (APAP) by intraperitoneal injection. Mice were harvested 18–20 h after injection and the levels of serum alanine aminotransferase (ALT) were measured in the blood by ELISA.

Statistical analysis. All results are presented as means ± standard errors. We performed statistical analysis using the two-tailed Student’s t-test. Differences were considered statistically significant when P < 0.05. P values are denoted by *P < 0.05, **P < 0.01, ***P < 0.001.

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