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Interleukin-10 does not contribute to the anti-contractile nature of PVAT in health

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Abstract

Perivascular adipose tissue (PVAT) is protective and reduces contraction of blood vessels in health. PVAT is composed of adipocytes, multiple types of immune cells and stromal cells. Interleukin (IL)-10, an anti-inflammatory cytokine usually produced by T cells, B cells and macrophages, was identified as one of the highly expressed (mRNA) cytokines in the mesenteric PVAT of healthy rats. One report suggested that exogenous IL-10 causes relaxation of mouse mesenteric arteries, also suggesting that IL-10 maybe a potential anti-contractile factor. Hence, we hypothesized that PVAT-derived IL-10 causes vasorelaxation and/or reduces vasoconstriction, thus contributing to the anti-contractile nature of PVAT in health. Mesenteric arteries from rats and mice expressed the receptor for IL-10 (in tunica intima and media) as determined by immunohistochemistry. Mesenteric resistance arteries for rats and superior mesenteric artery for mice were used for isometric contractility studies. Increasing concentrations [0.4– 100 ng/mL] of recombinant rat/ mouse (rr/ mr) IL-10 or vehicle was directly added to half-maximally constricted (phenylephrine, PE) vessels (without PVAT, with endothelium). IL-10 did not cause a direct vasorelaxation. Further, the ability of rrIL-10 to cause a rightward or downward shift of a vasoconstriction-response curve was tested in the rat. The vessels were incubated with rrIL-10 [100 ng/mL or 10 ng/mL] or vehicle for 1.5 hours in the tissue bath followed by a cumulative PE [10^{-8}–10^{-4} M] or U46619 [10^{-10}–10^{-5} M] response curve. The maximal contractions and EC_{50} values were similar in IL-10 incubated vessels *vs* vehicle. Thus, acute exposure of exogenous IL-10 did not reduce local vasoconstriction. To further test if endogenous IL-10 from PVAT was anti-contractile, superior mesenteric arteries from IL-10 WT and KO mice, with and without PVAT, were subjected to increasing concentrations of PE. The anti-contractile nature of PVAT was preserved with both

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9.0 Author Contributions:

RK: conceptualized and performed experiments, designed the studies, analyzed and interpreted the data, wrote the manuscript; **LK:** performed experiments, edited the manuscript; **CER:** provided resources, edited the manuscript; **SWW:** conceptualized, guided the study design and data interpretation, provided funding and resources, and edited the manuscript. All the authors proofread the final version of the manuscript.

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None.

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

short-term and prolonged depletion (using younger and older mice, respectively) of endogenous IL-10 in males and females. Contrary to our hypothesis, PVAT-derived IL-10 neither caused vasorelaxation nor reduced local vasoconstriction directly/ indirectly. Therefore, IL-10 does not contribute to the anti-contractile nature of PVAT in healthy rodents.

Keywords

IL-10; vascular tone; vasorelaxation; anti-contractile; perivascular adipose tissue

1.0 Introduction

In health, the presence of PVAT inhibits agonist-induced vasoconstriction in vessels from humans and rodents [1,2]. This is called the classic ‘anti-contractile’ nature of PVAT, experimentally observed as a rightward or downward shift in an agonist contractile response curve. PVAT also directly promotes vasorelaxation [2,3], which would reduce blood pressure in healthy subjects.

The molecules responsible for the anti-contractile/ vasorelaxing functions of PVAT in health may include adiponectin, nitric oxide, and angiotensin (1–7). These factors have been thought of as primarily derived from PVAT adipocytes [4]. But in addition to adipocytes and stromal cells, multiple groups including ours have discovered that a community of innate and adaptive immune cells co-exist in PVATs [5, 6, 7,8]. These immune cells may either directly release factors that are anti-contractile or promote other cells in PVAT (*e.g.* adipocytes) to release vasorelaxants. Recent work supports eosinophils as critical to the anti-contractile actions of PVAT in health [10]. Unlike the considerable literature which supports a pathological function of immune cells in cardiovascular disease [11], this paper raised the provocative idea that under healthy conditions, the immune cells in PVAT may actively contribute to the anti-contractile nature of PVAT.

Macrophages and T cells are the dominant immune cells in PVATs in health [5]. They are the major producers of the anti-inflammatory master regulatory cytokine interleukin (IL)-10 [12]. IL-10 was reported to be a direct vasorelaxant in the presence of endothelium in mesenteric arteries of wildtype (WT) male mice [13]. This led us to hypothesize that PVAT-derived IL-10 causes vasorelaxation and/ or reduces local vasoconstriction, thus contributing to the anti-contractile nature of PVAT in healthy rodents. The term ‘healthy’ was used as no purposeful disease induction was invoked. Experiments were designed to test: (1) the contribution of IL-10 to the anti-contractile nature of PVAT in two species: rats and mice; (2) exogenous *vs* endogenous (or direct *vs* indirect effects) IL-10 to cause vasorelaxation/ reduce vasoconstriction and (3) if the anti-contractile nature of PVAT was lost with short-term or prolonged depletion of IL-10 in males and females. Both 13 week-age young and 26 week-age older IL-10 KO/ WT mice were used because prolonged genetic depletion of IL-10 (26 week-age older KO) leads to activation of systemic inflammation in KO mice [14] and the vascular effects of depleting IL-10 might take longer to appear without disease induction. Contrary to our hypothesis, we discovered that PVAT-derived IL-10 neither

caused vasorelaxation nor reduced vasoconstriction in healthy rodents. These studies support that IL-10 is not involved in the anti-contractile nature of PVAT in health.

2.0 Materials and Methods

2.1 Animals

Animal maintenance and experimental protocols were approved by the Michigan State University Institutional Animal Care and Use Committee, and compiled with the National Institutes of Health Guide for Animal Care and Use of Laboratory Animals (2011). All the animals were maintained on a 12/12 light/dark cycle at 22–25 °C, fed on *ad-libitum.*

Rats: Male Sprague Dawley rats (SD; 8–12 weeks of age, Charles River, IN, USA; RRID: RGD_10395233) were used. Prior to all dissections, the rats were anesthetized with sodium pentobarbital (60–80 mg/kg, i.p.) and death was assured by creating a bilateral pneumothorax.

Mice: Male and female IL-10 knockout KO mice on C57BL/6 background (13 weeks of age and 26 weeks of age; RRID: IMSR_JAX:002251) and age-matched male and female C57BL/6 wildtype WT mice (~13 weeks old and ~26 weeks old; RRID: IMSR_JAX:000664) were obtained from Jackson Laboratories, ME, USA. Mice were euthanized in compliance with American Veterinary Medical Association recommendations. IL-10 KO mice were validated as described in section 2.7.4 (data in Supplementary figure S1).

2.2 Tissue handling and dissection

Third order mesenteric arteries from rats and superior mesenteric artery from mice were isolated and, where appropriate, cleaned of PVAT in a silastic-impregnated dish filled with physiological salt solution [PSS; in mM; 130 NaCl; 4.7 KCl; 1.17 $MgSO_4 \cdot 7H_2O$; 1.18 K_2HPO_4; 14.8 $NaHCO_3$; 5.5 dextrose; 0.03 $CaNa_2$ ethylenediametetraacetic acid; 1.6 $CaCl_2$ (pH 7.2)]. In other instances, PVAT was dissected from vessels and retroperitoneal (RP) fat was cleaned off of small blood vessels and used for qPCR.

2.3 RNA isolation and RT-qPCR

MRPVAT (PVAT around the mesenteric resistance vessels) and RP (retroperitoneal) fat from male WT SD rats, and SMPVAT (PVAT around the superior mesenteric vessels) and RP fat from male WT mice were homogenized in 2 mL tubes (Omni, Kennesaw, GA) with 1.4 mm ceramic bead media (cat #19–645, Omni) using the Omni Bead Ruptor. RNA was isolated with the Zymo Quick-RNA Mini Prep Kit (Cat # R1054, Zymo Research, CA, USA), then quantified on a NanoDrop 2000c (Thermo Scientific).

Rat: cDNA was reverse transcribed using the RT^2 First Strand Kit (cat# 330404, Qiagen, MD, USA). RT-qPCR was performed on a QuantStudio 7 Flex Real-Time PCR system using RT^2 SYBR Green ROX Master Mix (Cat #330521, Qiagen). RT2 profiler PCR array kit (#PARN-053ZE-4, Qiagen) was used to measure IL-10 and housekeeping gene beta-2-microglobulin, according to manufacturer's protocol.

Mouse: cDNA was reverse transcribed using the High Capacity cDNA kit (cat# 4368814, Thermo Fisher Scientific). RT-qPCR was performed on a QuantStudio 7 Flex Real-Time PCR system using Fast SYBR Green Master Mix (Cat # 4385612, Thermo Fisher Scientific). All primer sequences were retrieved from Primer Depot (http://primerdepot.nci.nih.gov/) and synthesized by Integrated DNA Technologies. The primer sequences are: IL-10 forward primer: 5'-ACCAGCTGGACAACATACTGC-3'; IL-10 reverse primer: 3'-ATTTCTGGGCCATGCTTCTCT-5' and RPL13A (ribosomal housekeeping gene) forward primer: 5'-GTTGATGCCTTCACAGCGTA-3', RPL13A reverse primer: 5'-AGATGGCGGAGGTGCAG-3'.

qPCR for both the rat and mouse samples was performed with the following parameters: 95 °C for 20 s; 95 °C for 1 s and 60 °C for 20 s for 40 cycles, followed by a melt curve to determine the presence of a single PCR product. Relative IL-10 expression for the rat/mouse was normalized to the respective housekeeping gene and the data were analyzed with the 2^{-dC_t} method in which C_t is the threshold cycle.

2.4 Immunohistochemistry

All tissue sections were formalin-fixed and paraffin embedded. Mesenteric vessels with MRPVAT from male WT SD rats, superior mesenteric vessels with SMPVAT from male WT mouse and male WT mouse spleen were embedded and sectioned (8 μm thick) by Michigan State University's Investigative Histopathology Lab. WT male SD rat spleen sections (5 μm thick) were purchased from Zyagen (San Diego, CA, USA). The rat/ mouse spleens were used as biological positive controls for IL-10RA. Using standard immunohistochemistry protocols, slides were de-waxed and antigens unmasked. All the slides were then blocked with a species-specific blocking serum (1.5% goat serum in PBS; Vector Laboratories, CA, USA) for 60 minutes at room temperature. IL-10 RA primary antibody (1:100, RRID:AB_2847938, Abcam) was used in both the rat and the mouse (it has proven reactivity to both). After overnight incubation at 4°C with primary antibody in blocking buffer or blocking buffer alone (for negative control), all the sections were exposed to anti-rabbit secondary antibody (1:1000, #PK6101; Vector Laboratories, CA, USA) for 45 minutes. ImmPACT NovaRED HRP substrate kit (#SK-4805) for rat slides and DAB with HRP substrate kit (#SK-4100) for mouse slldes from Vector Laboratories (Burlingame, CA) were used. Hematoxylin (#H2404, Vector Laboratories, CA, USA) was used as a nuclear stain. Nikon Eclipse TE2000 inverted microscope (Nikon, Otowara, Japan) was used to capture bright field images using the 100X objective. Images were captured using a Nikon Digital Sight DS-Qil camera and Nikon NIS Elements BR 3.0 software. Adjustments in brightness and contrast for all the images were applied uniformly across each image. Images representative of five animals were selected for the figures.

2.5 Isometric contraction

Third order mesenteric arteries from rats and superior mesenteric artery from mice, cleaned of fat (−PVAT) or with fat intact (+PVAT) and endothelium intact, were mounted into a multi wire myograph system 620 (Danish Myo Technology, Denmark). Data were acquired using a PowerLab Data Acquisitions unit (ADInstruments, CO, USA). Baths contained warmed, oxygenated PSS. Rings were pulled to optimum resting tension (13.3 kPa) and equilibrated

for 30 minutes with washes every 10 min. The vessels were exposed to an initial concentration of 10 μM phenylephrine (PE), an α−1 adrenergic agonist, to test viability and to elicit maximum contraction. Tissues were washed and returned to baseline. Presence of the endothelium was verified with a tissue relaxation caused with addition of 1 μM acetylcholine (ACh) after inducing a half-maximal PE-induced contraction. The tissues were washed and returned to baseline. It was ensured that all the vessels (from both rats and mice) that were exposed to exogenous IL-10 had demonstrated at least 80% relaxation with 1μM ACh. All the cumulative response curves were performed with sufficient time necessary for a response to plateau prior to addition of the next concentration. However, if no change in response was recorded within 4 minutes, the next concentration was applied. Tissues were taken through one of the protocols below.

Protocol I: Direct relaxation caused by IL-10—Male SD rat mesenteric resistance arteries without PVAT and 13 week-old male WT mouse superior mesenteric artery without PVAT were used. The arteries were half-maximally constricted with PE and then mouse (mr)/ rat (rr) recombinant IL-10 (0.4 ng/mL to 100 ng/ mL; mr: #575802 Biolegend, CA, USA; rr: #522-RLB R&D Systems, MN, USA) or their respective vehicle controls (mouse: 20 mM Tris and 150 mM NaCl pH 7.5 in PBS; rat: PBS+0.2% BSA) were applied in a cumulative fashion. Same volume of vehicle as mr/ rrIL-10 was added to the vessels from the respective species.

Protocol II: Ability of IL-10 to shift a contractile curve—Rat mesenteric resistance arteries with and without PVAT were used. Either rrIL-10 (10 ng/ mL or 100 ng/ mL) or vehicle (PBS+ 0.2% BSA; same volume as rrIL-10) was added for 90 minutes without washing, following which a cumulative PE (10^{-8} to 10^{-4} M) or U46619 a thromboxane A_2 agonist (10^{-10} to 10^{-5} M; #16450 Cayman Chemical Company, MI, USA) response curve was performed. PE and U46619 curves were performed on different rats.

Protocol III: Vascular effects with genetic loss of IL-10—Two ages of IL-10 KO and WT mice were used: 13 week and 26 week-old males and females. The superior mesenteric artery with or without PVAT were subjected to a cumulative PE (10^{-8} to 10^{-4} M) concentration response curve.

2.6 Cytokine assays (IFN-γ and IL-10) and ELISA

2.6.1 Splenocyte preparation—

The spleens isolated from rats/ mice were mechanically disrupted by a syringe plunger and filtered through a 40 μm filter in complete Dulbecco's Modified Eagle Medium (cDMEM) supplemented with 10% fetal bovine serum (Biowest LLC, MO, USA), 25 mM HEPES, 50 μM 2-mercaptoethanol, nonessential amino acids (1X final concentration from 100X stock solution), 100 U/ml penicillin, and 100 μg/ml streptomycin (cDMEM). The single cell suspension obtained was then washed with cDMEM and then, red blood cell lysis was performed by adding ammonium-chloride-potassium lysis buffer and incubating for 2 minutes on ice. The splenocytes were further washed twice with cDMEM and counted using a hemocytometer.

2.6.2 IFN-γ ELISA in rat splenocytes—Wells of a 96 well flat-bottom plate were pre-coated with anti-CD3 antibody in PBS (1: 200; #14003085, eBiosciences, CA, USA) or PBS alone and incubated at 4 °C overnight. The wells were emptied and 200,000 splenocytes per well were added, with and without 100 ng/mL rat recombinant IL-10. Simultaneously, the wells that received anti-CD3 antibody also received anti-CD28 antibody (1:100; #200902, Biolegend, CA, USA). After 72 hours, the supernatants were collected and rat IFN-γ ELISA (#BMS621, Thermo Fisher Scientific, CA, USA) was performed according to the manufacturer's protocol. This is the standard assay to evaluate the activity of IL-10 [15].

2.6.3 IFNγ ELISA in mouse splenocytes—Wells of a 96 well flat-bottom plate received 1 million splenocytes per well. The cells received either purified hamster anti-mouse CD3 (1.5 μg/ mL; #14003386 eBiosciences, CA, USA), purified hamster anti-mouse CD28 (1.5 μg/ mL; #14028186, eBiosciences, CA, USA) and an $F(ab')_2$ fragment specific for anti-Syrian hamster IgG crosslinker (#107006142, Jackson ImmunoResearch Laboratories, PA, USA) or nothing, with and without mouse recombinant IL-10 (100 ng/ mL). After 72 hours, the supernatants were collected and mouse IFNγ ELISA (#430801; Biolegend, CA, USA) was performed as per manufacturer's protocol.

2.6.4 IL-10 ELISA in mouse splenocytes—One million murine splenocytes at a concentration of 2×10^6 cells/mL were seeded out in a 48 well flat bottom plate. The cells were activated using cell activation cocktail phorbol myristate acetate (PMA; 40 nM)/ ionomycin (Io; 0.5 μM) (#423301; Biolegend, CA, USA) or vehicle (Dimethyl Sulfoxide, #D2650; Sigma Aldrich, MO, USA) for 48 hours. It was necessary to activate the splenocytes with PMA/Io because basal level of IL-10 protein is challenging to be detected with the commercially available ELISA kits. Subsequently, IL-10 concentration in the supernatants was assessed using ELISA (#431411; Biolegend, CA, USA).

2.7 Data Presentation and Statistics

Data are reported as mean ± SEM for the number of animals N. Statistical analysis was performed with GraphPad Prism 8.0 (GraphPad Software Inc., La Jolla CA; RRID: SCR_002798). mRNA measures were reported as 2^{-dCt} and unpaired t-test was performed. For all ELISAs, one-way ANOVA with Tukey's multiple comparison's test was performed. Contraction was reported as a percentage of the initial contraction to 10 μM PE and relaxation was reported as a percentage of half-maximal PE-induced contraction. Two-way ANOVA with posthoc Tukey test was performed for isometric contractility studies. For all the data presented, a $P < 0.05$ was considered statistically significant. Potencies ($-\log EC_{50}$) and maximal effect were calculated using GraphPad Prism 8.0. When a response didn't plateau, ($-\log EC_{50}$) and maximal effect values automatically estimated by GraphPad Prism was used.

3.0 Results

3.1 IL-10 mRNA was present in PVATs of male SD rats (MRPVAT) and WT mice (SMPVAT)

IL-10 mRNA was present in MRPVAT in male SD rats, in 10 times greater quantity *vs* RP fat, a non-PVAT white fat (Fig 1A). IL-10 mRNA was also present in SMPVAT and RP fat in male WT mice (Fig 1B).

3.2 The receptor for IL-10 was present in mesenteric resistance arteries (male rats) and superior mesenteric artery (male wildtype mice)

For IL-10 to be potentially vasoactive, the receptor for IL-10, IL-10RA, needs to be present in the mesenteric arteries. IL-10 RA (pointed by green arrows) was expressed in tunica intima and tunica media of mesenteric resistance arteries, the red blood cells and MRPVAT in male rats (Figure 2C, D) and in the tunica intima and tunica media of superior mesenteric artery and SMPVAT in male WT mice (Figure 2G, H). Spleen from rats (Fig 2A, B) and mice (Fig 2E, F) served as biological positive controls.

3.3 Acute exogenous IL-10 did not cause direct vasorelaxation in both wildtype rats and mice.

Cytokines have been used in the range of 10 to 100 ng/ mL concentrations in vascular contractility studies with rat aorta (IL-8, IL-2) and in *ex vivo* studies with adipose tissue (IL-10) [16,17]. Hence, 10 ng/ mL and 100 ng/ mL of rr/ mr IL-10 or 0.4 to 100 ng/ mL for IL-10 cumulative concentration response curves were chosen for the current study.

Both rrIL-10 and mrIL-10 (used in respective species) reduced IFN-γ secreted by cultured splenocytes activated for 72 hours by anti-CD3/anti-CD28; this reduction was statistically significant in the mouse but not in the rat (Figure 3A, D). Hence, the rr and mr IL-10 proteins used in the current study were biologically active molecules at the concentrations used in contractility experiments. Vehicle controls or rr/mr IL-10 was added to half-maximally constricted arteries (constricted with PE) in a cumulative concentration-dependent manner to the respective species. Representative tracings demonstrated the inability of rr/ mr IL-10 to relax PE-induced half-maximal contraction (Figure 3B, E). Tracings from multiple experiments were then quantified and are shown in Figure 3C and F.

3.4 Acute exogenous IL-10 did not directly reduce vasoconstriction in mesenteric resistance arteries of wildtype rats.

Third order mesenteric resistance arteries were incubated with rrIL-10 (10 ng/mL or 100 ng/mL) or vehicle to test if rrIL-10 caused a rightward shift of an agonist response curve. But neither the PE concentration response curve (Figure 4A, B) nor the U46619 concentration response curve (Figure 4C, D) shifted rightward in the presence of either low (10 ng/mL) or high (100 ng/mL) concentrations of IL-10. The potencies ($-\log EC_{50}$ [M]) and the maximal contractions of the curves were similar in the presence of IL-10 and vehicle (Table 1).

Together, exogenous acute exposure of IL-10 neither caused a direct vasorelaxation nor was anti-contractile. We further tested if endogenous IL-10 derived from PVAT was anti-

contractile. IL-10 KO mice were used to assess if the anti-contractile nature of PVAT was lost with short-term or prolonged depletion (using younger and older mice, respectively) of endogenous IL-10.

3.5 PVAT still remained anti-contractile with short-term genetic depletion of endogenous IL-10 in mice.

Stimulation of cultured splenocytes with a pan-immune activator such as PMA/Io for 48 hours increased IL-10 secretion in both male and female WT mice but not in the IL-10 KO mice. Thus, IL-10 KO mice were validated using an IL-10 ELISA. (Supplementary Figure S1). In isolated mouse superior mesenteric artery, the anti-contractile nature of PVAT was present in both WT and IL-10 KO mice at 13 weeks of age (younger mice) in males (Figure 5A) and females (Figure 5B). The rightward/downward shift in the contractile response curve in arteries with PVAT was not lost even with IL-10 depletion (Table 2).

3.6 The anti-contractile nature of PVAT was still intact with prolonged genetic depletion of endogenous IL-10 in mice.

The anti-contractile function of PVAT was intact (Table 3) in response to PE-induced vasoconstriction in both sexes of WT mice at 26 weeks of age (older mice), as expected. However, surprisingly, the anti-contractile nature of PVAT was also still evident in both males (Figure 6A) and females (Figure 6B) of IL-10 KO mice at 26 weeks of age. However, there was a leftward shift in the KO *vs* WT PE-response curves in females only, in the absence of PVAT (indicated by # in Table 3).

There were no differences in EC_{50} values of PE-induced contraction and maximal PE contractions between 13 and 26 weeks-old mice (−PVAT/ +PVAT, male/ female, WT/KO; comparing Tables 2 and 3).

4.0 Discussion

The current study tested the hypothesis that PVAT-derived IL-10 causes a direct vasorelaxation and/ or reduces vasoconstriction, thus contributing to the anti-contractile function of PVAT in health. New and Novel: This is the first study to report testing of the idea that PVAT-derived IL-10 may contribute to the anti-contractile nature of PVAT in health. This study has evaluated: (1) direct *vs* indirect effects of IL-10 on vascular tone regulation; (2) the anti-contractile nature of PVAT with short-term *vs* prolonged depletion of endogenous IL-10 in both sexes; and (3) vasoactive potential of IL-10 in two rodent species.

IL-10 as a direct vasorelaxant in health: a conundrum

Our results revealed that IL-10 neither caused direct vasorelaxation nor reduced vasoconstriction in arteries from healthy rats and mice (Figures 3–6). This is in agreement with the finding that preincubation with 20 ng/mL of IL-10 did not affect the ability of rat cremaster artery to constrict to PE [18]. But our finding is in disagreement with another published study [13] that reported IL-10 to cause a direct vasorelaxation in mesenteric arteries (−PVAT, with endothelium) of 8 week-old male WT mice. These suggested the possibility of a species difference in the vasoactive potential of IL-10. Thus, we performed

experiments using both the rat and the mouse in the current study. However, one reason for the controversy could be that while Kassan *et. al.* showed that 60 ng of IL-10 could directly vasorelax, the exact concentration used was not reported, thus making a direct comparison difficult. We used no higher than 100 ng/ mL because physiological range of endogenous IL-10 protein in serum of healthy human adults has been reported to be only 13 pg/mL [19]. In addition, the source and activity of the recombinant IL-10 protein were not reported in the Kassan 2011 paper.

The pleiotropic effects of IL-10 in health

IL-10 KO mice exhibit chronic systemic inflammation, colitis and frailty, all of which are associated with increased sensitivity to adrenergic agonists [20]. Studies suggest that IL-10 KO mice have enhanced sensitivity to β-adrenergic signaling that is responsible for enhanced thermogenic gene induction *vs* WT [17]. In the current study, there was a leftward shift in the PE curve in tissue without PVAT from 26 week females (as determined by EC_{50} values in Table 3) *vs* respective WT, suggesting that the arteries of IL-10 KO mice in females only became more sensitive to the α1-adrenergic agonist, PE, with age. IL-10 KO females exhibited stronger immune responses elicited to infections *vs* IL-10 KO males [21]. Hence, although these sex-differences in the current study were unexpected, other sex differences in IL-10 KO mice are well known [21].

Along with the existing literature and our finding, IL-10 receptor signal on red blood cells was unexpected and surprising (Figure 2C). We could not find any evidence for or against IL-10R expression on red blood cells. It maybe be a non-specific signal such as the antibody binding to hemoglobin in the red blood cells. Previous findings have identified IL-10RA in mature adipocytes of inguinal white adipose tissue in mice [17]. This aligns with our finding that IL-10RA was present not just in the mesenteric arteries but also in adipocytes in PVAT in both mice and rats (Figure 2). This also suggests that IL-10 is likely not indirectly triggering release of a substance from PVAT that could cause vasorelaxation. Bone marrow derived IL-10 acts on adipocytes *via* IL-10RA to limit thermogenesis and confer resistance to obesity [17]. IL-10 may also be involved in maintaining PVAT-immune homeostasis and insulin sensitivity in health. Future studies are needed to better understand the biological purpose of IL-10 in PVATs in health.

Could IL-10 be vasoprotective in disease?

The current study supports that the anti-contractile nature of PVAT does not depend on short-term or prolonged absence of endogenous IL-10 (Figure 5, 6). One possibility may be that physiological compensation occurred when IL-10 was knocked out, such that the anti-contractile nature of PVAT was maintained even when systemic inflammation occurred in IL-10 KO mice. However, the use of exogenous IL-10 in the current study helped rule out physiological compensation and supported that IL-10 is not a vasorelaxant or an anticontractile factor. However, future *in vivo* IL-10 infusion studies in the IL-10 KO mice would be helpful.

The only known receptor for IL-10 is IL-10R. IL-10 homodimer binds to IL-10 RA, activates JAK/STAT and Akt cascades to exhibit immunoregulatory functions [22]. In mice,

IL-10 limits multiple pathologies such as angiotensin-II induced hypertension, diabetes, endothelin-1-induced vascular injury. In these pathologies, IL-10 either rescues or aids endothelium-dependent vasorelaxation. It does so by increasing eNOS expression/ activity or inhibiting NADPH oxidase activity by p38 MAPK-dependent mechanism [23, 24, 25, 13]. Hence, care was taken to preserve the endothelium in vessels that were exposed exogenous IL-10 in the present study. Additionally, treatment with the anti-inflammatory cytokine IL-10 impaired production of pro-inflammatory cytokines such as TNF-α, IL1-β and IL-6 [26]. These together suggest that IL-10 may act like brakes to the immune system such that the vascular functions of IL-10 are visible and better appreciable when the immune system is actively responding to an insult *vs* in a healthy state.

6.0 Limitations

We acknowledge several limitations in the current study. First, the protein level of endogenous IL-10 in PVATs in health was not measured as its levels are below the detection limits of any reliable commercially available kit. However, studies have reported obese human white adipose tissue a source of IL-10 protein [27]. Thus, adipose tissue is capable of synthesizing IL-10 protein. Second, *in vivo* infusion of exogenous IL-10 or disease induction was not done, because the intent of this study was to understand if the biologically available IL-10 in PVATs was vasoactive (anti-contractile) in health. Third, the vasoactive potential of endogenous IL-10 in rat could not be evaluated due to lack of availability of an IL-10 KO rat. Fourth, different arteries for mice (superior mesenteric) and rats (mesenteric resistance) were used. This was purposeful and ensured confidence that IL-10 was not vasoactive, using two different types of blood vessels (superior mesenteric artery=surrounded by a mix of white and brown PVATs; mesenteric resistance artery=surrounded by white PVAT) across two species. However, our findings cannot be directly extended beyond the mesenteric arteries, as the structure, composition, function and the surrounding PVAT vary profusely between the different blood vessels along the vascular tree. Finally, IL-10 receptor antagonists could not be used in the rats due to lack of availability of reliable antagonists.

5.0 Conclusion

IL-10 (mRNA) was present in the mesenteric PVAT and its receptor IL-10RA was present in the mesenteric arteries of both mice and rats. However, in both species, IL-10 did not cause a direct vasorelaxation and IL-10 in PVAT also did not cause a reduction in vasoconstriction directly or indirectly. Hence, IL-10 does not contribute to the anti-contractile nature of PVAT in health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. IL-10 (mRNA) was expressed in mesenteric PVATs of rats and mice in health.
2. IL-10 receptor was present in the mesenteric arteries of both rats and mice.
3. Exogenous IL-10 did not directly vasorelax mesenteric arteries.
4. IL-10 in PVAT did not reduce vasoconstriction directly or indirectly.
5. IL-10 does not contribute to the anti-contractile nature of PVAT in health.

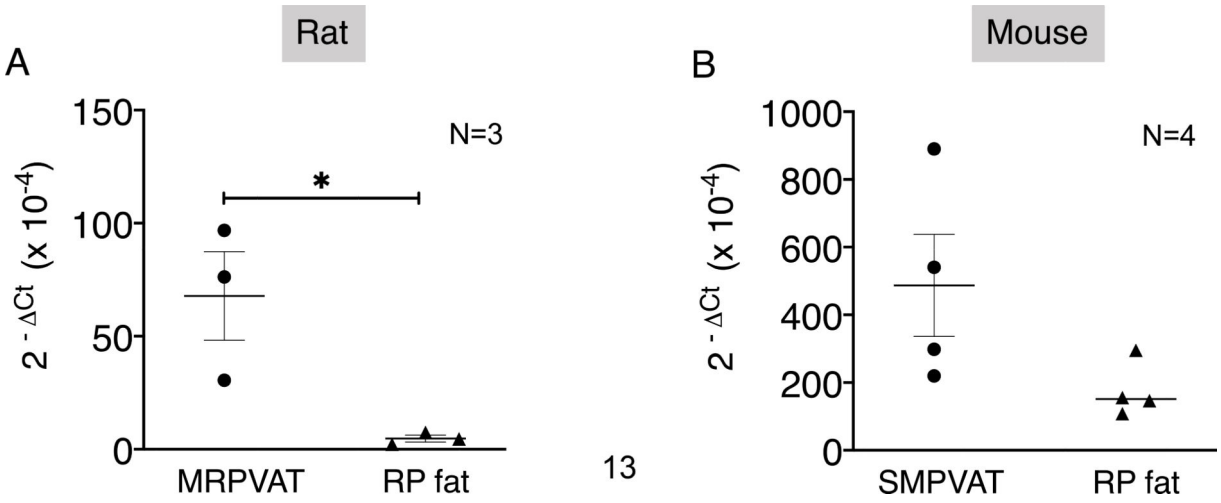

Figure 1. IL-10 mRNA was present in male wildtype rats (MRPVAT) and mice (SMPVAT).

IL-10 mRNA quantified by real time-qPCR in MRPVAT and RP fat from male SD rats (A) and in SMPVAT and RP fat from male WT mice (B). Reference gene: beta-2-microglobulin for the rat and RPL13a for the mouse. Data were normalized to the respective reference genes. Each dot represents an animal and means±SEM is plotted in the graphs. * represents $p< 0.05$ by an unpaired student t-test for the number of animals indicated by N. Mean C_t ±SEM for reference genes: rat MRPVAT= 17.6±0.2; rat RP fat= 16.8±0.3; mouse SMPVAT= 32.4±0.4; mouse RP fat= 24±0.4.

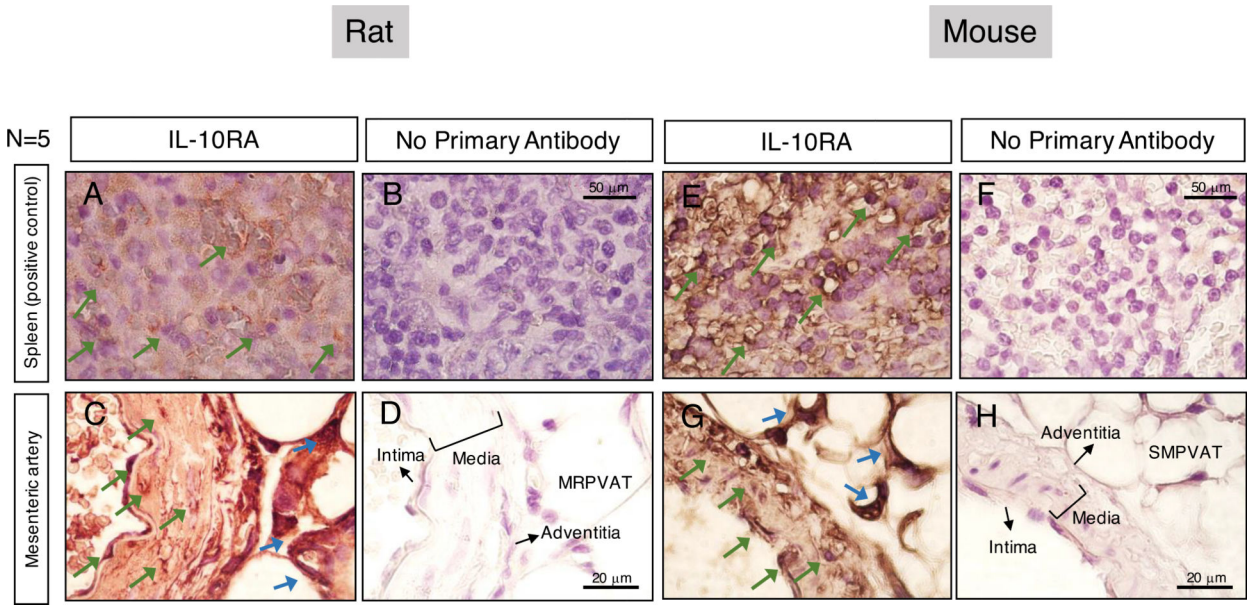

Figure 2. IL-10 receptor was present in male rat mesenteric and male mouse superior mesenteric arteries.

Brightfield images (100X objective) using a primary antibody against IL-10RA (pointed with green arrows) on the spleen (A, B) and mesenteric arteries (green arrows) + MRPVAT (blue arrows) (C, D) from male WT SD rats; on the spleen (green arrows) (E, F) and superior mesenteric artery (green arrows) + SMPVAT (blue arrows) from male WT mice (G, H). Images are representative of 5 animals (indicated by N). Spleen: biological positive control; No primary antibody: technical negative control; Tunica intima, media, adventitia and MRPVAT (D) or SMPVAT (H) are marked in tissues without primary antibody for reference.

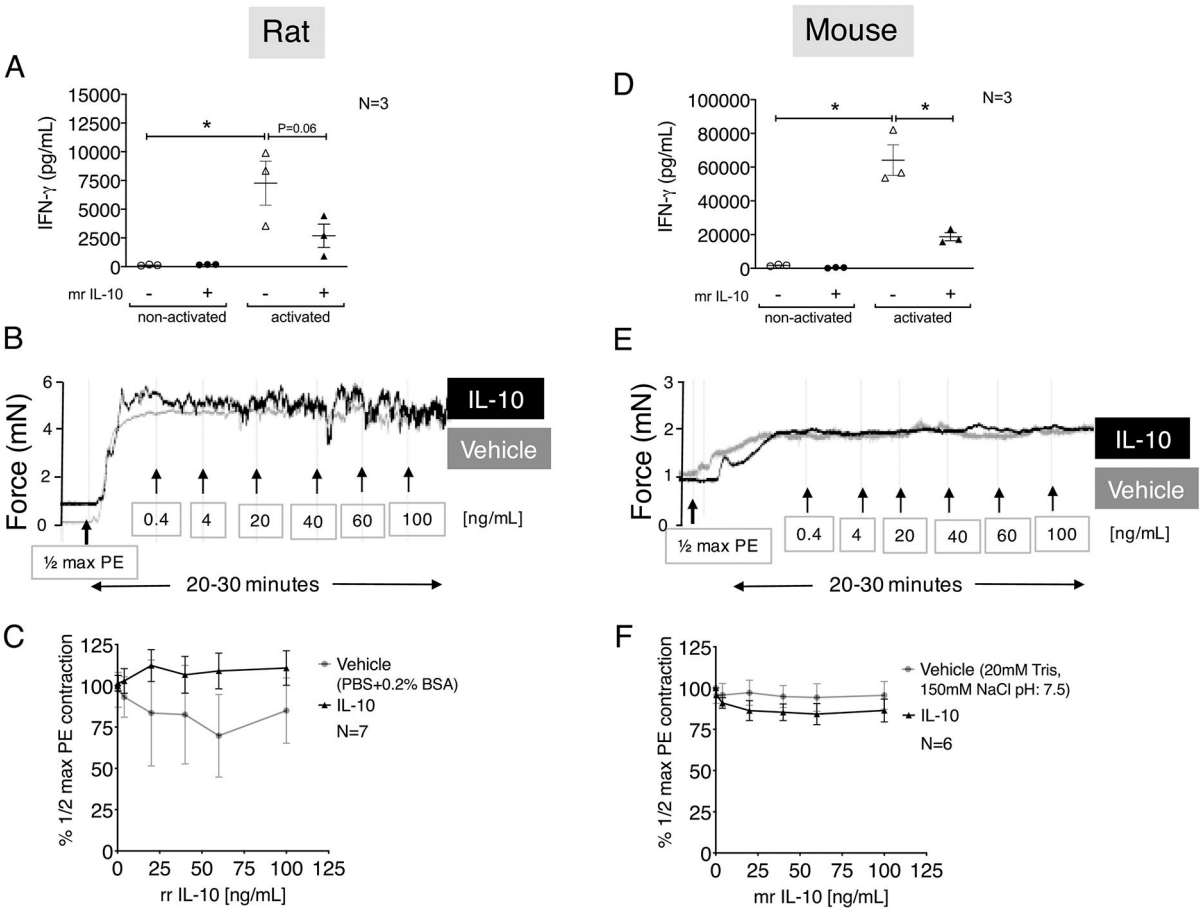

Figure 3. Acute exposure to exogenous IL-10 did not cause direct vasorelaxation in mesenteric arteries from both wildtype male rats and mice.

IFN-γ measures by ELISA with/ without activation, with/ without mr/ rr IL-10 (100 ng/ mL; added to respective species) using splenocytes isolated from male SD rats (A) and male WT mice (D). Representative tracings of half maximally constricted (with PE) rat third-order mesenteric resistance arteries (no PVAT, with endothelium; B) and mouse superior mesenteric artery (no PVAT, with endothelium; E) subject to a cumulative rr/ mr IL-10 (0.4 ng/ mL to 100 ng/ mL; added to respective species) concentration response curve and their quantification (C, F). * represents $p<0.05$ by one-way ANOVA with Tukey's multiple comparisons test. Points represent means±SEM and the response to IL-10 is normalized to the respective half-maximal PE-induced contraction. Half-maximal PE-induced contraction (mN): Vehicle (rat)= 5.8±0.7; rrIL-10= 5.1±0.5; Vehicle (mouse)= 1±0.08; mrIL-10= 1.8±0.3. N= number of animals.

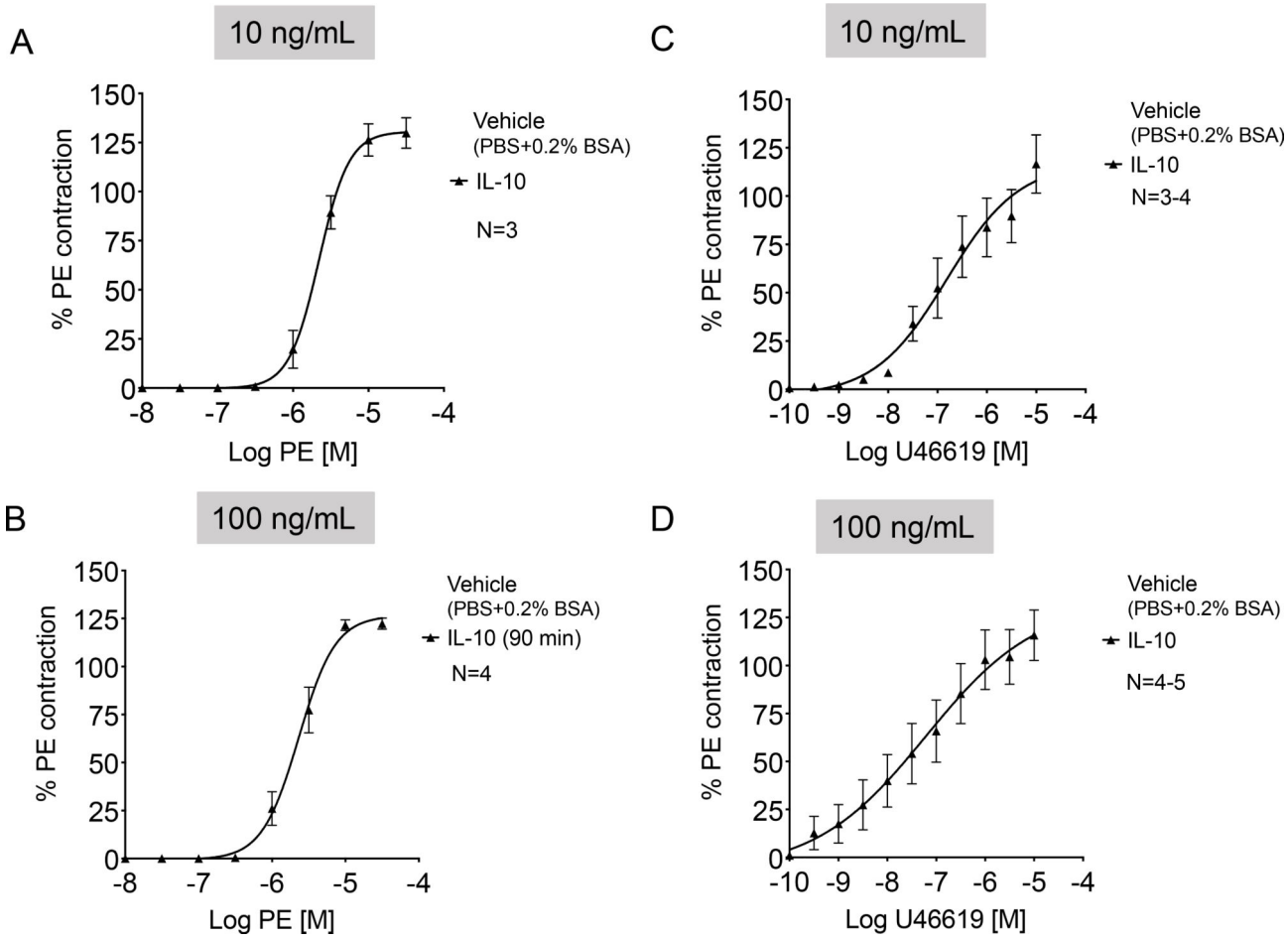

Figure 4. Acute exposure to exogenous IL-10 was not anti-contractile in rat small mesenteric artery

PE-induced cumulative contraction (10^{-8} to 10^{-4} M) of isolated rat third order mesenteric resistance artery without PVAT (with endothelium) after incubation with vehicle (PBS+0.2% BSA) or rrIL-10 (10 ng/mL: **A**; 100 ng/mL: **B**) for 90 minutes in the tissue bath. U46619-induced contraction (10^{-10} to 10^{-5} M) of the rat third order mesenteric resistance artery without PVAT (with endothelium) after incubation with vehicle (PBS+0.2% BSA) or rrIL-10 (10 ng/mL: **C**; 100 ng/mL: **D**) for 90 minutes in the tissue bath. Points represent means ±SEM. N represents the number of animals (and 2 vessels per animal).

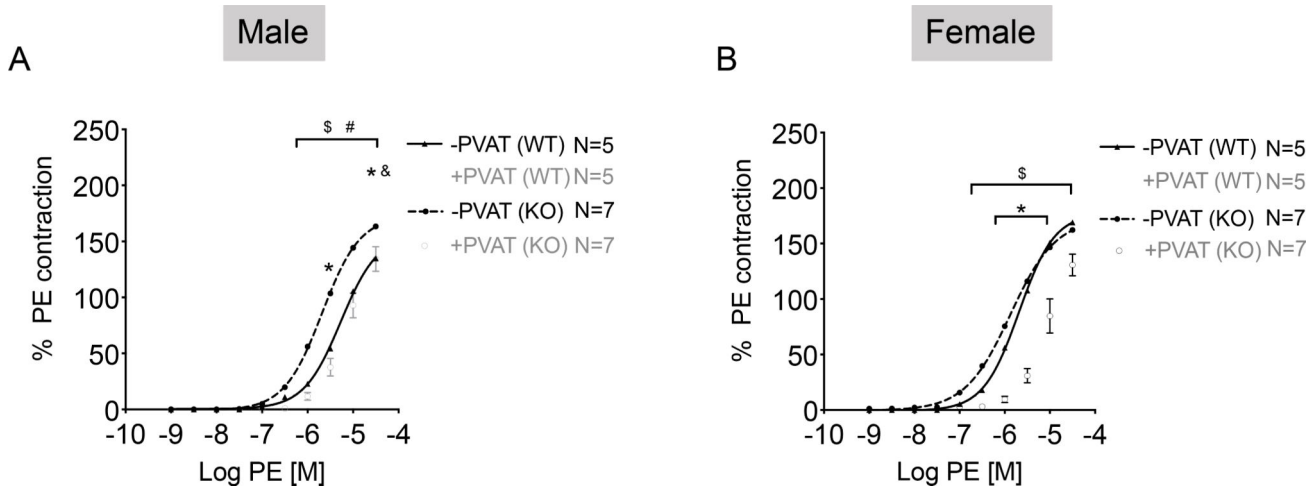

Figure 5. PVAT was anti-contractile with short-term genetic depletion of endogenous IL-10

Cumulative PE-concentration response curves (10^{-8} to 10^{-4} M) in superior mesenteric arteries with/ without PVAT from 13 week-old male **(A)** and female **(B)** IL-10 KO and respective age-matched WT mice. Points represent means±SEM for the number of animals N. Two-way ANOVA with posthoc Tukey test was used and a $P<0.05$ was considered statistically significant. Symbols denote significant differences in contraction between: with and without PVAT in WT (*); with and without PVAT in KO ($); WT and KO in tissues without PVAT (#); WT and KO in tissues with PVAT (&).

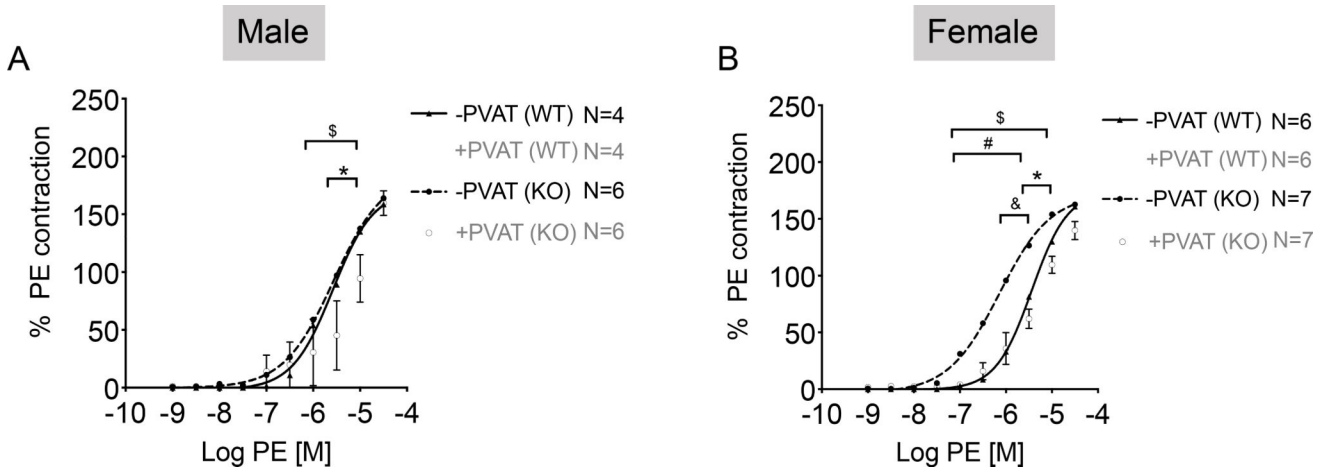

Figure 6. PVAT was anti-contractile even with prolonged genetic depletion of endogenous IL-10

Cumulative PE-concentration response curves (10^{-8} to 10^{-4} M) in superior mesenteric arteries with/ without PVAT from 26 week-old male **(A)** and female **(B)** IL-10 KO and respective age-matched WT mice. Points represent means±SEM for the number of animals N. Two-way ANOVA with posthoc Tukey test was used and a $P<0.05$ was considered statistically significant. Symbols denote significant differences in contraction between: with and without PVAT in WT (*); with and without PVAT in KO ($); WT and KO in tissues without PVAT (#); WT and KO in tissues with PVAT (&).

Table 1:

Pharmacological parameters of data presented in Figure 4.

Concentration of rrIL10 (ng/ mL)	Agonist	Exposure condition	$-\log EC_{50}$ [M]	Maximum contraction (%10 μM PE)	Initial PE contraction (mN)
10	PE	Vehicle	5.7±0.03	135.3±3.8	8.4±1.90
		IL-10	5.7±0.04	130.5±4.8	9.2±1.20
	U46619	Vehicle	7.1±0.12	100.0±6.1	8.2±1.20
		IL-10	7.2±0.30	112.0±7.0	8.0±0.80
100	PE	Vehicle	5.7±0.05	125.4±4.9	8.8±1.50
		IL-10	5.6±0.05	126.6±5.3	9.8±1.70
	U46619	Vehicle	7.7±0.28	126.3±13.2	5.3±0.70
		IL-10	7.2±0.60	133.5±41.0	7.3±1.60

Table 2:

Pharmacological parameters of data presented in Figure 5.

Mouse strain	Tissue type	$-\log EC_{50}$ [M]	Maximum contraction (as %10 μM PE)	Initial PE contraction (mN)
WT (13 wk)	−PVAT	M: 5.3±0.14	156.2±20.2	1.9±0.50
		F: 5.7±0.06	177.6±7.7	2±0.20
	+PVAT	M: 4.8±0.26	245.2±87	1.8±0.40
		F: 4.6±0.30 *	259.3±116.7	2.8±0.08
KO (13 wk)	−PVAT	M: 5.7±0.06	172.8±7.2	2.6±0.30
		F: 5.9±0.10	172.4±12.7	2.9±0.40
	+PVAT	M: 5.1±0.09	153.8±15.4	2.8±0.50
		F: 5.1±0.10 $	152.5±19.0	3.5±0.70

A $P < 0.05$ by one-way ANOVA with Tukey's posthoc test was considered statistically significant.

* and $ represent significant differences in $-\log EC_{50}$ values between −PVAT and +PVAT in WT females and KO females respectively; M=male; F=female.

Table 3:

Pharmacological parameters of data presented in Figure 6.

Mouse strain	Tissue type	$-\log EC_{50}$ [M]	Maximum contraction (as %10 μM PE)	Initial PE wake up (mN)
WT (26 wk)	−PVAT	M: 5.6±0.15	171.2±20.2	2.6±0.50
		F: 5.4±0.08	174.6±12.3	2.3±0.60
	+PVAT	M: 4.8±0.25	209.7±66.7	1.8±0.30
		F: 5.0±0.08 *	169.7±16.5	3.4±0.60
KO (26 wk)	−PVAT	M: 5.6±0.14	185.7±18.6	3.1±0.70
		F: 6.1±0.13 #	171.7±13.1	3.3±0.60
	+PVAT	M: NC	NC	3.5±1.00
		F: 5.2±0.20 $	172.6±26.8	5.7±1.20

A $P < 0.05$ by one-way ANOVA with Tukey's posthoc test was considered statistically significant.

* and $ represent significant differences in $-\log EC_{50}$ values between −PVAT and +PVAT in WT females and KO females respectively

# denotes significant difference in $-\log EC_{50}$ values between WT and KO in −PVAT tissues in females. M=male; F=female; NC= not calculable (curve not plateaued and value could also not be estimated by GraphPad Prism 8.1)