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RESEARCH ARTICLE | *Vascular Biology and Microcirculation*

Interleukin-33 rescues perivascular adipose tissue anticontractile function in obesity

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Saxton SN, Whitley AS, Potter RJ, Withers SB, Grencis R, Heagerty AM. Interleukin-33 rescues perivascular adipose tissue anticontractile function in obesity. *Am J Physiol Heart Circ Physiol* 319: H1387–H1397, 2020. First published October 9, 2020; doi:10.1152/ajpheart.00491.2020.—Perivascular adipose tissue (PVAT) depots are metabolically active and play a major vasodilator role in healthy lean individuals. In obesity, they become inflamed and eosinophil-depleted and the anticontractile function is lost with the development of diabetes and hypertension. Moreover, eosinophil-deficient ΔdblGATA-1 mice lack PVAT anticontractile function and exhibit hypertension. Here, we have investigated the effects of inducing eosinophilia on PVAT function in health and obesity. Control, obese, and ΔdblGATA-1 mice were administered intraperitoneal injections of interleukin-33 (IL-33) for 5 days. Conscious restrained blood pressure was measured, and blood was collected for glucose and plasma measurements. Wire myography was used to assess the contractility of mesenteric resistance arteries. IL-33 injections induced a hypereosinophilic phenotype. Obese animals had significant elevations in blood pressure, blood glucose, and plasma insulin, which were normalized with IL-33. Blood glucose and insulin levels were also lowered in lean treated mice. In arteries from control mice, PVAT exerted an anticontractile effect on the vessels, which was enhanced with IL-33 treatment. In obese mice, loss of PVAT anticontractile function was rescued by IL-33. Exogenous application of IL-33 to isolated arteries induced a rapidly decaying endothelium-dependent vasodilation. The therapeutic effects were not seen in IL-33-treated ΔdblGATA-1 mice, thereby confirming that the eosinophil is crucial. In conclusion, IL-33 treatment restored PVAT anticontractile function in obesity and reversed development of hypertension, hyperglycemia, and hyperinsulinemia. These data suggest that targeting eosinophil numbers in PVAT offers a novel approach to the treatment of hypertension and type 2 diabetes in obesity.

NEW & NOTEWORTHY In this study, we have shown that administering IL-33 to obese mice will restore PVAT anticontractile function, and this is accompanied by normalized blood pressure, blood glucose, and plasma insulin. Moreover, the PVAT effect is enhanced in control mice given IL-33. IL-33 induced a hypereosinophilic phenotype in our mice, and the effects of IL-33 on PVAT function, blood pressure, and blood glucose are absent in eosinophil-deficient mice, suggesting that the effects of IL-33 are mediated via eosinophils.

adipose tissue; diabetes mellitus; hypereosinophilia; hypertension; obesity

INTRODUCTION

The majority of peripheral blood vessels are invested in a layer of perivascular adipose tissue (PVAT) (1, 19, 24). In healthy lean individuals, it exerts an anticontractile effect on vascular tone via endocrine and paracrine mechanisms, thereby having an important hemodynamic influence on glucose uptake and blood pressure. In addition to adipocytes, nerves, and stem cells, PVAT contains a diverse and highly plastic immune cell population (2, 4, 46). We have shown previously that the PVAT anticontractile effect is lost in obesity, which may be contributing to the development of hypertension and type II diabetes (T2D) in this condition (2, 7, 19, 56). Interestingly, not all obese patients are hypertensive, but a common feature between obese hypertensives and lean hypertensive patients is inflammation of adipose tissues (28, 40).

In obesity, excess caloric intake results in hypertrophic expansion of adipocytes, without an accompanying increase in angiogenesis (7, 9, 18). This results in areas of local hypoxia and inflammation, and indeed, the loss of PVAT function in obesity can be replicated in healthy isolated vessels incubated under hypoxic conditions (19). Previously, we have found that eosinophil numbers in PVAT are reduced in a mouse model of obesity (7). In addition, the PVAT anticontractile effect is lost in the eosinophil-deficient ΔdblGATA-1 mouse and can be rescued by eosinophil reconstitution (52). Moreover, this mouse model is hypertensive and hyperglycemic. These studies indicate an interesting avenue of research, whereby the manipulation of eosinophil number in PVAT could present a therapeutic target in obesity. This proposal is supported by the low incidence of cardiovascular disease in countries where helminth infections are prevalent (5, 26).

Numerous studies are beginning to emerge which explore the therapeutic potential of helminth infection in inflammatory diseases, such as asthma and inflammatory bowel disease (14, 30). Of particular interest in the context of this study, Wu et al. (54) demonstrated that helminth-induced eosinophilia improves glucose tolerance in a mouse model of obesity. Interleukin-33 (IL-33) has been shown to directly activate eosinophils and play a role in eosinophil development (25). IL-33, which is a member of the interleukin-1 cytokine family, has been shown to drive the Th2 pro-inflammatory immune response (35, 44). Therefore, the present study was designed to examine the effects of IL-33 in a mouse model of obesity. We tested the hypothesis that IL-33

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injections would restore depleted eosinophil numbers in obese PVAT and rescue the PVAT anticontractile effect. We anticipated that the restoration of the PVAT anticontractile effect would be accompanied by reversal of hypertension and T2D in our obese model. We report that both effects were observed, with evidence of benefits also in lean chow-fed mice.

MATERIALS AND METHODS

Animal care and handling. All animal procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 under the appropriate Home Office License (P3A97F3D1), with ethical approval from the University of Manchester Ethics Committee. Male C57BL/6J mice (Charles River Laboratories, UK) were housed under a 12-h light/dark cycle and provided with food and water ad libitum. Mice were euthanized at 18–20 wk old by CO_2 asphyxiation followed by exsanguination. Animals were randomly assigned to experimental groups. Control mice were fed a standard chow diet (7.42% fat, Cat No. BK001, SDS Diets, UK). Obese mice were fed from 8 wk old with a 60% kcal from fat diet (Cat No. 824054, SDS Diets, UK) until euthanized. The effects of IL-33 were investigated in both control and obese mice by daily intraperitoneal injection of 0.1 μg of IL-33 (12) (Bio-Techne) reconstituted in saline (0.9% NaCl, Baxter Healthcare Ltd, UK) for five consecutive days before euthanasia. The last dosage was administered 24 h before euthanasia. To confirm the effects of IL-33 are mediated via eosinophils, IL-33 injections were also administered to the eosinophil-deficient ΔdblGATA-1 mouse [C.129S1(B6)-Gata1tm6Sho/J, RRID: IMSR_JAX:005653, Jackson Laboratories]. Endothelial nitric oxide synthase knockout mice (*eNOS*$^{-/-}$; strain B6.129P2-Nos3tm1Unc/J, RRID: IMSR_JAX:002684) were kindly provided by Dr. Elizabeth Cottrell. There were eight mice in control, obese, control + IL-33, obese + IL-33, and *eNOS*$^{-/-}$ groups, and seven mice in the ΔdblGATA-1 + IL-33 group.

Immediately before euthanasia, conscious mice were restrained, and systolic and diastolic blood pressure was measured using a CODA tail-cuff blood pressure monitoring system (Kent Scientific). Although not the gold standard, this method has been validated as showing good agreement with radiotelemetry recordings (13). Mixed blood samples were taken upon exsanguination, and blood glucose concentration was measured immediately by the application of a small drop of blood to an automatic blood glucose system (Contour; Bayer Consumer Care AG, Basel, Switzerland). All remaining blood was collected in BD Vacutainer blood collection tubes coated with the anticoagulant K_2EDTA (Thermo Fisher Scientific, UK). Plasma was retrieved by centrifuging blood samples at 3,000 *g* for 10 min at 4°C and stored at −80°C until required.

Immediately following euthanasia, the body weights of the mice were recorded. The spleen, epididymal fat pads, and mesenteric beds were dissected out and placed in ice-cold physiological salt solution (PSS) (119 mM NaCl, 4.7 mM KCl, 1.17 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.17 mM KH_2PO_4, 0.03 mM K_2EDTA, 5.5 mM glucose, and 1.6 mM $CaCl_2$; Thermo Fisher Scientific, UK) until required. Dry weights of spleens were recorded.

Eosinophil cationic protein, adiponectin, and insulin assays. The concentration of plasma insulin, adiponectin, and eosinophil cationic protein was measured using commercially available ELISA kits according to manufacturer instructions (Table 1). All samples and standards were measured in duplicate, and the optical density of the zero standard was subtracted from each value. Standard curves were fitted using nonlinear regression analysis with a sigmoidal four-parameter logistic curve to interpolate values.

Wire myography. Second-order mesenteric artery segments (<250 μm) with or without PVAT intact were isolated from the mesenteric bed by fine dissection in ice-cold PSS. Arteries were mounted onto 40-μm-diameter wires using a wire myograph system (Danish MyoTech, Denmark). Mounted arteries were allowed to equilibrate for 30 min in the myograph at 37°C in PSS perfused with 95% air-5% CO_2 to maintain pH 7.4. Vessel wall tension was normalized according to a standardized procedure (36), and the arteries were allowed to equilibrate for a further 30 min. Vessels were subjected to 60 mM high [K^+]PSS (KPSS) (63.7 mM NaCl, 60 mM KCl, 1.17 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.17 mM KH_2PO_4, 0.03 mM K_2EDTA, 5.5 mM glucose, and 1.6 mM $CaCl_2$; Thermo Fisher Scientific, UK) to establish a maximal constriction response. The KPSS was washed out and replaced with fresh PSS. Endothelial integrity was tested by preconstricting with 1×10^{-5} M noradrenaline (NA) and applying 1×10^{-7} M acetylcholine (ACh) (both obtained from Sigma-Aldrich, UK, and dissolved in PSS).

Cumulative concentration-response curves were generated using increasing doses of NA (1×10^{-9}–3×10^{-5} M), with traces continuously generated using LabChart 7 (ADInstruments, UK) in arteries from control, obese, control + IL-33, obese + IL-33, and the ΔdblGATA-1 + IL-33 mice. An example trace of a NA concentration response performed in a control -PVAT vessel is given in Supplemental Fig. S1 (see https://doi.org/10.6084/m9.figshare.12962774).

To determine whether IL-33 has a direct effect on vasculature contractility, arteries from control and *eNOS*$^{-/-}$ mice were first preconstricted with 1×10^{-5} M NA, and once stabilized, 3 ng/mL of IL-33 was added directly to the myograph bath. To determine the role of the endothelium, arteries from control mice with and without endothelium were used. To remove the endothelium, equine hair is inserted into the lumen for mechanical removal. Endothelial integrity was tested as described above. Vessels with less than a 20% relaxation in response to ACh were considered to be endothelium denuded.

Immunohistochemistry. PVAT samples (n = 5) were fixed in 4% ice-cold paraformaldehyde for 1 h. Samples were washed in 0.1 M phosphate buffer solution before embedding in KP-CryoCompound (Klinipath BV, The Netherlands). Samples were stored at −80°C until sectioned using a Leica CM 3050 cryostat (12-μm slices, Leica Microsystems, Germany). Heat-induced antigen retrieval was performed in a citrate buffer for 10 min at 95°C, followed by incubation with 3% hydrogen peroxide. Sections were incubated with 10% goat serum at room temperature for 1 h in combination with Triton X-100 (0.1%). Samples were incubated with the primary polyclonal antibody for eosinophil peroxidase (10 μg/mL, Abcam, UK; ab65319, RRID: AB_10712964) overnight at 4°C. Slides were incubated for 1 h with a biotinylated goat antirabbit secondary antibody (2 μg/mL, Abcam, UK; Cat No. ab6720, RRID: AB_954902) at room temperature. Vectastain ABC complex (Vector Laboratories, UK) followed by the addition of 3,3′-diaminobenzidine (DAB) solution (Vector Laboratories, UK) was used for the detection of antibody binding. Positive controls were conducted in sections of mouse spleen (Supplemental Fig. S2*A*, see https://doi.org/10.6084/m9.figshare.12497930). Negative controls were conducted in PVAT (Supplemental Fig. S2*B*). These were incubated with phosphate buffer solution in place of the primary antibody, and incubation with the secondary antibody and detection methods were conducted as normal. Images were captured using a color camera (Leica

Table 1. *Kits and suppliers*

Antigen	Name of Kit	Supplier	Catalogue No.	Sample Dilution
Adiponectin	Adiponectin mouse ELISA kit	Life Technologies Ltd	KMP0041	1 in 20,000
Eosinophil cationic protein	Mouse eosinophil cationic protein (ECP) ELISA kit	Cusabio Technology LLC	CSB-E11799M	Control and obese: none IL-33 injected control and obese: 1 in 4
Insulin	Insulin mouse ELISA kit	Invitrogen	EMINS	1 in 2

DFC450, Leica Microsystems, Germany) mounted on a microscope (Leica DM5000, Leica Microsystems, Germany).

Statistics. All data are reported as means ± standard error (SE). Normal distribution was confirmed using Shapiro–Wilk normality test, supported by skewness and kurtosis coefficients (using an acceptable range of ±2). Body, epididymal fat pad and spleen weights, blood pressure, blood glucose, plasma insulin, plasma adiponectin, and plasma eosinophil cationic protein were analyzed using one-way analysis of variance (ANOVA) with a Tukey's post hoc test. Consistent with previous studies (17, 19, 43), vessel contractility ± PVAT was expressed as a percentage of the maximum contraction evoked by KPSS and these data were analyzed by two-way ANOVA with a Bonferroni post hoc test. Relaxation in response to IL-33 or ACh was expressed as a percentage of the preconstriction elicited with NA and was analyzed with a one-way ANOVA followed by Tukey's post hoc test or an unpaired *t* test, respectively. Statistical analysis was carried out using GraphPad Prism 8.0, and a P value <0.05 was considered to be significant.

RESULTS

IL-33 injections induce eosinophilia and splenomegaly. Following euthanasia body, epididymal fat pads and spleen weights of all mice were recorded (Fig. 1, *A–C*). High-fat feeding induced significant elevations in body weight and epididymal fat pad weight (body weights: control vs. obese $P < 0.01$, control + IL-33 vs. obese + IL-33 $P < 0.0001$; epididymal fat pad weights: control vs. obese $P < 0.0001$, control + IL-33 vs. obese + IL-33 $P < 0.0001$), which was unaffected by IL-33 (for both body weight and epididymal fat pad weights: control vs. control + IL-33 $P > 0.05$, obese vs. obese + IL-33 $P > 0.05$, n = 8 all groups). However, spleen size was significantly larger in control and obese mice injected with IL-33 (control vs. control + IL-33 $P < 0.001$, obese vs. obese + IL-33 $P < 0.0001$). Eosinophilia was confirmed using an eosinophil cationic protein ELISA (Fig. 1*D*). Concentration of eosinophil cationic protein was significantly increased in the plasma of injected control and obese mice (control vs. control + IL-33 $P < 0.0001$, obese vs. obese + IL-33 $P < 0.01$), more so in control-injected mice (control + IL-33 vs. obese + IL-33 $P < 0.01$, n = 8 all groups). Using immunohistochemistry, we confirmed eosinophil infiltration into mesenteric PVAT after IL-33 (Fig. 2, n = 5).

IL-33 reverses hypertension, hyperglycemia, and hyperinsulinemia T2D. Immediately before euthanasia, blood pressure was recorded using the CODA tail-cuff method (Fig. 3*A*). High-fat feeding resulted in significant elevations in both systolic and diastolic blood pressures (control vs. obese $P < 0.05$). However, there was no difference in either systolic or diastolic blood pressure between obese + IL-33 mice and control/control + IL-33 mice ($P > 0.05$, n = 8 all groups), indicating a reversal of hypertension in IL-33-injected obese mice. Previously, we have shown that the ΔdblGATA-1 mice are hypertensive (52). Similarly, the ΔdblGATA-1 mice injected with IL-33 were

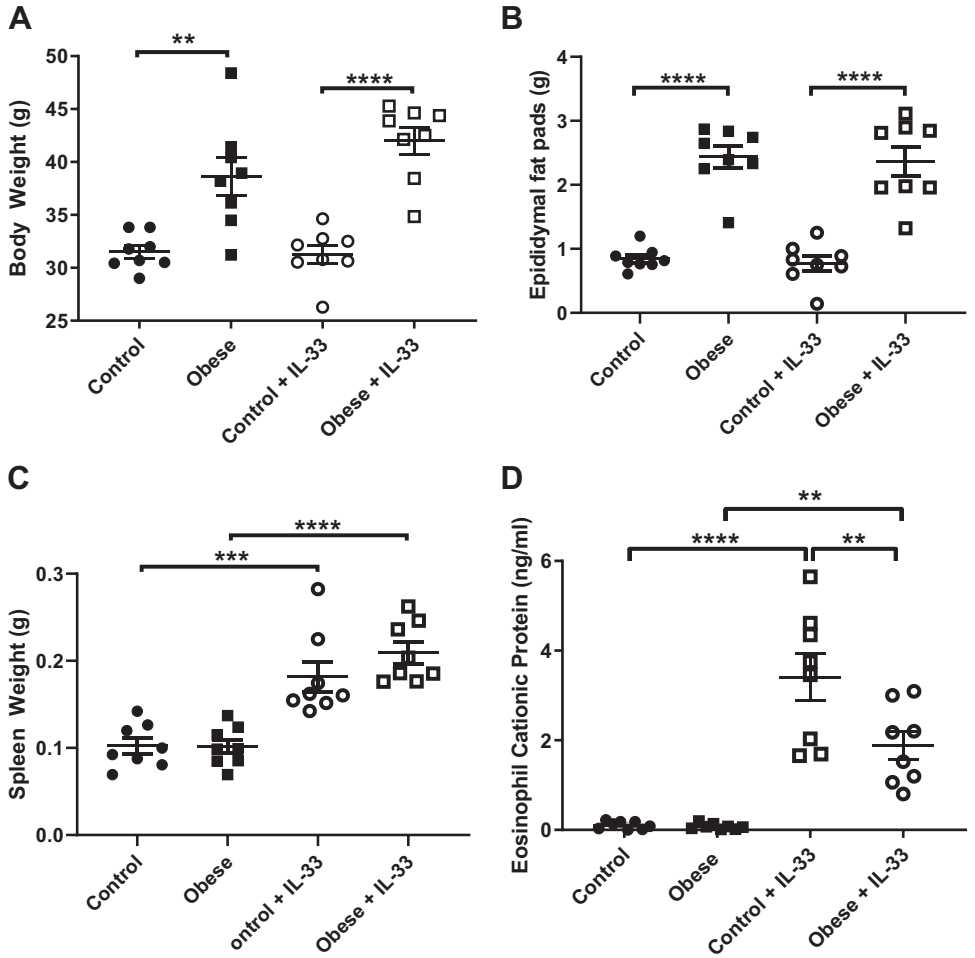

Fig. 1. IL-33 induces splenomegaly and eosinophilia. Body (*A*), epididymal fat pads (*B*), and spleen (*C*) weights were recorded. *D*: blood was collected and centrifuged to separate plasma. The concentration of eosinophil cationic plasma was measured using an ELISA kit. Data shown are means ± SE. One-way ANOVA followed by Tukey's post hoc tests (all groups n = 8, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$). ANOVA, analysis of variance; IL-33, interleukin-33.

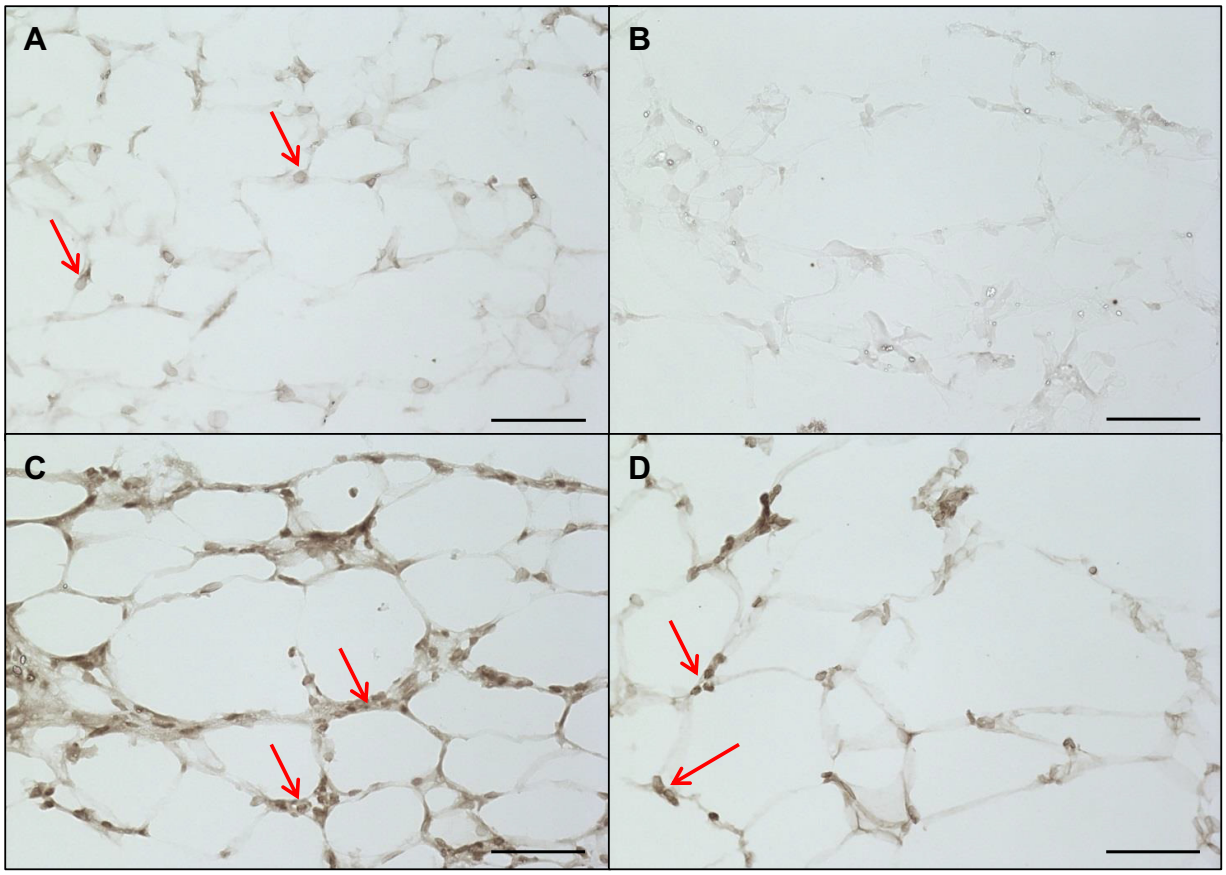

Fig. 2. Eosinophils are restored in obese PVAT by IL-33 treatment. The 12-μm sections were stained with an anti-eosinophil peroxidase antibody. *A*: control mouse mesenteric PVAT. *B*: obese mouse mesenteric PVAT. *C*: mesenteric PVAT from control mice injected with IL-33. *D*: mesenteric PVAT from obese mice injected with IL-33. Red arrows indicate eosinophils. Scale bar = 50 μm (representative of $n = 5$). IL-33, interleukin-33; PVAT, perivascular adipose tissue.

hypertensive compared with our control C57 mice (Fig. 3, control vs. ΔdblGATA-1 + IL-33 $P < 0.05$, control $n = 8$, ΔdblGATA-1 + IL-33 $n = 7$).

Immediately after euthanasia, blood glucose was measured using an automatic monitor (Fig. 3*B*). Similar to the blood pressure measurements, blood glucose concentration was significantly increased in the obese group (control vs. obese $P < 0.0001$) and was reduced to a level comparable with controls in the obese + IL-33 group ($P > 0.05$, $n = 8$ all groups). Interestingly, blood glucose was reduced in the control + IL-33 group versus controls ($P < 0.01$, $n = 8$ all groups). The same pattern was observed in plasma insulin measurements using an ELISA (Fig. 3*C*); insulin was significantly increased in the obese group (control vs. obese $P < 0.01$) and returned to control levels in the obese + IL-33 group ($P > 0.05$, $n = 8$). The mean insulin concentration from control mice was ~51 ng/mL, whereas in control + IL-33 mice, the mean concentration was ~23 ng/mL. Although this was not statistically significant, this may indicate at least a trend toward reduced plasma insulin in lean controls. Nonetheless, these data indicate that IL-33 injections reversed hyperinsulinemia and hyperglycemia in obese mice. Previously, we have shown that the ΔdblGATA-1 mice exhibit hyperglycemia (52). Similarly, the ΔdblGATA-1 mice injected with IL-33 were hyperglycemic compared with our control C57 mice (Fig. 3, control vs. ΔdblGATA-1 + IL-33 $P < 0.05$, control $n = 8$, ΔdblGATA-1 + IL-33 $n = 7$).

PVAT exerts a reproducible anticontractile effect, which is lost in obesity. Second-order mesenteric arteries ±PVAT were dissected from control and obese mice. Vascular contractility to increasing concentrations of NA was measured (Fig. 4). Consistent with previous findings, arteries contracted significantly less when PVAT was left intact ($P < 0.01$, $n = 8$); i.e., PVAT exerts an anticontractile effect on the vasculature. In obesity, the PVAT anticontractile effect was lost and the contractility of ±PVAT arteries was no different ($P > 0.05$, $n = 8$).

IL-33 injections restore PVAT function in obesity and enhance PVAT function in health. Second-order mesenteric arteries ±PVAT were dissected from control and obese mice injected with IL-33 and subjected to a NA concentration response (Fig. 5). In control + IL-33 mice, PVAT exerted a significantly larger anticontractile effect (Fig. 5*A*, $P < 0.0001$, $n = 8$). In Fig. 5, *C*–*F*, we have combined data from Figs. 4*A* and 5*A*, and Figs. 4*B* and 5*B*, to compare the responses of the same vessel types from mice with and without IL-33. Figure 5, *C* and *D*, is a comparison of -PVAT vessels from control mice with and without IL-33, and obese mice with and without IL-33, respectively. There is no significant difference between these responses ($P > 0.05$, $n = 8$ all groups). Figure 5, *E* and *F*, is a comparison of +PVAT vessels from control mice with and without IL-33, and obese mice with and without IL-33, respectively. Using this comparison, there is a significant difference in the responses of +PVAT arteries both within the control and obese

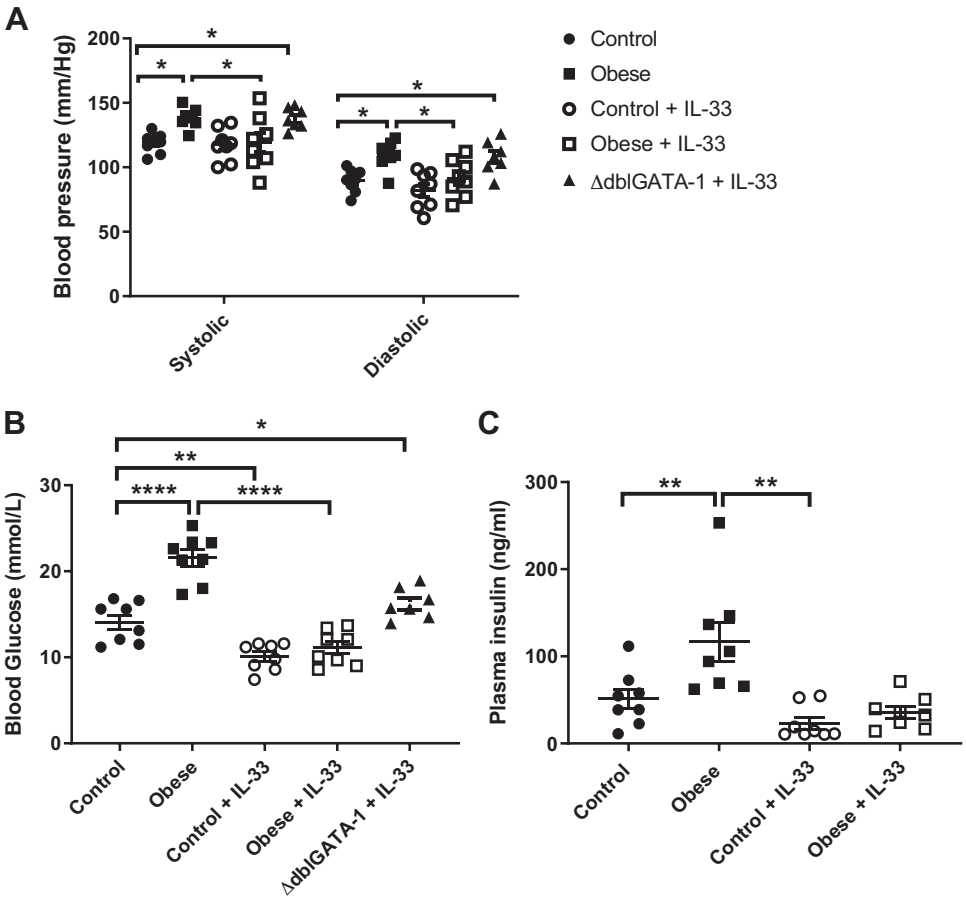

Fig. 3. IL-33 reverses hypertension, hyperglycemia, and hyperinsulinemia in obesity. *A*: blood pressure was recorded using the CODA tail-cuff system in conscious restrained mice. *B*: blood glucose was measured following euthanasia using an automatic glucose monitor. *C*: blood was collected and centrifuged to separate plasma. The concentration of insulin was measured using an ELISA kit. Data shown are means ± SE. One-way ANOVA followed by Tukey's post hoc tests (control, obese, control + IL-33, and obese + IL-33, $n = 8$; ΔdblGATA-1 + IL-33, $n = 7$; *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$). ANOVA, analysis of variance; IL-33, interleukin-33; NA, noradrenaline.

groups with and without IL-33 (both $P < 0.0001$, $n = 8$ all groups), suggesting that the effects of IL-33 on vascular contractility are dependent upon PVAT.

IL-33 is an acute endothelium-dependent vasodilator; however, endothelial function is impaired in obesity. Second-order mesenteric arteries from *eNOS*$^{-/-}$ and control mice with PVAT removed were preconstricted with 1×10^{-5} M NA, before adding 3 ng/mL IL-33 (example trace Fig. 6*B*—control vessel with endothelium intact). To examine the contribution of the endothelium, control vessels with endothelium intact and endothelium removed were tested and compared with vessels from *eNOS*$^{-/-}$ mice (Fig. 6*A*). IL-33 induced vasorelaxation, which was reduced in the absence of endothelium and in vessels from *eNOS*$^{-/-}$ (control + endothelium vs. control – endothelium, $P < 0.001$; control + endothelium vs. *eNOS*$^{-/-}$, $P < 0.0001$; control – endothelium vs. *eNOS*$^{-/-}$, $P > 0.05$, $n = 8$ all groups); therefore, this effect must be endothelium dependent. As can be seen in example trace Fig. 6*B*, the vasodilation is acute and rapidly decaying.

Endothelial integrity of control versus obese vessels was compared by preconstricting vessels with 1×10^{-5} M NA, before adding 1×10^{-7} M ACh (Fig. 6*C*). In obese arteries, relaxation to ACh is impaired ($P < 0.0001$, $n = 8$), indicating endothelial dysfunction in obesity.

Circulating adiponectin is increased in obesity and reduced by IL-33. The concentration of adiponectin in plasma was measured using a commercially available kit (Fig. 7). Plasma adiponectin was significantly increased in obesity compared with the control group, and this was reduced back to control levels with IL-33 (control vs. obese, $P < 0.0001$; control vs. control + IL-33, $P > 0.05$; control vs. obese + IL-33, $P > 0.05$; obese vs. obese + IL-33, $P < 0.001$; control and obese, $n = 8$; control + IL-33, $n = 5$; obese + IL-33, $n = 7$).

IL-33 injections have no effect on PVAT function in ΔdblGATA-1 mice. Previously, we have indicated that in the eosinophil-deficient mouse, ΔdblGATA-1 mice, the PVAT anticontractile effect is absent. Therefore, to confirm that the restorative/enhancing effects of IL-33 on PVAT function are mediated via eosinophils, we injected ΔdblGATA-1 mice with IL-33. Second-order mesenteric arteries ±PVAT were dissected from ΔdblGATA-1 + IL-33 and subjected to a NA concentration response (Fig. 8). Consistent with our previous findings (52), the PVAT anticontractile effect was absent, indicating that IL-33 has had no effect ($P > 0.05$, $n = 7$).

DISCUSSION

The aim of this study was to investigate the potential for targeting eosinophil numbers in obesity. The main findings of this study were that IL-33-induced eosinophilia enhanced anticontractile function in lean mice and rescued it in obesity and this was associated with a lowering of blood pressure and amelioration of glucose intolerance. In addition, we have shown that the eosinophil is the most likely immune cell responsible for this effect.

Consistent with previous studies, we have shown that IL-33 induced eosinophilia (12, 44), which was quantified using a

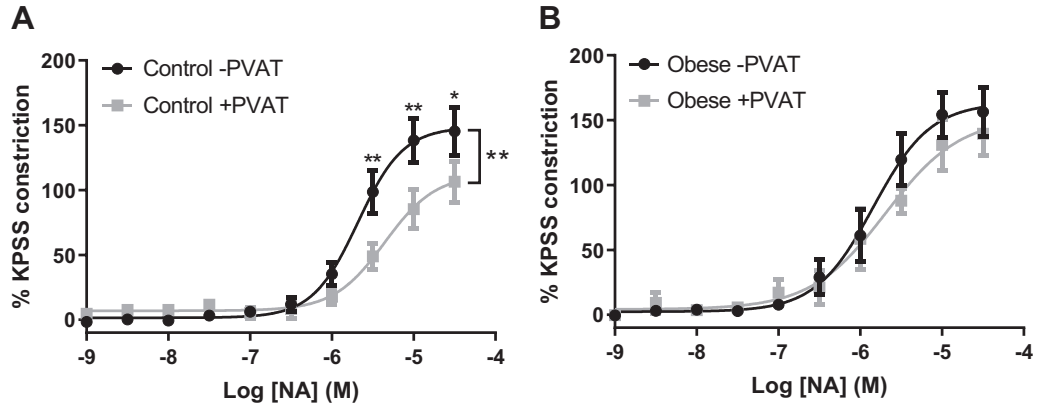

Fig. 4. PVAT exerts an anticontractile effect in health, which is absent in obesity. Small mesenteric arteries were isolated from control (*A*) and obese (*B*) mice and subjected to increasing concentrations of NA. Data shown are means ± SE. Vessel contractility is expressed as a percentage of the maximum contraction evoked by KPSS. Two-way ANOVA followed by Bonferroni post hoc tests ($n = 8$ both groups, $^*P < 0.05$, $^{**}P < 0.01$). ANOVA, analysis of variance; IL-33, interleukin-33; KPSS, [K^+]PSS; NA, noradrenaline; PVAT, perivascular adipose tissue.

plasma eosinophil cationic protein ELISA. The degree of eosinophilia was increased in control mice injected with IL-33 compared with obese mice injected with IL-33. In addition, using immunohistochemistry we demonstrated that the eosinophil numbers in PVAT, which we have previously shown to be reduced in obesity (7), are increased following IL-33 injections. This is consistent with another study where IL-33 was given over a longer period and eosinophil numbers were increased in subcutaneous depots (11). However, our data shown here are only qualitative. The aim was to demonstrate that eosinophil infiltration in PVAT is restored following IL-33 treatment. In the future studies, eosinophil number in PVAT could be quantified using flow cytometry. In addition, future studies to characterize the eosinophils present in PVAT (by cytokine receptor expression) are essential.

Interestingly, although there are multiple studies reporting reduced eosinophil numbers in mouse fat depots in models of obesity (7, 11, 22, 32), the opposite has been found in blood from human obese patients; i.e., circulating eosinophils are increased. To the best of our knowledge, there is only one study, which has looked at eosinophil numbers in obese human fat, but there are a number of limitations to this study including the absence of diabetes and hypertension (34), therefore shedding no light on the role of eosinophils in these diseases. Changes in eosinophil number following dietary intervention in mice are tissue specific (6); therefore, we hypothesize that during the development of obesity-associated diseases, changes in eosinophil recruitment signals and anchoring integrins result in eosinophils moving out of fat depots and into the bloodstream. These future studies will be vital in understanding the mechanistic links between obesity and cardiovascular and metabolic diseases.

IL-33 is expressed in numerous cell types including adipocytes (53), fibroblasts (42), and endothelial and epithelial cells (35), and its receptors are expressed on basophils (47), mast cells (3), and eosinophils (8). Therefore, to confirm that the beneficial effects of IL-33 observed in this study were mediated via eosinophils, eosinophil-deficient ΔdblGATA-1 mice were used. Previously, we have shown that these mice lack a PVAT anticontractile effect and are hypertensive and hyperglycemic (52). The lack of effect of IL-33 injections in this mouse model suggests that the effects of IL-33 are dependent on eosinophils. In addition to a potential direct effect of IL-33 on eosinophils, it is likely that IL-33-induced upregulation of IL-5 also contributes to development of eosinophilia in this model (12, 44), although this was not measured in the current study.

Paradoxically, IL-33 expression has been shown to be increased in the adipose tissues of severely obese humans (57). Despite this, multiple studies have demonstrated a beneficial role of IL-33 treatment in obesity as recently reviewed (10). Studies in the genetically obese *ob*/*ob* mouse have shown that treatment with recombinant IL-33 three times a week, for 3 wk, induced the production of the Th2 cytokines including IL-5, and the polarization of adipose tissue macrophages toward an M2 phenotype (31). Moreover, treatment reduced adiposity and reduced blood glucose concentration in vivo. However, this study did not describe the treatment route or exact dosage; therefore, comparisons with our study are difficult. Nonetheless, the results of these studies combined with our own may indicate that exogenous IL-33 will beneficially regulate the inflammatory process in obesity.

Interestingly, as briefly discussed, hypertrophy of adipocytes results in local regions of hypoxia, and studies have indicated that IL-33 expression in cultured human adipocytes is not affected by hypoxia; however, there is a threefold increase in the expression of IL-33 in hypoxic pre-adipocytes (53). Adipose tissue has a limited ability to recruit new adipocytes, and it is when this limit is reached that existing adipocytes will undergo hypertrophy, and it is hypertrophy of adipocytes, which is associated with increased cardiovascular risk (21). Therefore, targeting the differentiation of these hypoxic pre-adipocytes with increased IL-33 may present another target by which eosinophil number could be increased.

We are not the first to demonstrate improvements in glucose and insulin following IL-33 treatment. Duffen et al. (11) reported an improvement in the metabolic profile of their obese model when IL-33 was administered over a longer period. However, in their model, IL-33 induced gastrointestinal effects including diarrhea, resulting in weight loss. This weight loss may have played a role in the metabolic improvements observed. We did not observe any gastrointestinal irregularities in our model, which may indicate that the results of our study are a direct effect of immune cell regulation rather than gastrointestinal upset.

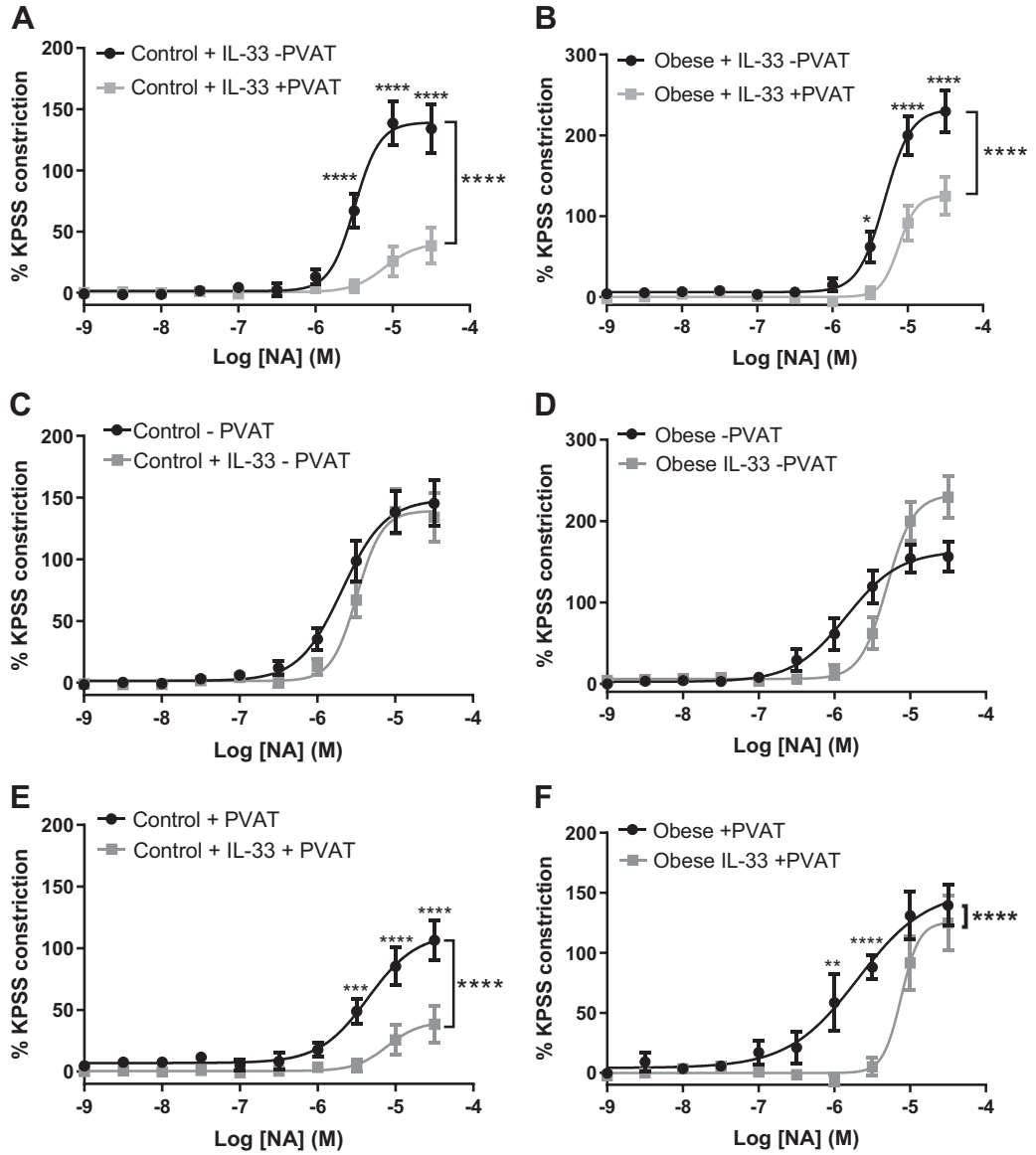

Fig. 5. IL-33 treatment enhances PVAT function and restores the anticontractile effect in obesity. Small mesenteric arteries were isolated from control (*A*) and obese (*B*) mice injected with IL-33 and subjected to increasing concentrations of NA. *C*–*F*: data from Fig. 4, *A* and *B*, have been combined with data from Fig. 5, *A* and *B*, to compare responses of ±PVAT vessels from mice with and without IL-33 treatment (*C* and *D*: -PVAT vessels from control and obese mice, respectively, *E* and *F*: +PVAT vessels from control and obese mice, respectively). Data shown are means ± SE. Vessel contractility is expressed as a percentage of the maximum contraction evoked by KPSS. Two-way ANOVA followed by Bonferroni post hoc tests ($n = 8$ all groups, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$). ANOVA, analysis of variance; IL-33, interleukin-33; KPSS, [K^+]PSS; PVAT, perivascular adipose tissue.

This is the first study to report a beneficial effect of IL-33 on blood pressure and the role of PVAT role in regulating vascular tone. Moreover, the effects of IL-33 were not limited to obesity, and these studies revealed a beneficial effect of IL-33 on blood glucose concentrations in lean mice. The mechanism by which IL-33-induced eosinophilia confers metabolic benefits in obesity may involve the recent finding that eosinophils produce catecholamines (52). Previously, we have demonstrated that the anticontractile effect is mediated via sympathetic nerve-derived NA, which activates adipocyte β_3-adrenoceptors, stimulating the release of the vasodilator adiponectin (43). In addition, we have found that eosinophils directly modulate the PVAT anticontractile function via a β_3-adrenoceptor and adiponectin-dependent mechanism (52). Therefore, catecholamines from eosinophils likely feed into the sympathetic nerve-mediated mechanism and promote vasodilation. This hypothesis is supported by our previous studies in the ΔdblGATA-1 mouse. As already discussed, the PVAT anticontractile effect is absent in these mice, but could be restored using eosinophil reconstitution (51). This restored PVAT effect could be reduced using either a β_3-adrenoceptor antagonist or a blocking peptide for adiponectin receptor 1. In obesity, PVAT function is lost, and it is widely accepted that autonomic dysfunction occurs in obesity (29, 45). Therefore, eosinophils may provide an alternative source of catecholamines to regulate PVAT function.

Plasma adiponectin levels were high in obese mice and were restored to normal levels following IL-33 injection. It is likely that increased plasma adiponectin in obesity is a result of

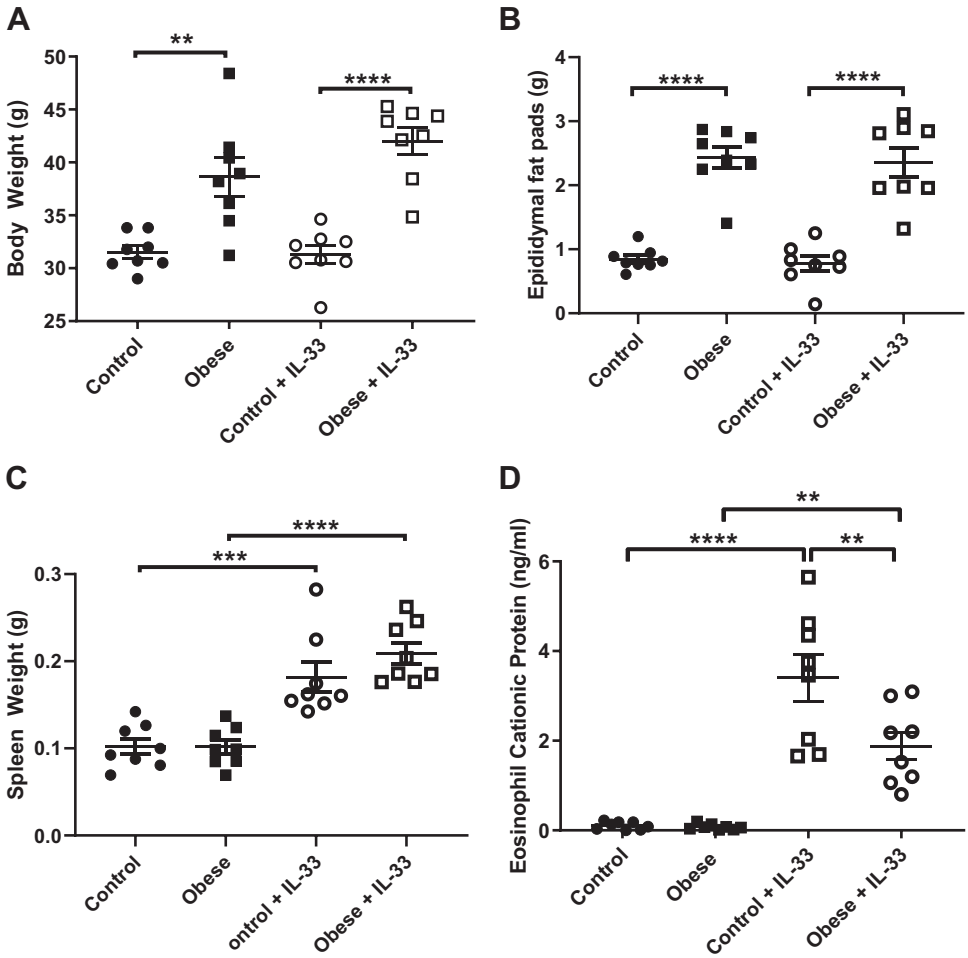

Fig. 6. IL-33 stimulates a rapidly decaying endothelium-dependent vasorelaxation. *A*: small mesenteric arteries were isolated from control and *eNOS*$^{-/-}$ mice. To examine the role of endothelium, it was mechanically removed from some control arteries. All arteries were preconstricted with NA (1×10^{-5} M) before applying a single dose of IL-33 (3 ng/mL). *B*: example trace for a control artery with endothelium intact. *C*: small mesenteric arteries were isolated from control and obese mice. To test endothelial integrity, vessels are preconstricted with NA (1×10^{-5} M), before adding ACh (1×10^{-7} M). Data shown are means ± SE. *A*: one-way ANOVA followed by Bonferroni post hoc tests. *C*: unpaired *t* test ($n = 8$ all groups, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$). Ach, acetylcholine; ANOVA, analysis of variance; *eNOS*$^{-/-}$, endothelial nitric oxide synthase knockout mice; IL-33, interleukin-33; NA, noradrenaline.

impaired uptake and/or clearance. Adiponectin binds to both AdipoR1 and AdipoR2 receptors to mediate its pleiotropic biological actions, and studies have reported decreased adiponectin receptor expression in obese mice (48) and decreased AdipoR1/R2 expression in obese mice are directly related to insulin resistance and elevated blood glucose (39). Furthermore, another study has reported a prolonged clearance rate of adiponectin by the liver in obese mice and a dramatically increased half-life (20). Therefore, if adiponectin clearance and metabolism is impaired in obesity, this may result in an accumulation in the blood. As such, further investigations into AdipoR1/AdipoR2 expression and adiponectin clearance in healthy and obese mice are required to understand these results. Nonetheless, IL-33 injections reduced adiponectin levels in obesity, indicating a restorative effect of IL-33 on adiponectin levels in obesity.

Interestingly, in this study, to our knowledge, we report for the first time that IL-33 is an acute vasodilator in small mesenteric arteries. IL-33 was tested in vessels with endothelium intact, and endothelium removed following mechanical disruption, which indicated that the vasodilator effect is endothelium dependent. However, this method of endothelium removal is limited; therefore, we further tested IL-33 in vessels from *eNOS*$^{-/-}$ mice; without eNOS, there can be no endothelium-dependent vasorelaxation, and indeed, IL-33 had very little effect on arteries from these mice. As such, IL-33 exerts a direct effect on the vasculature, which is mediated by nitric oxide in the endothelium. However, this effect was rapidly decaying and therefore is unlikely to contribute to the changes in blood pressure observed in this study, particularly given that the last dosage of IL-33 was given 24 h before measuring blood pressure. Moreover, this effect is endothelium dependent, and we have demonstrated that the endothelium is impaired in our obese model. Therefore, the results of this study are not mediated by a direct effect of IL-33 on the vasculature.

Some studies have indicated that beneficial role of eosinophils is via promoting anti-inflammatory M2 macrophage polarization in adipose tissue (54, 55). Macrophages represent the largest proportion of immune cells in PVAT, and pro-inflammatory M1 macrophages are increased in number in obesity (49, 50). In addition, M1 macrophages are associated with the development of insulin resistance and hypertension (23, 37, 41), whereas M2 macrophages play a role in adipose tissue homeostasis, in particular through their role in adipocyte turnover and lipid buffering (16, 27). Studies on M2 macrophages as a source of catecholamines are conflicting, with some suggesting a role for M2-derived catecholamines in adaptive thermogenesis (38), and others disputing the ability of M2 macrophages to synthesize catecholamines (15). These studies are further complicated by

the subtle differences between macrophage subpopulations and make clearly classifying macrophages difficult and perhaps currently unreliable (33).

Although the effectiveness of IL-33 injections in treating hypertension and T2D is promising, the resultant splenomegaly does significantly limit its therapeutic potential, as an enlarged spleen is susceptible to rupture. Of note, the splenomegaly in this study is consistent with previous studies of IL-33-injected mice, in which the enlarged spleens were attributed to increased numbers of eosinophils, mononuclear cells, and plasma cells (44). However, we did not conduct any long-term studies to determine how long the beneficial effects of IL-33 (or the effects on spleen size) persist following five consecutive daily injections. It is possible that alterations to the dosage, or frequency of injections, could induce beneficial effects in hypertension and T2D, without significant effects on the spleen. Indeed, another study using IL-33 treatment three times a week for 3 wk did not report any effects on the spleen (31). In addition, as previously discussed another study reported significant gastrointestinal effects (11), which reinforces the potential harmful effects of an intense forced immune response. Nonetheless, these studies open an exciting avenue to be explored, whereby targeting eosinophil number presents a novel and highly useful therapeutic target in obesity.

Limitations and future directions. The results of this study demonstrate a clear effect of IL-33 on blood pressure, blood glucose, and plasma insulin homeostasis. However, the mechanism of these effects remains unclear. As discussed above, we believe that eosinophils will factor into the sympathetic nerve-mediated anticontractile effect which we have previously described (43). Therefore, future studies are needed to investigate the levels of catecholamines, β_3-adrenoceptors, and adiponectin receptors in our model. In addition, eosinophil numbers and their phenotype in multiple tissues within our model need to be fully characterized to determine whether the effects of obesity and IL-33 on eosinophil number are tissue dependent and whether it is a certain type of eosinophil, which is required to mediate beneficial effects in obesity. Furthermore, future studies are needed to further characterize the effects of IL-33 on the obese phenotype, including using radiotelemetry to measure blood pressure, glucose tolerance testing, and characterization of changes to other circulating lipids and adipokines (25).

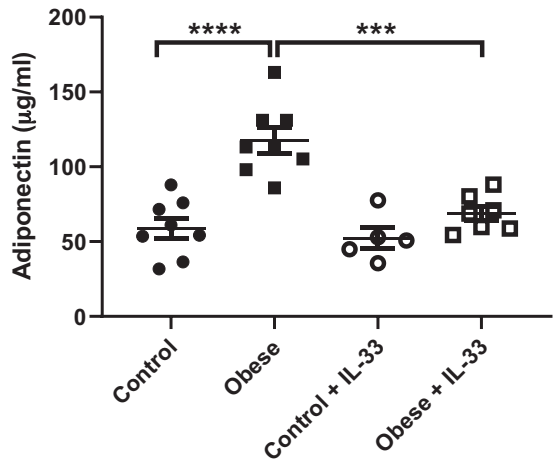

Fig. 7. Plasma adiponectin is increased in obesity and returned to control levels with IL-33. Blood was collected and centrifuged to separate plasma. The concentration of adiponectin was measured using an ELISA kit. Data shown are means ± SE. One-way ANOVA followed by Tukey's post hoc tests (control and obese, $n = 8$; control + IL-33, $n = 5$; obese + IL-33, $n = 7$; $^{***}P < 0.001$, $^{****}P < 0.0001$). ANOVA, analysis of variance; IL-33, interleukin-33.

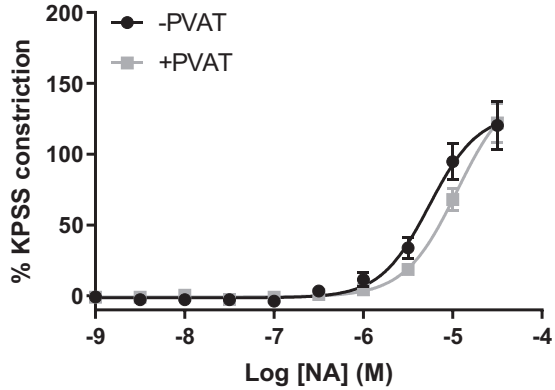

Fig. 8. The PVAT anticontractile effect is absent in eosinophil-deficient mice injected with IL-33. Small mesenteric arteries were isolated from ΔdblGATA-1 mice injected with IL-33 and subjected to increasing concentrations of NA. Data shown are means ± SE. Vessel contractility is expressed as a percentage of the maximum contraction evoked by KPSS. Two-way ANOVA followed by Bonferroni post hoc tests ($n = 7$). ANOVA, analysis of variance; IL-33, interleukin-33; KPSS, $[K^+]$PSS; NA, noradrenaline; PVAT, perivascular adipose tissue.

In summary, IL-33 induced eosinophilia in both lean and obese mice and restored PVAT function in obesity and enhanced the anticontractile function of PVAT in healthy animals. In addition, hypertension, blood glucose, and plasma insulin were normalized in obese mice. These data suggest that targeting the eosinophil numbers in PVAT presents a novel approach by which to influence cardiovascular and metabolic diseases in obesity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.N.S., S.B.W., R.G., and A.M.H. conceived and designed research; S.N.S., A.S.W., and R.J.P. performed experiments; S.N.S., A.S.W., and R.J.P. analyzed data; S.N.S., S.B.W., R.G., and A.M.H. interpreted results of experiments; S.N.S. prepared figures; S.N.S. drafted manuscript; S.N.S., S.B.W., and A.M.H. edited and revised manuscript; S.N.S., A.S.W., R.J.P., S.B.W., R.G., and A.M.H. approved final version of manuscript.

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