Beiging of perivascular adipose tissue regulates its inflammation and vascular remodeling

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Although inflammation plays critical roles in the development of atherosclerosis, its regulatory mechanisms remain incompletely understood. Perivascular adipose tissue (PVAT) has been reported to undergo inflammatory changes in response to vascular injury. Here, we show that vascular injury induces the beiging (brown adipose tissue-like phenotype change) of PVAT, which fine-tunes inflammatory response and thus vascular remodeling as a protective mechanism. In a mouse model of endovascular injury, macrophages accumulate in PVAT, causing beiging phenotype change. Inhibition of PVAT beiging by genetically silencing PRDM16, a key regulator to beiging, exacerbates inflammation and vascular remodeling following injury. Conversely, activation of PVAT beiging attenuates inflammation and pathological vascular remodeling. Single-cell RNA sequencing reveals that beige adipocytes abundantly express neuregulin 4 (Nrg4) which critically regulate alternative macrophage activation. Importantly, significant beiging is observed in the diseased aortic PVAT in patients with acute aortic dissection. Taken together, vascular injury induces the beiging of adjacent PVAT with macrophage accumulation, where NRG4 secreted from the beige PVAT facilitates alternative activation of macrophages, leading to the resolution of vascular inflammation. Our study demonstrates the pivotal roles of PVAT in vascular inflammation and remodeling and will open a new avenue for treating atherosclerosis.

Inflammation is deeply involved in the development of cardiovascular diseases, particularly in atherosclerosis as responses to vascular injury1–3. Temporal inflammation and its resolution are required for wound healing and repair after vascular injury, and prolonged excessive inflammation causes pathological vascular remodeling that manifests as neointimal hyperplasia4–7. It is widely accepted that the
pathogenesis of vascular remodeling starts from the impaired endothelial cell function, leading to recruitment of circulating inflammatory cells to the intima–media layer. The effects of potent vascular protective agents such as estrogen, statins and microRNAs have been mostly attributed to their beneficial functions on endothelial cells. There is also growing evidence suggesting that perivascular adipose tissue (PVAT), which accounts for the majority of the outer matrix surrounding systemic blood vessels, contributes to the pathophysiological vascular response in an ‘outside-in’ manner. Adipose tissues are classified into two distinct phenotypes; white adipose tissue (WAT), which stores energy as triglycerides, and brown adipose tissue (BAT), which dissipates energy as heat. A brown adipocyte-like phenotype has been reported to emerge within WAT in response to various stimuli such as cold exposure and β3-adrenergic receptor (β3AR) agonist. This unique phenomenon, called ‘browning’ or ‘beiging’, is controlled by transcriptional regulators, such as Prdm16, Nfat1 and Ebf2, and observed in humans as well as in rodents, contributing to energy homeostasis. Recent basic and clinical studies reported that activation of the PVAT surrounding coronary arteries show phenotypic changes in response to vascular inflammation, which can be detected by newly innovated computerized tomography-based methods. Although these findings suggest an association between PVAT phenotypic changes and vascular diseases, the causality and mechanisms involved remain unclear.

In this work, we examined the roles of PVAT in vascular inflammation and subsequent pathological remodeling. We show that vascular injury induces the beiging of PVAT, which fine-tunes inflammatory response and thus vascular remodeling as a protective mechanism.

Results
Endovascular injury induces PVAT beiging
We generated endovascular injury by inserting a wire into the femoral arteries (FAs) and examined inflammatory response in PVAT. Given the considerable effects of estrogen on the vasculature, male or ovariectomized female mice were used in the study. Genes of various types of immune cells were upregulated in PVAT at 24 h after vascular injury, including the macrophage-macrophage marker genes, Cd11b, Cd11c, Mrc1, and Adgre1 (F4/80) (Fig. 1a). Immunohistochemical staining revealed that F4/80+ macrophages accumulated predominantly in the outer tissues surrounding the vasculature, which were mainly comprised of PVAT, rather than in the vascular tissues, at the early phase (3 days) after injury (Fig. 1b, c). These F4/80+ macrophages then spread into the vascular tissues at the late phase (14 days) after injury (Fig. 1b, c). Concurrently, the expressions of inflammatory cytokine genes such as Tnf (TNFα), Serpinel1 (PAI-1), Ifnα and Il1b, were significantly upregulated in the surrounding tissues after vascular injury (Fig. 1d).

Transcriptome analysis of PVAT surrounding injured or sham-operated FAs showed that in addition to the increase in expression levels of several genes encoding immune cell markers and inflammatory cytokines, vascular injury induced a significant increase in expression levels of BAT marker genes including Ucp1, Cidea, Cox5b, Pparγc1a, Elovl3 and Dio2 in the PVAT (Supplementary Fig. 1a), which was confirmed by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis (Fig. 1e). Upregulation of Ucp1 in PVAT after vascular injury was confirmed by in situ hybridization analysis (Fig. 1f) and immunohistochemical analysis (Fig. 1g, h). Morphologically, adipocytes in PVAT after vascular injury possessed the beige/BAT characteristics including smaller cell size and multilocular lipid droplets compared with those after sham operation (Fig. 1g, i). Western blot analysis also showed the increase in Ucp1 expression in the outer tissues denuded from vessels rather than the remained arteries (Fig. 1j).

Then, we examined the role of infiltrated macrophages in the upregulation of Ucp1. Reduction of macrophages by the administration of clodronate liposome significantly attenuated upregulation of Ucp1 after vascular injury (Fig. 1k, l). The effects of ovariectomy on the expression levels of BAT markers, such as Ucp1 and Elovl3, were marginal, while the upregulation of these markers after vascular injury was more prominent in ovariectomized mice than in controls (Supplementary Fig. 1b). A significant upregulation of Ucp1 and the morphological shrinkage of adipocyte cell size in PVAT after vascular injury were observed in intact gonadal male mice (Supplementary Fig. 1c, d). Taken together, these results suggest that in male and ovariectomized female mice, vascular injury elicits the infiltration of macrophages in PVAT, which induces the change of PVAT into BAT-like phenotypes, resembling the ‘beiging’-phenomenon that is observed in subcutaneous and visceral WAT following exposure to cold or β3AR agonists.

PVAT beiging alters vascular inflammatory response
We next investigated the pathophysiological roles of the PVAT beiging in the development of vascular remodeling after injury using mice with an adipocyte-specific deletion of Prdm16 gene, an essential regulator of beiging, using Adipoq (Adiponectin) Cre−/−;Prdm16fl/fl mice, referred to as Adipoq−/−;Prdm16 mice. Prdm16 mRNA expression was >90% lower in adipose tissues such as thoracic aorta PVAT and intra-ocular BAT compared with Adipoq−/−;Prdm16 control littermates, despite comparable expression in other tissues such as lung and intestine (Supplementary Fig. 2a). Vascular injury induced more prominent remodeling accompanied by marked inflammation and suppressed beiging in the PVAT in both males and ovariectomized females of Adipoq−/−;Prdm16 mice compared with Adipoq−/−;Prdm16 mice at 14 days after injury (Fig. 2a, b and Supplementary Fig. 2b, c). To rule out the effects of Prdm16 deletion in adipose tissues of the whole body, we locally repressed Prdm16 by applying pluronic gel containing small interfering RNA (siRNA) against Prdm16 around FAs (Fig. 2a). A significant reduction of Prdm16 mRNA expression was confirmed in the outer tissues surrounding FA of wild type mice after siRNA treatment, whereas no difference was observed in contralateral untreated FA (Supplementary Fig. 2d). The Prdm16 siRNA treatment abolished PVAT beiging after vascular injury (Supplementary Fig. 2e) and exacerbated pathological intimal thickening 14 days after vascular injury (Fig. 2c).

Macrophages change their phenotypical and functional characteristics, termed macrophage polarization, in response to pro- or anti-inflammatory stimuli. Although the accumulation of F4/80+ cells was comparable between locally beiging-suppressed FA and control FA (Fig. 2c), the ratio of inducible nitric oxide synthase (iNOS)+ activated inflammatory macrophages to CD206+ alternatively activated anti-inflammatory macrophages was significantly increased in the beiging-suppressed FA PVAT as compared with that of control FA PVAT 14 days after vascular injury (Fig. 2c). These significant differences were not observed during the early phase (3 days) after injury (Supplementary Fig. 3a, b), suggesting that inhibition of PVAT beiging exacerbates pathological remodeling after vascular injury by prolonged activation of pro-inflammatory macrophages.

The effects of systemic activation of WAT beiging by exposure to cold or β3AR agonists on vascular pathogenesis in vivo remain controversial and depend on the experimental models studied. These conflicting results are considered to come from direct and/or indirect effects of systemic activation of beiging on vascular remodeling, such as brown/beige fat-mediated lipolysis, release of anti-inflammatory adipokines and glucose homeostasis. Therefore, we locally activated beiging of the FA PVAT using pluronic gel containing the specific β3AR agonist, CL316243 and evaluated the specific effects of PVAT beiging on the phenotypic transition of macrophage and vascular remodeling. Local β3AR activation significantly induced PVAT beiging in the outer surrounding tissue of FA, but not in the contralateral untreated FA, visceral WAT or BAT (Supplementary Fig. 3c), and significantly attenuated vascular intimal thickening 14 days after injury with a shift of macrophage polarization to anti-inflammatory phenotype in FA PVAT (Fig. 2d and Supplementary Fig. 3d). These significant
differences were not observed during the early phase after injury (Supplementary Fig. 3a, b). In contrast, the beiging in PVAT elicited by vascular injury was attenuated by a β3AR antagonist (SR59230A) with pluronic gel (Supplementary Fig. 3e). These data suggest that pharmacological beiging stimulation exerts additional protection against pathological remodeling after vascular injury by suppression of prolonged activation of pro-inflammatory macrophages, and that the beiging in PVAT after the injury is mediated by the β3AR signaling.

Then, we examined the interactions between PVAT-derived beige adipocytes and macrophages. When RAW 264.7 macrophage cells or bone marrow-derived macrophages (BMDMs) were cultured in medium conditioned by beige adipocytes differentiated from the stromal
vascular fraction of PVAT, expression levels of Mrc1 (CD206) were increased and those of inflammatory cytokines such as Tnf, Serpinel, and Il6b were decreased (Fig. 3a–d). These changes were abrogated by inhibition of PVAT beiging by siRNA knockdown of Prdm16 (Fig. 3e, f and Supplementary Fig. 3f). We further examined the effects of PVAT beiging on cell growth of macrophages. Culture medium conditioned by PVAT-derived beige adipocytes significantly decreased the number of RAW 264.7 macrophages activated by interferon-gamma (IFNγ) and lipopolysaccharide (LPS), but not that of macrophages alternatively activated by interleukin (IL) 4 (Fig. 3g and Supplementary Fig. 3g). Culture medium conditioned by PVAT-derived adipocytes with knockdown of Prdm16 did not decrease the number of classically activated macrophages induced by IFNγ and LPS (Fig. 3g). Taken together, these results suggest that factors secreted from PVAT-derived beige adipocytes selectively inhibit the growth of classically activated macrophages and shift macrophage phenotypes into an alternatively activated state, resulting in anti-inflammatory effects.

NRG4 as an anti-inflammatory factor

Some studies have shown the contributions of UCP1 to atherogenesis. Therefore, we first examined UCP1 as a possible regulator of vascular remodeling after injury. Unexpectedly, vascular wall thickening 14 days after injury was comparable between Ucp1+/− and littermate wild type (Ucp1+/+) mice (Fig. 3h), suggesting that UCP1 is not involved in the beneficial effects of PVAT beiging on vascular remodeling.

To identify the key factors that control endovascular injury-induced inflammation in PVAT, we performed transcriptional RNA profiling using single-cell RNA sequencing (scRNA-seq) and subsequent computational analysis using publicly available scRNA-seq datasets of murine inguinal WAT (iWAT) treated with CL316243 or control (GSE 133486). Cluster analysis of uniform manifold approximation and projection (UMAP) dimensionality reduction after integration with Seurat revealed that the cells of iWAT were classified into 15 clusters (C0–14) and that C7(seurat) mostly consisted of CL316243-treated cells (Fig. 4a, b and Supplementary Fig. 4a). Beige/brown adipocyte-specific genes such as Ucp1, Cidea and Pparpg1a, were predominantly expressed in C7(seurat) (Fig. 4c and Supplementary Figs. 4b, 5), indicating that this cluster represents beige adipocytes induced by β3AR activation. The emergence of the beige adipocyte cluster by β3AR activation with CL316243 was also confirmed using another integration method, Harmony (C0(harmony), Supplementary Figs. 6a–f and 7). In addition, the cell type deconvolution analysis of the bulk RNA-seq dataset of iWAT (GSE129083) showed that the beige adipocyte population appeared in iWAT treated with CL316243 (Supplementary Table 2). Furthermore, the analysis of another dataset (GSE 133486) demonstrated that β3AR activation with cold stimuli also induced the emergence of a beige adipocyte population (C9(cold)), Supplementary Figs. 5a–e and 9). Gene ontology analysis for the significantly regulated genes in C7(seurat) identified Nrg4 as a highly expressed secretory factor involved in the molecular function of receptor-binding (Fig. 4c, d). The enrichment of Nrg4 was also detected in C6(harmony) and C9(cold) ( Supplementary Figs. 6d, e and 8c, d).

Consistent with the findings from the comprehensive data analysis, we observed that Nrg4 was upregulated in PVAT after vascular injury (Fig. 4e) and in cultured PVAT-derived preadipocytes stimulated by beige adipocyte differentiation factors, accompanied by other beige genes in a time-dependent manner (Fig. 4f). The upregulation of Nrg4 in PVAT surrounding injured FAs was also observed 14 days after injury by in situ hybridization analysis (Supplementary Fig. 10a), where Nrg4 was expressed in beige adipocytes of PVAT expressing Ucp1 (Supplementary Fig. 10b). Nrg4 upregulation was significantly attenuated in AdipoCre+;Prdm16 mice and PVAT-derived preadipocytes transduced with siRNA against Prdm16 or Nfia (Fig. 4g, h), whereas it occurred independently of UCP1 (Supplementary Fig. 10c). NRG4 has recently emerged as a brown fat-enriched secreted factor that ameliorates diet-induced metabolic disorders, including insulin resistance and hepatic steatosis. We thus examined whether NRG4 was involved in beige adipocytes-induced resolution of macrophage inflammation. Culture media conditioned by PVAT-derived beige adipocytes induced phenotypic changes from classical to alternative activation and downregulated expression of inflammatory genes in RAW 264.7 macrophages or BMDMs, but these changes were not observed by the culture media conditioned by PVAT-derived beige adipocytes with knockdown of Nrg4 by siRNA (Supplementary Fig. 10d and Fig. 4i–l). Consistently, recombinant murine NRG4 attenuated the Cd68/Mrc1 ratio and inflammatory cytokine levels in macrophages classically activated (Supplementary Fig. 10e, f). The knockdown of Erb-B2 receptor tyrosine kinase 4 (ErbB4), a receptor of NRG4, in macrophages abolished the alternative activation and anti-inflammatory effects of culture media conditioned with PVAT-derived beige adipocytes (Supplementary Fig. 10g–l). Furthermore, culture media conditioned by PVAT-derived beige adipocytes with Nrg4 knockdown no longer inhibited the increase in the number of RAW 264.7 cells activated by IFNγ and LPS (Fig. 4m). In mice, Nrg4 siRNA treatment with pluronic gel was found to exacerbate pathological intimal thickening 14 days after vascular injury (Fig. 4n). Taken together, these results suggest that NRG4 secreted from PVAT-derived beige adipocytes and immunohistochemical staining for UCP1 in injured (3 and 14 days after injury) or sham-operated FAs and outer tissue. Scale bars represent 100 μm (thick bars) and 50 μm (thin bars). Images are representative of three independent experiments. h Time course of UCP1+ cell accumulation in PVAT (n = 3, 4, 7, 7, 6 at each group, respectively, one-way ANOVA followed by Tukey–Kramer post-hoc test). i Histograms of adipocyte area of sham-operated or injured PVAT (14 days after injury). Three images of each biological replicate were analyzed and combined to create the histogram. The size distribution between each group was compared using Kolmogorov-Smirnov test. Each bin was normalized to a percent of the total count for that individual tissue. Adipocytes of the bin size in the range of 20-500 μm² were included for the analysis. j Representative images of western blots for UCP1 in arteries (vessel) and outer tissues mainly composed of PVAT harvested 48 h after injury. Intra-scapular BAT was used as a positive control. GAPDH was used for internal control (n = 4 for each group, representative images are shown). k Immunohistochemical staining for F4/80 and UCP1 in outer tissue surrounding FAs 14 days after injury was performed in mice treated with either clodronate or vehicle. Scale bars represent 50 μm. Images are representative of three independent experiments. fF4/80+ and UCP1+ area in PVAT (vehicle, n = 9; clodronate, n = 4, unpaired two-tailed Student’s t test). Data represent mean ± SEM. Source data are provided as a Source Data file.

Fig. 1 | Endovascular injury-induced macrophage accumulation and inflammation in PVAT followed by upregulation of BAT markers. a Gene expressions of immune cell markers in PVAT 24 h after vascular injury (sham, n = 4 for Cdl, 6 for others; injury, n = 9, two-tailed t tests with Holm-Sidak’s correction for multiple comparisons). b Haematopoietin and eosin (H&E), Elastica van Gieson (EVG) and immunohistochemical staining for F4/80 in the early (day 3) and late (day 14) phases after injury. Blue dashed lines indicate the external elastic lamina. Scale bars represent 100 μm (thick bars) and 50 μm (thin bars). Images are representative of three independent experiments. c Time course of F4/80+ cell accumulation in arteries (vessel) and outer tissues mainly composed of PVAT (n = 3, 4, 7, 7, 6 (from left to right) at each group, respectively, one-way analysis of variance (ANOVA) and Tukey–Kramer post-hoc test). d Gene expressions of inflammatory cytokine markers in PVAT 48 h after vascular injury (sham, n = 12, 14, 12, 12, 8, 8, respectively; injury, n = 10, 12, 7, 7, 8, 8, respectively; two-tailed t tests with Holm-Sidak’s correction for multiple comparisons). e Gene expressions of BAT (Ucp1 and Cldut) and VAT (Rtn3 (Resistin) and Cdf (Adipsin) markers in PVAT at 24 and 48 h after injury (sham, n = 6, 8, 8, respectively; injury 24 h, n = 6, 6, 8, 8, respectively; injury 48 h, n = 11, 7, 6, 6, respectively, one-way ANOVA followed by Tukey–Kramer post-hoc test). f In situ hybridization showing Ucp1 mRNA expression 14 days after vascular injury in wild type mice. Scale bars represent 50 μm (thick bars) and 20 μm (thin bars). Images are representative of three independent experiments. g H&E and immunohistochemical staining for UCP1 in injured (3 and 14 days after injury) or sham-operated FAs and outer tissue. Scale bars represent 100 μm (thick bars) and 50 μm (thin bars). Images are representative of three independent experiments. h Time course of UCP1+ cells accumulation in PVAT (n = 3, 4, 7, 7, 6 at each group, respectively, one-way ANOVA followed by Tukey–Kramer post-hoc test). i Histograms of adipocyte area of sham-operated or injured PVAT (14 days after injury). Three images of each biological replicate were analyzed and combined to create the histogram. The size distribution between each group was compared using Kolmogorov-Smirnov test. Each bin was normalized to a percent of the total count for that individual tissue. Adipocytes of the bin size in the range of 20-500 μm² were included for the analysis. j Representative images of western blots for UCP1 in arteries (vessel) and outer tissues mainly composed of PVAT harvested 48 h after injury. Intra-scapular BAT was used as a positive control. GAPDH was used for internal control (n = 4 for each group, representative images are shown). k Immunohistochemical staining for F4/80 and UCP1 in outer tissue surrounding FAs 14 days after injury was performed in mice treated with either clodronate or vehicle. Scale bars represent 50 μm. Images are representative of three independent experiments. fF4/80+ and UCP1+ area in PVAT (vehicle, n = 9; clodronate, n = 4, unpaired two-tailed Student’s t test). Data represent mean ± SEM. Source data are provided as a Source Data file.

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inhibits the growth of classically activated macrophages and induces phenotypic shift to an alternatively activated state, leading to accelerated resolution of macrophage inflammation, resulting in attenuation of pathological vascular remodeling after injury.

**PVAT beiging is detectable in human AAD**

Acute aortic dissection (AAD) is the acute destruction of aortic wall that accompanies severe local inflammation\(^42\). We examined whether beiging was recognized in PVAT of human aorta with AAD. In addition to the accumulation of macrophages, protein expression levels of markers for beige cells, such as UCP1 and CIDEA, and NRG4 were significantly increased likely as a compensatory response in the PVAT of AAD lesions compared with the PVAT without AAD (Fig. 5a, b and Supplementary Fig. 11a, b). To elucidate the role of PVAT beiging in AAD, we used an AAD murine model with the systemic administration of angiotensin II, β-aminopropionitrile, and N-nitro-L-arginine methyl ester\(^43\)–\(^45\). CL316243 administration significantly reduced death due to dissection or rupture of the aorta (Fig. 5c), concomitant with the shift
of macrophage phenotypes in PVAT to the alternatively activated state evaluated by fluorescence-activated cell sorting (FACS) analysis (Fig. 5d, e and Supplementary Fig. 12). These findings suggest that beiging occurs in the PVAT of the human aorta during acute dissection and may regulate the inflammatory response during the development of AAD.

**Discussion**

Vascular damage provokes regional inflammation and prolonged inflammation leads to pathological vascular remodeling. The balance of pro- and anti-inflammation is critical for successful vascular wound healing and clinical outcomes. The primary process of inflammation resolution is accomplished by the phenotypic conversion of pro-inflammatory macrophages into anti-inflammatory macrophages. However, the mechanisms involved in this conversion have not yet been fully elucidated. This study revealed that endovascular injury induced 'beiging' of regional PVAT, and that inhibition of PVAT beiging exacerbated thickening of the intima after injury accompanied by an increased accumulation of pro-inflammatory macrophages, while activation of beiging conversely attenuated pathological vascular remodeling and accumulation of pro-inflammatory macrophages. These findings suggest that beiging of PVAT plays a pivotal role in changing the initial inflammation phase to its resolution after vascular injury. Various studies have suggested that endothelial dysfunction leads to the recruitment of circulating monocytes to the intima–medial layer. In our study, the marked accumulation of monocytes was also identified in the outer tissues surrounding the vasculature from the early phase after vascular injury. The infiltrated monocyte-macrophages elicited PVAT beiging, resulting in the suppression of the excessive vascular inflammatory response to the injury. These data indicate a pivotal role of the ‘outside-in’ manner in wound healing after vascular injury.

Local activation of PVAT beiging attenuated pathological vascular remodeling after injury, suggesting that PVAT acts directly on nearby arteries and mediates the pathophysiological response to vascular damage in a paracrine manner. We identified NRG4 as an anti-vascular remodeling factor derived from beige PVAT. NRG4 is a member of the NRG protein family, which acts via ErbB receptor tyrosine kinases. This molecule is highly expressed in the pancreas, skeletal muscles and BAT and protects against diet-induced insulin resistance and hepatic steatosis through regulating hepatic lipogenic and cytoprotective signaling.

Recent studies have shown the expression of functional ErbB receptors on innate immune cells such as macrophages, dendritic cells and neutrophils, and the NRG4-ErbB4 axis exerts anti-inflammatory effects in macrophages by promoting apoptosis of classically activated macrophages but not alternatively activated macrophages. Consistent with these findings, medium conditioned by PVAT-derived beige adipocytes significantly inhibited the number of classically activated macrophages, which was abolished by Nrg4 knockdown. Reduced levels of NRG4 have been reported to be associated with increased carotid intimal thickness, increased angiographic severity of coronary artery disease and acute coronary syndrome.

In this study, PVAT beiging was detected in human aorta with acute dissection, suggesting that NRG4 secreted from beige PVAT might protect human aorta against AAD. The lineage of the beige cells that emerged in the PVAT was not examined in this study. Reportedly, smooth muscle cell has been considered a possible lineage of origin for PVAT, and Angueira et al. have shown an increase in Myh11 smooth muscle cell-derived adipocytes and thoracic PVAT after treatment with rosiglitazone. Since rosiglitazone reportedly recruits beige cells in fat tissues, the beige cells in PVAT may be originated from smooth muscle cells. Lineage tracing studies using inducible genetic techniques may provide further biological and functional insights into the beiging phenomenon in the PVAT. Our data showed that the beiging after injury was suppressed by the macrophage depletion or the local β3AR inhibition, implying that the PVAT beiging was elicited via β3AR signaling mediated by infiltrated macrophages. Meanwhile, the involvement of other signaling pathways of beiging such as succinate metabolisms and BMP4 in the beiging process in PVAT after injury remains to be elucidated. In conclusion, the present study revealed that PVAT beiging plays a critical role in the vascular inflammatory response to injury by controlling macrophage inflammation and its resolution and suggests that PVAT beiging and NRG4 may be novel targets of vascular injury including AAD.

**Methods**

**Animal studies**

All animal procedures were approved by the University of Tokyo Ethics Committee for Animal Experiments and strictly adhered to the guidelines for animal experiments at the University of Tokyo. Male or, unless otherwise specified, ovariecotomized female mice were used for the study. All wild type C57BL/6J mice were purchased from CLEA Japan. Adipoq+/− mouse (B6.FVB-Tg[Adipox-Cre]Evd/J, #10803), Prdm16fl/fl homozygous mice (B6.129-Prdm16+/−J, #024992) and Ucp1tm1Kz/− mice (B6.129-Ucp1tm1Kz/J, #003124) were purchased from Jackson Laboratory. All mice were maintained in specific pathogen-free conditions in the animal facilities of the University of Tokyo. They were housed in a controlled environment with a 12 h light/12 h dark cycle at a maintained temperature and kept with free access to food and water throughout the whole experiment period. Ambient room temperature was regulated at 23 ± 2 °F and humidity was controlled at 50 ± 10%. Endovascular injury of femoral arteries (FA) was performed after being inserted. Arteries and outer tissues surrounding the vasculature were collected. Ovariectomies were performed in 8–10-week-old female mice 2 weeks before the endovascular injury, as previously described. To selectively remove the macrophages, mice were given intraperitoneally clodronate liposomes.
Fig. 3 | Beige-differentiated PVAT shifts macrophage phenotypes to alternatively activated state. a–d Cultured preadipocytes isolated from the PVAT stromal vascular fraction were differentiated into beige adipocytes, and media conditioned by the cells were added to RAW 264.7 macrophage cells (a, b) or bone marrow-derived monocytes (BMDMs) (c, d). After 48 h, mRNA was extracted from the RAW 264.7 cells or BMDMs and qRT-PCR was performed. The gene expression levels of macrophage phenotype markers [Cd86 (classically activated), Mcr1 (CD206, alternatively activated) and ratio of Cd86 to Mcr1, (a, c)] and inflammatory cytokines [Tnf, Serpine1, Ccl2, and Ifb] (b, d) were analyzed. Control cells were treated with media cultured in the absence of adipocytes (a n = 6 biological replicates, representative data of three different culture lines are shown, two-tailed t tests with Holm-Sidak’s correction for multiple comparisons, b–d n = 4 biological replicates, representative data of three different culture lines are shown, two-tailed t tests with Holm-Sidak’s correction for multiple comparisons). e–f PVAT-preadipocytes were introduced by siRNA of Prdm16 or scrambled and stimulated with beige differentiation factors. Culture media conditioned by these cells were added to RAW 264.7 cells and mRNA was extracted 48 h after treatment. The results of the qRT-PCR analyses of the gene expression levels of macrophage phenotype markers (e) and inflammatory cytokines (f) are shown (n = 4, biological replicates, representative data of three different culture lines are shown, two-tailed t tests with Holm-Sidak’s correction for multiple comparisons). g Cell growth analysis of RAW 264.7 cells 72 h after treatment with culture media conditioned by PVAT-derived beige adipocytes. RAW 264.7 cells were pre-treated with IFNγ (20 ng/ml) and LPS (20 ng/ml) or IL4 (10 ng/ml) for 24 h and treated with media cultured in the absence of adipocytes (gray dots), culture media conditioned by adipocytes (red dots) and culture media conditioned by adipocytes introduced by Prdm16 siRNA (blue dots) (n = 4 biological replicates, representative data of three different culture lines are shown, one-way ANOVA followed by Tukey-Kramer post-hoc test). h EVG staining in the FA 14 days after injury in Ucp1+/+ and wild type (Ucp1−/−) mice. Scale bars represent 100 μm. The ratio of intima to media area (intima/media) and % intima–media area in the area surrounded by the external elastic lamina (area of intima–media) were analyzed (Ucp1+/+, n = 8; Ucp1−/−, n = 10, unpaired two-tailed Student’s t test). Data represent mean ± SEM. Source data are provided as a Source Data file.
L-NAME (100 mg/kg per day; Cayman Chemical Co.) was administered orally with drinking water at the same time as pump implantation until the end of the experiment. For being activation studies in AAD murine model, β3AR agonist (CL316243, 1 mg/kg/day; Sigma-Aldrich) or vehicle PBS was administered daily for seven days using the Alzet osmotic minipump.

Local administration of reagents
Pluronic F-127 gel (Sigma-Aldrich) is a thermoreversible gel, which is liquid at refrigerated temperatures (4–5 °C), but gel upon warming to room temperature. The gelation is reversible upon cooling. For local gene knockdown studies, 0.2 nmol/mouse Ambion in vivo pre-designed siRNA against Prdm16 (s89042), Nrg4 (s206928), or...
negative control mixed with Inivofectamine 3.0 reagent (Thermo Fisher Scientific) was dissolved in pluronic gel on ice and administered locally in PVAT of the injured artery. For β3AR signal activation or inhibition studies, β3AR agonist (CL316243, 0.01 mg/kg; Sigma-Aldrich), β3AR antagonist (SR52930A, 0.02 mg/kg; Sigma-Aldrich), vehicle PBS, or vehicle dimethyl sulfoxide was dissolved in pluronic gel and administered in the PVAT.

**Histology and immunohistochemistry**

Arteries including outer tissues fixed in Ufx (Sakura Finetek), were embedded in paraffin and sectioned into 4-μm-thick sections. Specimens were then stained by hematoxylin and eosin (H&E), and Elastic van Gieson (EVG) methods. The lumen, internal elastic lamina (IEL), and external elastic lamina (EEL) were defined, and the intimal (the tissue between lumen and IEL) and medial (tissue between IEL and EEL) areas were recorded using National Institutes of Health (NIH) ImageJ software. To evaluate vessel remodeling, the ratio of intima to media area (intima/media) and the % intima–media area = (IEL area + (luminal area/EEL area) × 100 (%) (% area of intima–media) were calculated as previously described. To evaluate adipocyte area, PVAT samples were stained with H&E and imaged with DP70 (Olympus). Uncompressed tiff files were analyzed for adipocyte area by Adipo-soft plugin (v. 1.6) for NIH ImageJ.

**Cell culture**

RAW 264.7 cells (European Collection of Authenticated Cell Culture), BMDMs, and PVAT-derived adipocytes were cultured at 37°C in DMEM/F12 containing 1% streptomycin, 1% penicillin, and 10% fetal bovine serum (FBS) (Cosmo Bio) and in 40 ng/mL of recombinant mouse M-CSF (576406; BioLegend) for 7 days, as previously described. The differentiated BMDMs were detached from plates using TrypLE (Thermo Fisher Scientific) and replated into 12-well tissue culture dishes at a density of 5 × 10^5 cells per well prior to cell stimulation.

**Laser scanning confocal microscope**

Laser scanning confocal microscope (LSM880, Zeiss). The number of positive dots was calculated using NIH ImageJ.
Differentiation of PVAT stromal vascular fraction to beige cells

PVAT-derived preadipocytes were differentiated to beige cells according to the previous reports. Briefly, cells that reached confluence were treated for 48 h in medium containing 10% FBS, 850 nM insulin, 0.5 mM isobutylmethylxanthine, 125 nM indomethacin, 1 μM dexamethasone, 1 nM 3,3′,5-Triiodo-L-thyronine (T3) and 0.5 μM rosiglitazone. After 48 h treatment, the cell culture media were switched to media containing 10% FBS, 850 nM insulin, 1 nM T3 and 0.5 μM rosiglitazone and incubated for additional 5 days. Six to eight days after adding the induction medium, cells are fully differentiated to mature beige fat cells and are filled with oil droplets. Culture media conditioned by PVAT-derived beige adipocytes were added to RAW 264.7 cells, and mRNA expression of RAW 264.7 cells 24 h after stimulation was examined. For siRNA-mediated gene knockdown experiments in...
cultured PVAT-derived adipocytes, siRNAs against Prdm16, Nrg4, ErbB4 (s89042, s206928, s201303, respectively. Silencer select pre-designed siRNA, Thermo Fisher Scientific). Nfat (sc-36045, Santa Cruz Biotechnology) and negative control (Thermo Fisher Scientific) were transfected with Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific) 2 days before confluence and following every 48 h, according to the manufacturer’s instructions.

Western blotting
Tissues were lysed in RIPA buffer containing 0.1% sodium dodecyl sulfate, 1% Triton X-100, 0.5% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-Cl (pH 8.0), 1 mM EDTA supplemented with protease inhibitor (Roche). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride membrane, and detected with the antibodies of anti-UCP1 (Abcam, ab23841, 1:1000) and anti-GAPDH (Cell Signaling Technology, #2118, 1:500).

Quantitative reverse transcription-polymerase chain reaction
Total RNA from tissue samples was extracted using the RNeasy Mini Kit (Qiagen). Superscript VILO cDNA synthesis kit (Invitrogen) was used to generate cDNA, qRT-PCR was performed on QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific) using Thunderbird SYBR qPCR Mix (Toyobo). The specific primers are listed in Supplementary Table 1. Relative expression levels of target genes were calculated using the comparative CT method. Each sample was run in duplicate, and the results were systematically normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for experiments using cultured cells or β2 microglobulin (B2m) for experiments using mouse tissues.

Bulk RNA-seq data processing
Outer tissues surrounding the FAs 48 h after injury or sham operation (n = 2 for each group) were collected. Total RNA (480 ng) from each sample was used to generate RNA-seq libraries using TruSeq Strandless mRNA Library Prep Kit (Illumina). 150 bp paired-end sequencing reads were obtained using Illumina HiSeq 2500 platform. The Fastq sequences were initially checked for quality through the FastQC program v0.11.8. Adaptor sequences were removed by Cutadapt v1.18. Sequencing reads from RNA-seq libraries were then trimmed using Trimmomatic v0.39, where reads with alignment quality < Q33 were discarded, and aligned to the mouse reference genome (mm9) using STAR aligner v2.6.0a. RSubread-2.0.1-FeatureCounts software for quantification and DESeq2 v1.28.0 software for differential analysis were used on R v3.6.0.

Single-cell RNA-seq data analysis
scRNA-seq data from publicly available datasets of mouse inguinal white adipose tissue (iWAT) were used. Among the original database that contains two types of scRNA-seq data (AdipoCre-; Il10ra ox/ox mice and AdipoCre+; Il10ra ox/ox mice), the data from AdipoCre+; Il10ra ox/ox mice were used for the present analysis. The standard Seurat (V.4.0.1) for R (V.4.0.2) protocol ("FindIntegrationAnchors" and "IntegrateData") was used for the integration 76. To observe the effects of CL316243 treatment, an alternative integration step was performed with Harmony (V.0.1). 77 All sequenced cells were projected onto two dimensions using UMAP on R v.4.0.2. The optimal number of principal components used for UMAP dimensionality reduction was determined using the Jackstraw permutation approach and a grid-search of the parameters. Cells were divided into several clusters and color-coded according to cell types. Cells were also color-coded for the treatment group (CL316243 or cold stimulation) (pink) or control (turquoise). Marker genes were searched using the FindMarkers function in Seurat. Gene Ontology analysis was performed using Database for Annotation, Visualization, and Integrated Discovery ver. 6.8 (DAVID 6.8) 78. Furthermore, the gene signature from the scRNA-seq data was used to deconvolute public bulk RNA-seq data to confirm the validity of the cell type clustering performed in this scRNA-seq re-analysis. We confirmed that the beige adipocytes also exist in iWAT treated with CL316243 in another public bulk RNA-seq study (GSE129083) 79 using MeSiC, a method for predicting cell abundance based on single-cell data 80.

Fluorescence-activated cell sorting analysis
After the isolation of stromal vascular fraction of PVAT, cells were suspended in PBS containing 5% FBS, Zombie NIR Fixable Viability Kit (BioLegend) was used to label dead cells according to the manufacturer’s instructions. Nonspecific binding of the antibodies to Fc receptors was blocked by using Mouse Fc receptor-blocking agent (BioLegend). Cells were incubated at room temperature 30 mins with FITC anti-mouse CD45 Antibody (BioLegend, #103107, 1:50), PE/Cyanine7 anti-mouse F4/80 antibody (BioLegend, #123113, 1:50), PE anti-mouse CD11b antibody (BioLegend, #101207, 1:50), APC anti-mouse CD206 antibody (BioLegend, #141707, 1:50), and Brilliant Violet 421 anti-mouse CD11c antibody (BioLegend, #117329, 1:50), then centrifuged and fixed with PBS containing 2% PFA. Cells were analyzed using a FACSMelody cell sorter (BD Biosciences). For the analysis of flow cytometry data, CD11c+, CD11b+, and F4/80+ cells were defined as macrophages, which could be further differentiated into classically (M1; defined as CD11c−, CD206+) and alternatively activated (M2; defined as CD11c−, CD206+) macrophages 81. Data analyses were performed using FlowJo software (Tree Star).

Statistics and reproducibility
Data were presented as means ± standard error of mean (SEM). All statistics are described in figure legends. In general, comparisons between two groups were performed using the two-tailed Student’s t test or Mann–Whitney U-test, and multiple group comparisons were performed by one-way ANOVA followed by Tukey–Kramer post-hoc test. Survival curves were created using the Kaplan–Meier method and compared by a log-rank test. All statistical analyses were performed using the Prism software (GraphPad). P-values of <0.05 were considered statistically significant.

Human studies
Pathological records were reviewed, and formalin-fixed paraffin-embedded descending aorta tissues were obtained from the archives of the Department of Pathology, The University of Tokyo Hospital. The aortic specimens of AAD were obtained by the autopsy of six patients who died of AAD, five males and one female, aged between 45 and 84 years; four of these patients had hypertension, one patient had suffered from a previous stroke, and three had a smoking history. The aortic specimens of controls were autopsy specimens from five patients who died of non-aortic causes, four males and one female, aged between 44 and 78 years; one patient had hypertension, one patient suffered from dyslipidemia, one with chronic kidney disease, one suffered from a previous stroke, and one had a smoking history. Patients with an apparent history of inherited aortic diseases such as Marfan syndrome were not included in the AAD cases and controls. The inclusion and exclusion criteria for patients with AAD were as follows: AAD cases were selected from consecutive pathological autopsies from 2006 to 2017 with a description of aortic dissection as a finding in autopsy report. Controls were selected from consecutive pathological autopsies from 2014 to 2017 with no significant changes in the aortic wall based on the autopsy. The immunostaining using primary antibodies against CD204 (Transgenic, KT022, 1:500) was performed using a Ventana Benchmark automated stainer (Ventana Medical Systems). The study followed the principles outlined in the Declaration of Helsinki. This study was approved by the ethics committee of the University of Tokyo (approval ID-20200019NI), and
written informed consent was waived because this is a retrospective study using existing tissue blocks. Instead, we used an opt out approach to provide participants with an informed choice about participation, although no patient in the cohort for screening used the opt out option.

**Reporting summary**
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**
Source data are provided with this paper. The RNA-seq data have been deposited in GSE206399, GSE133486, and GSE129083. The information of mouse genome (mm9) is available on UCSC Genome Browser (http://genome.ucsc.edu). The authors declare that all data are available in the article file and Supplementary information files, or available from the authors upon reasonable request. Source data are provided with this paper.

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Author contributions
Y.A., K.U., and I.K. conceived the project, designed the study, and interpreted the results.; Y.A. and K.U. performed in vivo and in vitro experiments with the help of S.N., M.Katoh, M.Katagiri, M.Hashimoto, B.Z., G.N., A.O., Y.H., H.W., and E.T.; Y.A. and K.U. analyzed RNA-seq data under the supervision of S.N., K.I., and S.Y.; M.Hinata collected the human samples and clinical histories of the patients and helped immunohistochemistry under the supervision of T.U.; Y.A., K.U., and I.K. performed data analysis and wrote the manuscript under the supervision of S.N., K.I., N.T., H.M., T.Y., and E.T.; All authors contributed to the article and approved the submitted version.

Competing interests
The authors declare no competing interests.

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