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Role of chemokine RANTES in the regulation of perivascular inflammation, T-cell accumulation, and vascular dysfunction in hypertension.

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ABSTRACT **Recent studies have emphasized the role of perivascular inflammation in cardiovascular disease. We studied mechanisms of perivascular leukocyte infiltration in angiotensin II (Ang II)-induced hypertension and their links to vascular dysfunction. Chronic Ang II infusion in mice increased immune cell content of T cells (255 ± 130 to 1664 ± 349 cells/mg; $P < 0.01$), M1 and M2 macrophages, and dendritic cells in perivascular adipose tissue. In particular, the content of T lymphocytes bearing CC chemokine receptor (CCR) 1, CCR3, and CCR5 receptors for RANTES chemokine was increased by Ang II (CCR1, 15.6 ± 1.5% *vs.* 31 ± 5%; $P < 0.01$). Hypertension was associated with an increase in perivascular adipose tissue expression of the chemokine RANTES (relative quantification, 1.2 ± 0.2 *vs.* 3.5 ± 1.1; $P < 0.05$), which induced T-cell chemotaxis and vascular accumulation of T cells expressing the chemokine receptors CCR1, CCR3, and CCR5. Mechanistically, RANTES$^{-/-}$ knockout protected against vascular leukocyte, and in particular T lymphocyte infiltration (26 ± 5% in wild type Ang II *vs.* 15 ± 4% in RANTES$^{-/-}$), which was associated with protection from endothelial dysfunction induced by Ang II. This effect was linked with diminished infiltration of IFN-γ-producing CD8$^+$ and double-negative CD3$^+$CD4$^-$CD8$^-$ T cells in perivascular space and reduced vascular oxidative stress while FoxP3$^+$ T-regulatory cells were unaltered. IFN-γ *ex vivo* caused significant endothelial dysfunction, which was reduced by superoxide anion scavenging. In a human cohort, a significant inverse correlation was observed between circulating RANTES levels as a biomarker and vascular function measured as flow-mediated dilatation ($R = -0.3$, $P < 0.01$) or endothelial injury marker von Willebrand factor ($R = +0.3$; $P < 0.01$). Thus, chemokine RANTES is important in the regulation of vascular dysfunction through modulation of perivascular inflammation.—Mikolajczyk, T. P., Nosalski, R., Szczepaniak, P., Budzyn, K., Osmenda, G., Skiba, D., Sagan, A., Wu, J., Vinh, A., Marvar, P. J., Guzik, B., Podolec, J., Drummond, G., Lob, H. E., Harrison, D. G., Guzik, T. J. Role of chemokine RANTES in the regulation of perivascular inflammation, T-cell accumulation, and vascular dysfunction in hypertension. *FASEB J.* 30, 1987–1999 (2016). www.fasebj.org**

Key Words: blood pressure · endothelial function · vascular inflammation · immune activation · superoxide

Traditionally, adipose tissue (AT) has been considered a site for energy storage; however, there is increasing interest in the role of AT in inflammation (1–3). Cells of the innate and adaptive immune system, such as macrophages and lymphocytes, accumulate in visceral AT but minimally in subcutaneous AT (4). Visceral AT has often been viewed

Abbreviations: ACh, acetylcholine; Ang II, angiotensin II; AT, adipose tissue; BAT, brown adipose tissue; CCL, CC chemokine ligand; CCR, CC chemokine receptor; DC, dendritic cell; FMD, flow-mediated dilatation; met-RANTES, methionylated RANTES; PEG-SOD, polyethylene glycol superoxide dismutase; pVAT, perivascular adipose tissue; T_h, T helper; vWF, von Willebrand factor; WT, wild-type

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as a homogenous; however, AT adjacent to large- and medium-size vessels—that is, perivascular AT (pVAT)—seems to represent a specialized compartment from which release of fatty acids, adipokines, and other mediators exert both beneficial and untoward effects on the adjacent vessels (5–7). Alterations of pVAT structure and function in genetically hypertensive rats have been implicated in altering vasomotor tone (6), but the role of pVAT in regulation of vascular function and vascular inflammation in hypertension remains unclear.

Recently we found that hypertensive stimuli such as angiotensin II (Ang II) and excess salt promote infiltration of leukocytes, including T cells, into pVAT (8). Subsequent studies have shown that T-cell (9–11), monocyte (12), and NK-cell (13) activation and recruitment into target organs is very important in the pathogenesis of hypertension (8, 14, 15) and in target organ damage (16–18). Likewise, T cells accumulate in pVAT and adventitia of hypercholesterolemic mice (19). While these studies emphasize the role of the pVAT in vascular disease, the mechanisms involved in homing of inflammatory cells to pVAT are undetermined in hypertension. Interestingly, a large proportion of these cells bear RANTES CC chemokine receptor (CCR) 5 (8). RANTES, also referred to as CC chemokine ligand 5 (CCL5), is an important chemoattractant for inflammatory cells that has been implicated in the pathogenesis of atherosclerosis (20) and is present in AT of both humans and mice (4). RANTES can be produced by resident cells of the vessel and AT, and Ang II added *in vitro* increases its expression in arteriolar and venular endothelium (21). We hypothesized that RANTES plays a crucial role in the genesis of perivascular inflammation and can therefore affect the development of vascular dysfunction in hypertension.

In the present study, using RANTES$^{-/-}$ mice and wild-type (WT) controls, we identify a novel role of RANTES in regulation of vascular dysfunction in Ang II–induced hypertension. These effects of RANTES are particularly related to modulation of recruitment of IFN-γ to perivascular AT and, importantly, are independent of blood pressure changes. Finally, we demonstrate that pharmacologic modulation of this pathway can protect from vascular dysfunction development in hypertension.

MATERIALS AND METHODS

Animals

Male C57BL/6 (n = 58) and RANTES$^{-/-}$ (n = 24) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Twelve-week-old mice (body mass 27 ± 3 g) underwent either sham or Ang II (490 ng/min/kg s.c.) treatment for 14 d *via* a surgically implanted osmotic minipump (Alzet Model 2002; Alzet, Cupertino, CA, USA). Sham treatment involved infusion of the vehicle for Ang II. During treatment, all mice underwent noninvasive blood pressure measurement by tail cuff plethysmography after a period of training before commencement of the treatment protocol. For invasive measurements of blood pressure, radiotelemetry units were inserted 7 to 10 d before Ang II infusion, as previously described (8). The Institutional Animal Care and Use Committees at Jagiellonian University and at Emory University approved the protocols we used.

Met-RANTES treatment

Both sham- and Ang II–treated C57BL/6 mice also received the RANTES receptor antagonist met-RANTES (50 mg/kg i.p.; gift from A. Proudfoot, Merck Serono, Darmstadt, Germany) or vehicle (sterile PBS) every third day during the 14 d treatment period. Met-RANTES was first dissolved in sterile water to a concentration of 4 μg/L and was subsequently diluted in sterile PBS for *in vivo* use.

Measurement of mRNA expression

Tissue levels of mRNA of various chemokines and cytokines were quantified by real-time PCR with commercially available assays (TaqMan; Applied Biosystems, Foster City, CA, USA). Data were normalized to levels of 18S mRNA, and relative quantification was calculated as $2^{-\Delta\Delta Ct}$.

Immunohistochemical analysis of RANTES localization

After euthanasia, a cannula was placed in the right ventricle and mice were perfused with saline followed by 10% formaldehyde. Aortic tissue was embedded in paraffin. An anti-mouse/rat CCL5 (RANTES) antibodies (1:100, overnight, 4°C; eBioscience, San Diego, CA, USA) or anti-CCL5 (189841; Abcam) and staining was visualized using the Dako LSAB+ System HRP kit (Dako, Glostrup, Denmark) according to the manufacturer's protocol. Studies were performed at d 7 and 14 of Ang II infusion.

Measurements of vascular reactivity and superoxide production in aortic segments

Relaxation to the endothelium-dependent and -independent vasodilators acetylcholine (ACh) and sodium nitroprusside was measured in isolated 3 to 4 mm segments of aorta in organ chambers as previously described (8). In some experiments, aortic rings were preincubated for 24 h in RPMI containing 50 mg/ml IFN-γ or control buffer to assess the direct effects of this cytokine on endothelial function. Polyethylene glycol superoxide dismutase (PEG-SOD; 500 IU/ml) was used to dissect the role of superoxide in endothelial dysfunction. Aortic superoxide production was measured by quantifying formation of 2-hydroxyethidium from dihydroethidium (25 μM) by HPLC. This product specifically reflects the reaction of superoxide anion with dihydroethidium and has been validated previously (8). In validating studies vascular segments were preincubated with 100 U/ml PEG-SOD, which inhibited 80 ± 15% of Ang II–dependent increase in detected superoxide.

Analysis of leukocytes in tissues

pVAT was isolated from thoracic and abdominal aorta. This region of AT is invariably present on the anterior surface of the aorta and adheres when the aorta is removed from the mouse. Epididymal fat pads were used as representative of visceral fat. Subcutaneous fat was obtained from the inguinal fossae and the subscapular region. For analysis of cells in fat, AT was digested using collagenase type XI (125 U/ml), collagenase type IS (450 U/ml), and hyaluronidase IV-S (60 U/ml) that had been dissolved in PBS containing calcium and magnesium for 20 min at 37°C, with regular agitation. The digested tissue was then passed through a 70 μm sterile cell strainer (Falcon; BD Biosciences, San Jose, CA, USA) to yield a single-cell suspension. Cells were washed and resuspended in fluorescence-activated cell sorting buffer, counted, and stained, using multicolor flow cytometry as previously described (8, 19, 22), using a BD FACSCanto II flow cytometer with DIVA software (BD Biosciences). Macrophage subpopulations were defined in F4/80$^+$CD11b population by expression of CD11c and CD206 as previously described (23). Intracellular staining was performed as previously described (24). Dead cells were eliminated from analysis using 7-AAD (BD Biosciences). For each experiment, we performed fluorescence

minus one controls for each fluorophore to establish gates. In selected experiments, we confirmed accuracy of the fluorescence minus one gating strategy using isotype controls. Data were analyzed by FlowJo 8.8.1 software (FlowJo, Ashland, OR, USA).

Chemotaxis assay

Blood was obtained from either sham- or Ang II–infused mice and total peripheral blood mononuclear cells were isolated by a standard density gradient with LSM 1077 Lymphocyte Separation Medium (PAA Laboratories, Pasching, Austria). Either T cells or B cells were isolated from peripheral blood mononuclear cells by negative selection. Cell purity was confirmed to be ≥96%. After separation, the lymphocytes were resuspended in RPMI 1640 (Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 2 mM/ml L-glutamine, and 50 μg/ml gentamicin (Sigma-Aldrich, St. Louis, MO, USA). Cells (2×10^5) were added to the upper chamber of a 24-transwell apparatus (6.5 mm diameter, 5 μm pore size; Costar 3421) and incubated for 2 h at 37°C in 5% CO_2. Recombinant mouse CCL5/RANTES (10 ng/ml; R&D Systems, Minneapolis, MN, USA) or supernatant (conditioned medium) from an 18 h organ culture of pVAT (diluted 1:50) was placed in the lower chamber. The optimal concentration of RANTES was determined in preliminary experiments. In a subset of experiments, conditioned medium from pVAT–Ang II cultures was preincubated with 0.5 μg/ml anti-RANTES antibody (clone 53405; R&D Systems) chemotaxis was assessed. The percentage of cells that migrated to the lower chamber was determined by flow cytometry as described above.

Endothelial function studies in humans

Vascular function was measured in 129 subjects with type 2 diabetes. The clinical characteristics of the population are shown in **Table 1**. Major clinical risk factors were categorized as follows: hypercholesterolemia (total plasma cholesterol >4.8 mM or use of cholesterol-lowering medication); smoking (current or within last 6 mo); diabetes (fasting glucose >5.5 mM or current treatment with insulin or oral hypoglycemic agents); hypertension (blood pressure >140/90 mmHg or current treatment with antihypertensive agents), and overweight and obesity (body mass index >25 kg/m^2). Some of these subjects have been described in a prior publication (24). Flow-mediated dilatation (FMD) was used as a measure of endothelial function, as described in detail elsewhere (25). Images were analyzed by Vascular Tools 5 software by 2 independent blinded observers. Von Willebrand factor (vWF) was measured using sandwich immunoassay utilizing an anti-human vWF antibody (Dako) and expressed as percentage of reference sample (25). Serum RANTES levels were determined using a quantitative ELISA (CCL5/RANTES Quantikine ELISA kit; R&D Systems). The Jagiellonian University ethics committee approved all human studies. All subjects provided written informed consent before the study.

TABLE 1. *Clinical characteristics of high-cardiovascular-risk study cohort*

Characteristic	Patient cohort
No. of patients	129
Age (y)	56.6 ± 6.3
Sex (M:F)	64:65
Risk factors	
Current smoking	29 (22%)
Hypertension	115 (89%)
Diabetes	129 (100%)
Hypercholesterolemia	90 (70%)
BMI (kg/m^2)	32.5 ± 5.6
Cholesterol (mM)	5.4 ± 1.6
CAD	65 (50%)
Cardiovascular medications	
Aspirin	70 (54%)
Angiotensin-converting enzyme inhibitor	99 (77%)
β-Blocker	48 (37%)
Statin	62 (48%)
Calcium channel blocker	30 (23%)
Insulin	68 (52%)
Oral hypoglycemic drugs	83 (64%)

Data are presented as means ± SD or *n* (%). CAD, coronary artery disease; BMI, body mass index.

Statistical analysis

For comparison of 2 groups, unpaired 2-tailed Student's *t* tests were used. For comparison of 3 or more independent groups, 1-way ANOVA was used with a Student-Newman-Keuls *post hoc* test. For comparison of the effects of Ang II on parameters in different groups of mice, we used 2-way ANOVA with a Bonferroni *post hoc* test. For comparisons of vascular function in organ chamber experiments, repeated measures ANOVA was used. The relationship between RANTES and vWF levels with FMD and nitroglycerin-mediated dilatation were analyzed by Spearman's correlation tests. Values of $P < 0.05$ were considered significant.

RESULTS

Leukocyte content in pVAT and effect of Ang II

Leukocytes represented 2% of the vascular stromal fraction of pVAT in sham-treated mice. Ang II infusion significantly increased both the percentage and absolute quantity of leukocytes in pVAT (**Fig. 1*A*, *B***). Using antibodies against specific markers for T cells (CD3), B cells (CD19), macrophages (I-Ab/CD11b), and dendritic cells (DCs; CD11c/I-Ab), we found that pVAT contained all of these cell types (Fig. 1*C*). Ang II infusion markedly increased the number of T cells, macrophages, and DCs in pVAT (Fig. 1*C*) while not affecting the content of these in visceral or subcutaneous fat. Although there was a modest increase in B cells in the pVAT in response to Ang II, this was not statistically significant (Fig. 1*C*). Thus, macrophages, DCs, and T cells are the most prevalent leukocytes in the pVAT of Ang II hypertensive mice. Ang II caused greatest increase of T cells in pVAT percentage-wise (15 ± 2 to 24 ± 5%; $P < 0.05$).

Role of RANTES and its receptors in AT T-cell recruitment

We next focused on understanding the mechanisms responsible for T-cell accumulation in pVAT. Ang II infusion increased the percentage of total T cells expressing the RANTES receptors CCR1, CCR3, and CCR5 in pVAT (Fig. 1*D* and Supplemental Fig. S1*A*), but not in visceral fat (Supplemental Fig. S2). In keeping with the attraction of T cells bearing these surface receptors, Ang II increased RANTES mRNA in the perivascular fat already at d 7 of Ang

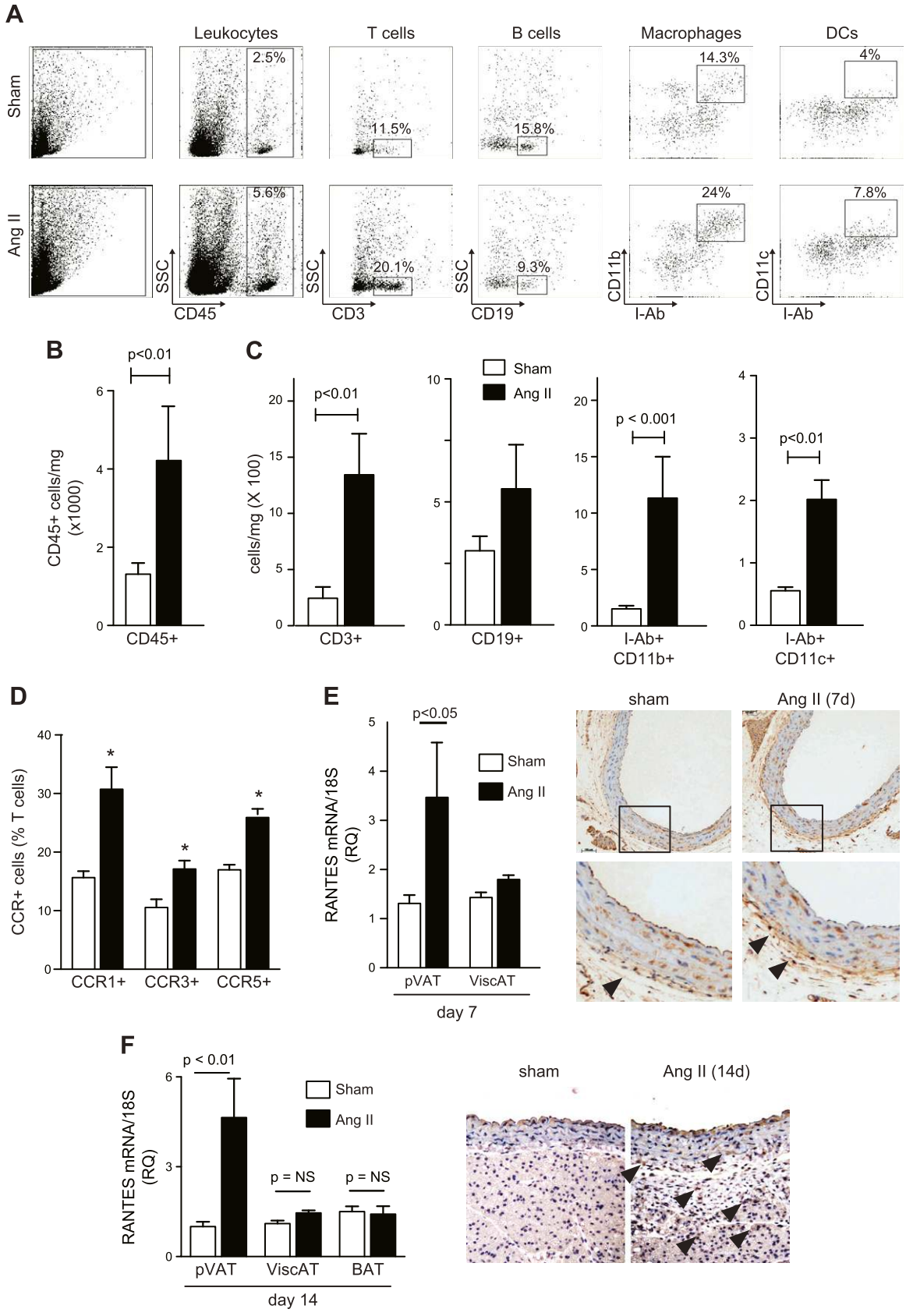

Figure 1. Leukocyte infiltration, chemokine receptors, and RANTES expression in pVAT during Ang II–dependent hypertension. Hypertension was induced by chronic 14 d Ang II infusion by osmotic minipump (490 ng/min/kg), and AT was obtained from periaortic fat pad (pVAT) and epididymal AT (visceral AT). *A*) Representative flow cytometric analysis of major leukocyte subpopulations in vascular stromal fraction isolated from periaortic AT of sham- and Ang II–infused mice. *B*) Effect of Ang II

(*continued on next page*)

II infusion while not changing RANTES mRNA in visceral AT (Fig. 1*E*). Moreover, at d 14 of Ang II infusion, when vascular pathology is fully developed in this model, RANTES expression was further up-regulated while no increase was observed in either visceral AT or brown AT (BAT). Immunohistochemical staining confirmed that RANTES protein was present in perivascular fat and adventitia after Ang II infusion at both d 7 (Fig. 1*E*) and d 14 (Fig. 1*F*). RANTES can be produced by both T cells and by resident cells within tissues; however, Ang II also increases RANTES mRNA in the pVAT of RAG-1$^{-/-}$ mice to an extent similar to that observed WT mice (3.1 ± 0.5 fold; $P < 0.01$), indicating that resident cells rather than lymphocytes are the major source of this chemokine in response to Ang II.

To gain additional insight into the role of RANTES, we performed chemotaxis assays of lymphocytes from sham- and Ang II–treated mice toward soluble RANTES in a Boyden chamber. As is evident in **Fig. 2*A***, T cells, but not B cells, from Ang II–infused mice were more avidly attracted to RANTES than T cells from sham-infused animals. This was true for both CD4 and CD8 cells (Fig. 2*B*). In further studies, we placed conditioned medium from organoid cultures of pVAT from sham- and Ang II–treated mice in the lower portion of a Boyden chamber and examined migration of T cells from Ang II–treated mice. There was minimal migration of T cells toward the conditioned medium of pVAT from sham-infused mice (Fig. 2*C*); however, CD4$^+$, CD8$^+$, and double-negative CD3$^+$CD4$^-$ CD8$^-$ T cells were stimulated to migrate toward the conditioned medium of pVAT from Ang II–treated mice (Fig. 2*D* and Supplemental Fig. S1*B*, respectively). Importantly, when conditioned media was preincubated with 0.5 μg/ml anti-RANTES antibody (clone 53405; R&D Systems), T-cell chemotaxis toward pVAT from Ang II–infused mice was significantly reduced (Fig. 2*E*).

Role of RANTES in vascular dysfunction and blood pressure elevation in response to Ang II

RANTES shows significant functional effects in the vasculature, as the vasodilatation evoked by ACh was impaired in WT mice but not in RANTES$^{-/-}$ mice that had received Ang II (**Fig. 3*A***). Endothelium-independent responses to sodium nitroprusside were not altered by Ang II in either WT or RANTES$^{-/-}$ mice (Fig. 3*B*). Vascular superoxide production did not differ between WT and RANTES$^{-/-}$ mice at baseline, but lack of RANTES was associated with abrogated Ang II–induced increase in vascular superoxide (Fig. 3*C*). We next examined the hypertensive response to Ang II in mice lacking RANTES using radiotelemetry (Fig. 3*D*). Ang II induced approximately equivalent degrees of hypertension in WT and RANTES$^{-/-}$ mice (Fig. 3*D*). Ang II-induced hypertension was associated with increased sensitivity to noradrenaline induced vasoconstriction and this effect was were unaltered in RANTES$^{-/-}$ mice (Supplemental Fig. S3)

To validate links between RANTES and endothelial function in humans, we examined the relationship between FMD and serum level of RANTES in a cohort of subjects with metabolic syndrome and other risk factors for coronary disease as described in Table 1. A statistically significant inverse relationship between FMD and RANTES was observed (Fig. 3*E*), while there was no relationship between the brachial artery response to nitroglycerin and RANTES (Fig. 3*F*). In line with this, levels of vWF, an independent measure of endothelial dysfunction, also positively correlated with levels of RANTES (Fig. 3*G*).

Role of RANTES in modulating perivascular inflammation in Ang II hypertension

While the infiltration of total leukocytes in pVAT of RANTES$^{-/-}$ mice was modestly reduced compared to that of WT mice, the infiltration of T cells was 50% less in RANTES$^{-/-}$ mice compared to WT mice (**Fig. 4*A*, *B***). Interestingly, RANTES deficiency was also associated with decreased pVAT macrophage content, but this effect was less pronounced (Fig. 4*C*). Within the F4/80$^+$CD11b$^+$ macrophages, both CD11c$^+$CD206$^-$ (corresponding mainly to M1 polarization) and CD11c$^-$CD206$^+$ (corresponding to M2 polarization) (Fig. 4*E*) were significantly increased in the pVAT by Ang II infusion (Fig. 4*F*). Both of these subpopulations in the pVAT were decreased in hypertensive RANTES$^{-/-}$ mice, although this RANTES-related effect was particularly pronounced in relation to CD11c$^-$CD206$^+$ cells (Fig. 4*F*).

RANTES' effect on relative content of T cells was, however, more pronounced than on other leukocyte subsets (Fig. 4*D*). Thus, in Ang II–dependent hypertension, RANTES plays an important role in homing of T cells, and particularly CCR5$^+$ cells, to pVAT (**Fig. 5*A***). CCR5$^+$ cells exhibited particularly high production of IFN-γ. Accumulation of cells bearing CCR6 remained unaffected (Fig. 5*B*). In line with this, recruitment of T-helper (T_h)17 cells (CD4 cells producing IL-17) upon Ang II infusion, predominantly modulated by CCR6, was not affected by RANTES$^{-/-}$ (Fig. 5*C*). CD8$^+$ T-cell production of IL-17 was negligible (data not shown).

RANTES is important in perivascular recruitment of IFN-γ-producing T cells, which may affect vascular dysfunction

Because CCR5 are often expressed in IFN-γ-producing T cells, we examined IFN-γ production by T cells within the pVAT. While CD4$^+$ cells did not produce IFN-γ in the pVAT of either sham- or Ang II–infused mice, a small number of CD8 T cells produced this cytokine at baseline,

infusion on absolute numbers of CD45$^+$ total leukocyte content in pVAT compartment expressed per mg of tissue ($n = 14$). *C*) Effect of Ang II infusion on content CD3$^+$ T cells, CD19$^+$ B cells, I-Ab$^+$CD11b$^+$ macrophages, and I-Ab$^+$CD11c$^+$ DCs in pVAT ($n =$ 12–14 for each). *D*) Effect of Ang II–dependent hypertension on content of CCR1, CCR3, and CCR5$^+$ T lymphocyte (CD3$^+$) in isolated pVAT ($n = 6$). *E*) Effect of 7 d Ang II–induced hypertension on mRNA expression of RANTES in pVAT and visceral AT ($n = 5$), and immunostaining of aortas from sham-treated and Ang II–infused C57BL/6J mice using anti-RANTES antibody (representative of 5 experiments). *F*) Effect of 14 d Ang II–induced hypertension on mRNA expression of RANTES in pVAT, visceral AT, and BAT ($n = 5$) and immunostaining of aortas from sham-treated and Ang II–infused C57BL/6J mice using anti-RANTES antibody (representative of 5 experiments).

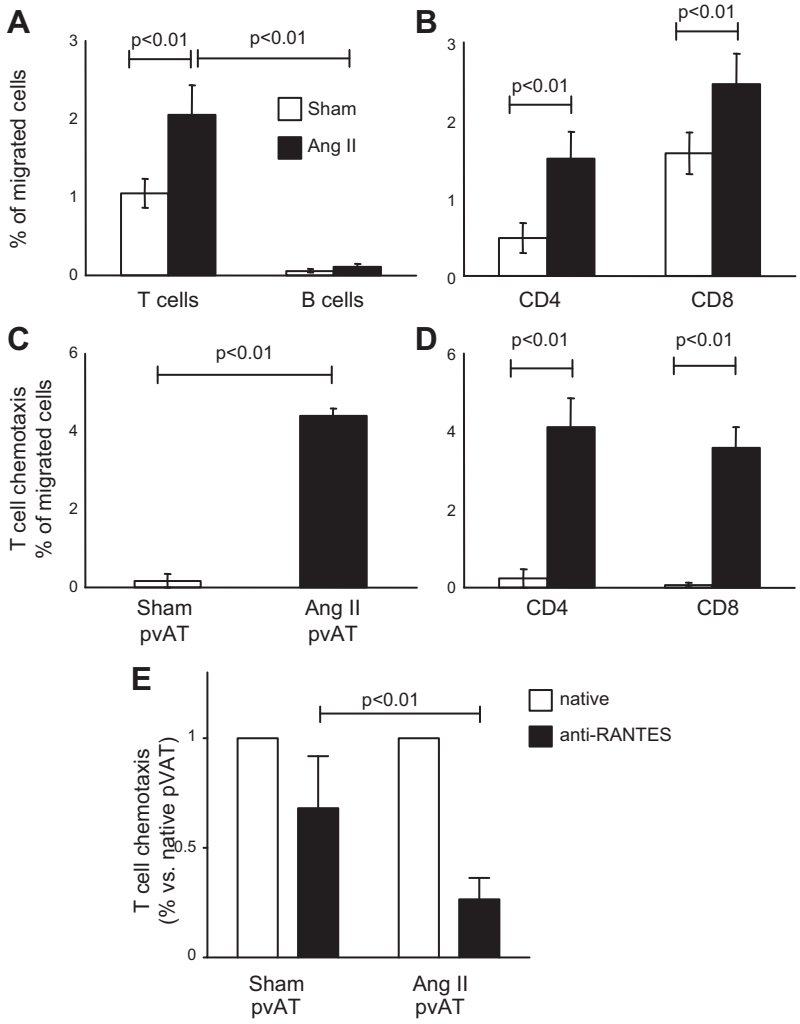

Figure 2. Role of RANTES in T lymphocyte migration in response to Ang II. Experiments were performed in modified Boyden chamber. *A, B*) Migration of T cells and their subsets and B cells (as reference) from sham- or Ang II–infused mice toward soluble RANTES (10 ng/ml, $n = 9$). *C, D*) Chemotaxis of T cells and $CD4^+$ and $CD8^+$ T cells toward conditioned medium from pVAT from sham- and Ang II–infused mice ($n = 6$ for each). *E*) Effects of anti-RANTES neutralizing antibody pretreatment on T-cell chemotaxis toward pVAT conditioned medium from sham- and Ang II–treated mice. Isotype antibody–pretreated medium was treated as 100%. Data are expressed as means ± SEM.

and this is increased in response to Ang II in WT mice. The Ang II–dependent increase in IFN-γ-producing $CD8^+$ T cells was not observed in $RANTES^{-/-}$ mice (Fig. 5*D*). mRNA expression of IFN-γ was not increased by Ang II in the pVAT of $RANTES^{-/-}$ mice (Fig. 5*E*). We have previously demonstrated that $CD3^+CD4^-CD8^-$ double-negative T cells were characteristic for hypertensive vasculature. Recruitment of $CD3^+CD4^-CD8^-$ to pVAT is significantly blunted in $RANTES^{-/-}$ mice (**Fig. 6*A***). Moreover, these cells can also produce significant amounts of IFN-γ in Ang II–infused WT mice but not in $RANTES^{-/-}$ mice (Fig. 6*B*).

To investigate a possible role of IFN-γ in causing endothelial dysfunction, we incubated aortic segments with IFN-γ and observed that it caused significant endothelial dysfunction that was partially reversed by preincubation with PEG-SOD (Fig. 5*F*). Thus, RANTES-dependent recruitment of IFN-γ-producing T cells may provide an important link between perivascular inflammation and endothelial dysfunction in hypertension.

RANTES in perivascular recruitment of T-regulatory cells

Because T-regulatory cells have been implicated in the pathogenesis of hypertension, we studied whether $RANTES^{-/-}$ was associated with alterations of recruitment of these cells into the pVAT. $CD4^+CD25^+FoxP3^+$ T-regulatory content in the pVAT was very low and remained unaltered upon Ang II infusion in both WT and $RANTES^{-/-}$ mice (Supplemental Fig. S4).

Pharmacologic modulation RANTES signaling in hypertension

To determine whether met-RANTES, a pharmacologic inhibitor of RANTES-dependent inflammation, exerts vasoprotective effects in Ang II–induced hypertension, we treated C57Bl/6 mice intraperitoneally with met-RANTES (50 mg/kg) every 3 d, beginning 3 d before initiation of Ang II infusion. Intraperitoneal injection of saline was used as a control. Met-RANTES treatment prevented the development of endothelial dysfunction in response to Ang II (**Fig. 7*A***) while not significantly affecting blood pressure increase (tail cuff blood pressure 152 ± 8 mmHg in vehicle-treated mice *vs.* 150 ± 9 mmHg in met-RANTES-treated mice). Similarly, the increase in vascular superoxide production caused by Ang II was inhibited by met-RANTES (Fig. 7*B*). These changes of vascular phenotypes were accompanied by significant reductions of leukocyte and T-cell recruitment into pVAT (Fig. 7*C*).

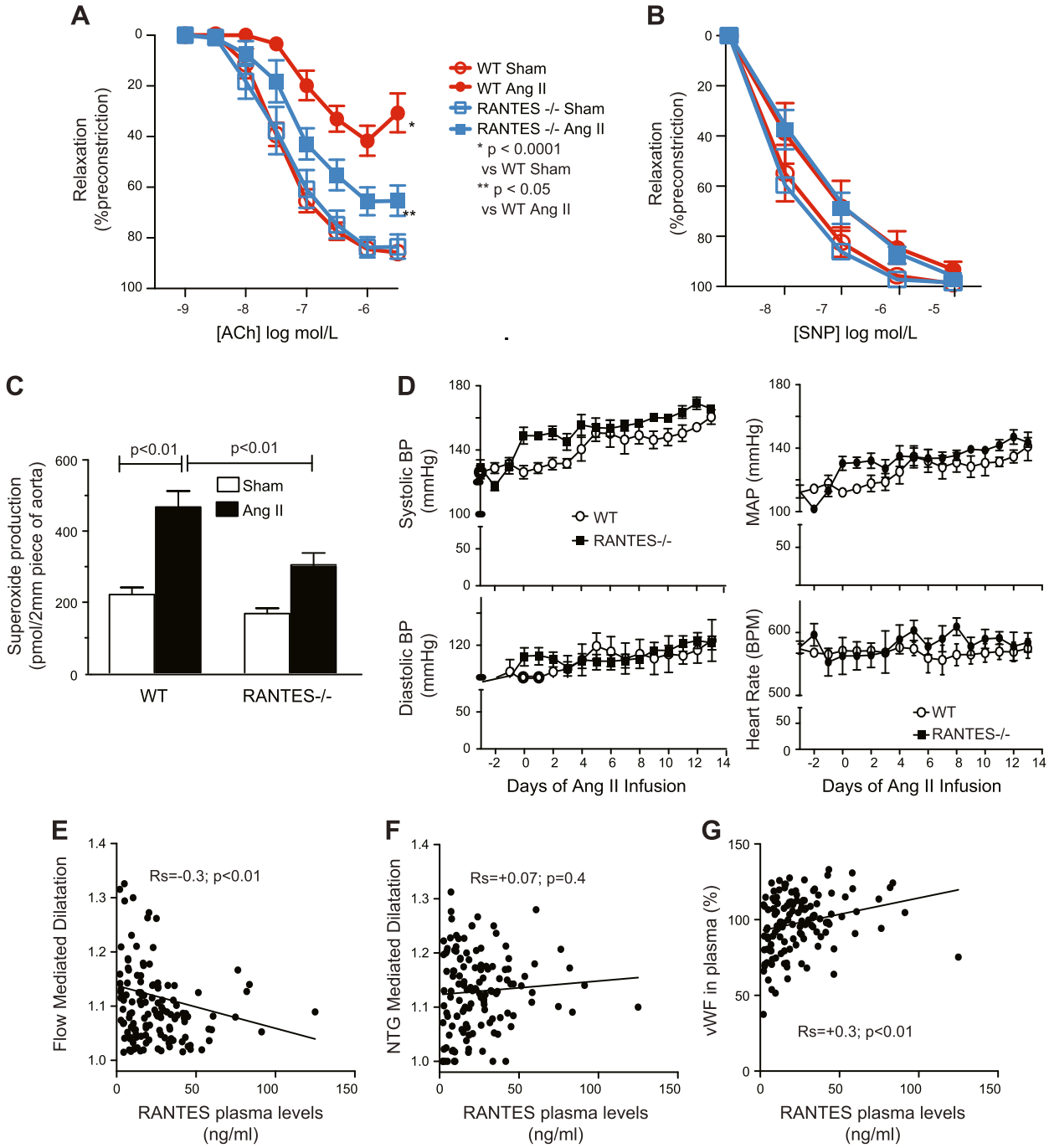

Figure 3. RANTES in Ang II–dependent hypertension and regulation of vascular dysfunction in animal model and in humans. *A*) Effect of Ang II–induced hypertension on endothelium-dependent vasodilatation to ACh in aortas of WT and RANTES$^{-/-}$ mice ($n = 6$ for each). *B*) Relaxations to sodium nitroprusside as measure of non-endothelium-dependent vasodilatation ($n = 6$ for each). Statistical analysis was performed by repeated measures ANOVA. *C*) Aortic superoxide levels measured by monitoring oxidation of dihydroethidium to 2-hydroxyethidium using HPLC in WT and RANTES$^{-/-}$ mice infused for 14 d with buffer (sham) or Ang II ($n = 5$ each group). *D*) Mean daily values of invasive telemetric measurements of systolic (top left), diastolic (bottom left), and mean arterial (top right) blood pressure and heart rate (bottom right) at baseline and during Ang II infusion in WT and RANTES$^{-/-}$ mice ($n = 6$). *E*) Correlation between serum RANTES levels and FMD in high-cardiovascular-risk cohort of 129 subjects. *F*) Relationship between RANTES serum levels and non-endothelium-dependent nitroglycerin-mediated dilatation induced vasodilatation this cohort. *G*) Relationship between RANTES and vWF (as biochemical marker for endothelial dysfunction) levels in serum of high-cardiovascular-risk cohort. *E–G*) Statistics for these relationships presented as Spearman's correlation tests.

DISCUSSION

There is an increasing body of evidence of the role of perivascular inflammation in atherosclerosis, although the mechanisms of this link are complex and poorly understood. In the present study, we identify the role of RANTES chemokine in mediating pVAT inflammation in Ang II–induced hypertension and show possible links to

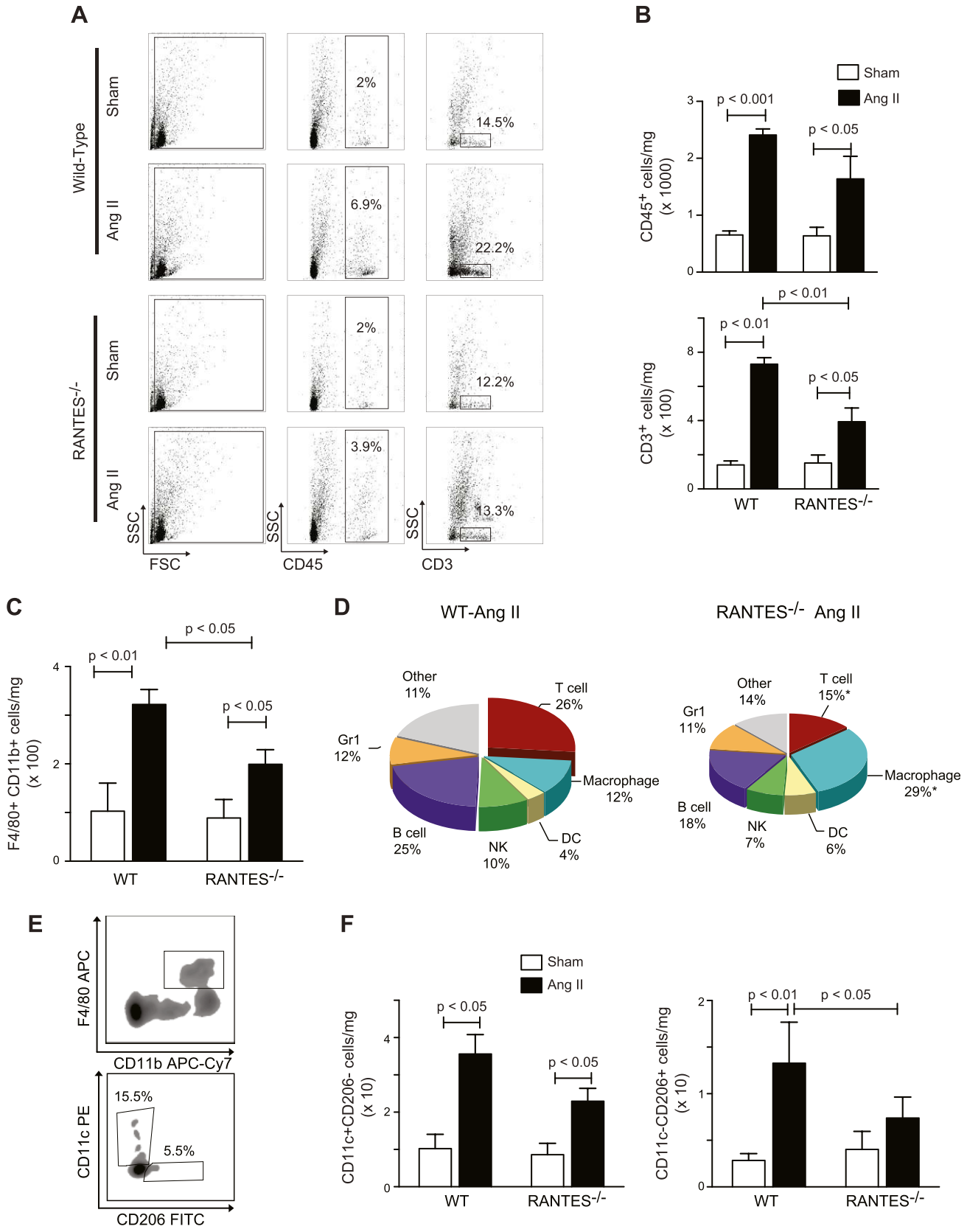

Figure 4. Role of RANTES in Ang II–dependent hypertension and T-cell perivascular infiltration. *A*) Examples of flow cytometric determination of effects of Ang II infusion on isolated pVAT (minus aorta) infiltration with total leukocytes ($CD45^+$) and T cells ($CD3^+$) in WT and $RANTES^{-/-}$ mice. *B*) Effect of Ang II–dependent hypertension on mean total leukocyte ($CD45^+$ cells) and T-cell ($CD3^+$) content in isolated pVAT in WT and $RANTES^{-/-}$ mice ($n = 5$ each). *C*) Effect of Ang II–dependent hypertension on mean macrophage

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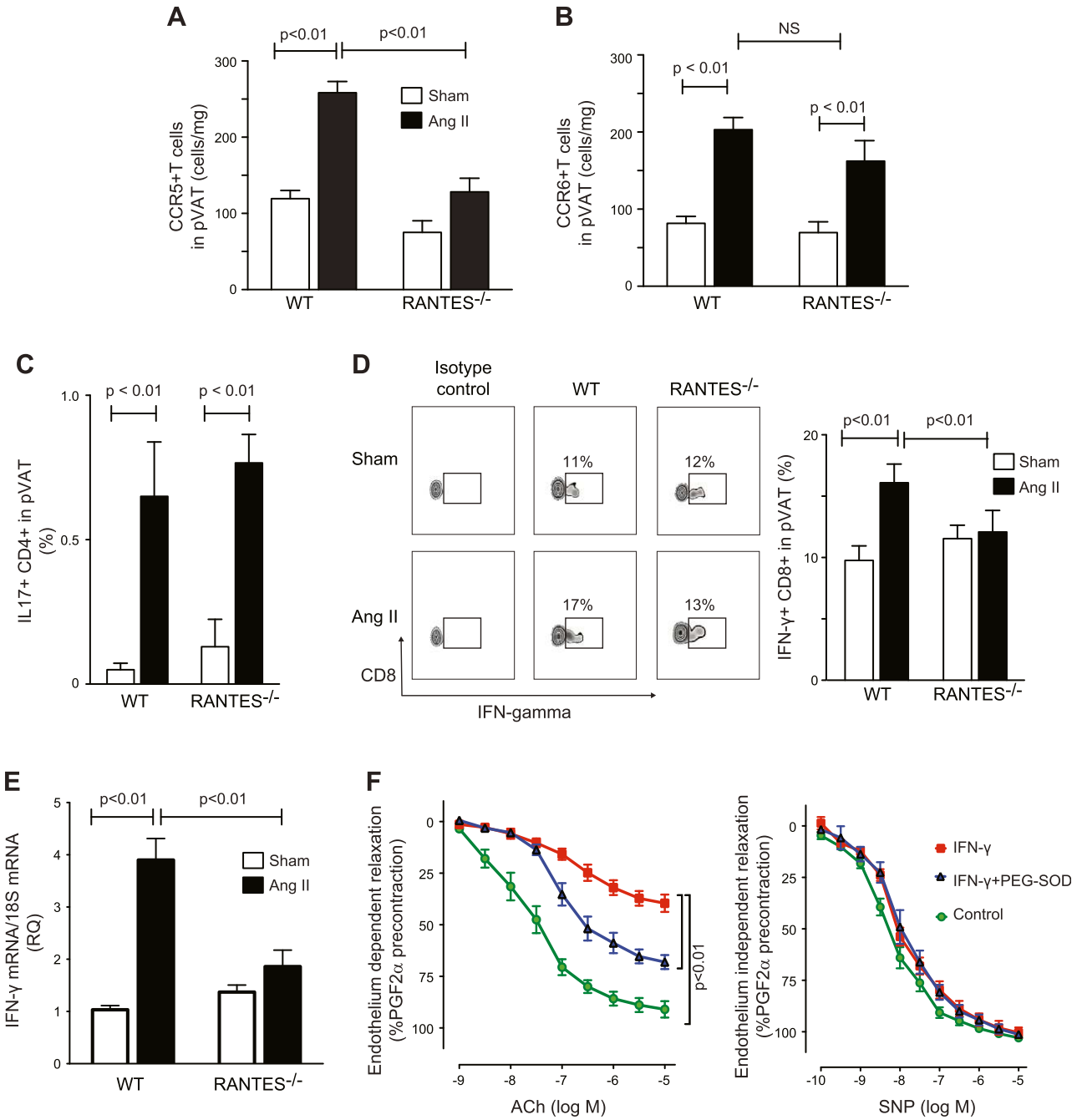

Figure 5. T-cell subsets in isolated pVAT are regulated by RANTES in hypertension; links to vascular dysfunction. *A, B*) Flow cytometric analyses were used to determine number of $CCR5^+$ T cells (*A*) and $CCR6^+$ T cells (*B*) in pVAT of sham- and Ang II–infused mice ($n = 5$). *C*) Ang II–dependent changes in IL-17-producing $CD4^+$ T cells in pVAT from WT and $RANTES^{-/-}$ mice ($n = 5$). *D*) Ang II–dependent changes in IFN-γ-producing $CD8^+$ T cells in pVAT from WT and $RANTES^{-/-}$ mice ($n = 5$). *E*) Effect of Ang II on mRNA expression (real-time PCR) of IFN-γ in pVAT from WT and $RANTES^{-/-}$ mice ($n = 5$). *F*) Effects of IFN-γ (50 ng/ml) on endothelium-dependent and -independent relaxations in mouse aorta. Role of reactive oxygen species was examined using PEG-SOD (500 IU/ml) preincubation ($n = 6$; *P*, repeated measures ANOVA).

endothelial and vascular dysfunction and oxidative stress. Ang II infusion stimulates accumulation of T cells, macrophages, and DCs in the pVAT but not in other visceral or subcutaneous AT. This perivascular inflammatory response is accompanied by increased expression of inflammatory cytokines such as IFN-γ or IL-17, which have been implicated in the genesis of hypertension (8, 24, 26). Using mice lacking RANTES, we further show that at least 2 pathways

infiltration in pVAT ($n = 5$ each). *D*) Differences in leukocyte subpopulation composition of pVAT upon Ang II infusion in WT and $RANTES^{-/-}$ mice showing notable reduction of T-cell content ($n = 5$ each). *E*) Gating strategy for detection of M2 ($CD11c^-CD206^+$) and M1 type AT macrophages ($CD11c^+CD206^-$) within $F4/80^+CD11b^+$ cells. *F*) Effect of Ang II–dependent hypertension on mean M1 (left) and M2 (right) macrophage infiltration in pVAT upon Ang II infusion in WT and $RANTES^{-/-}$ mice ($n = 5$ each).

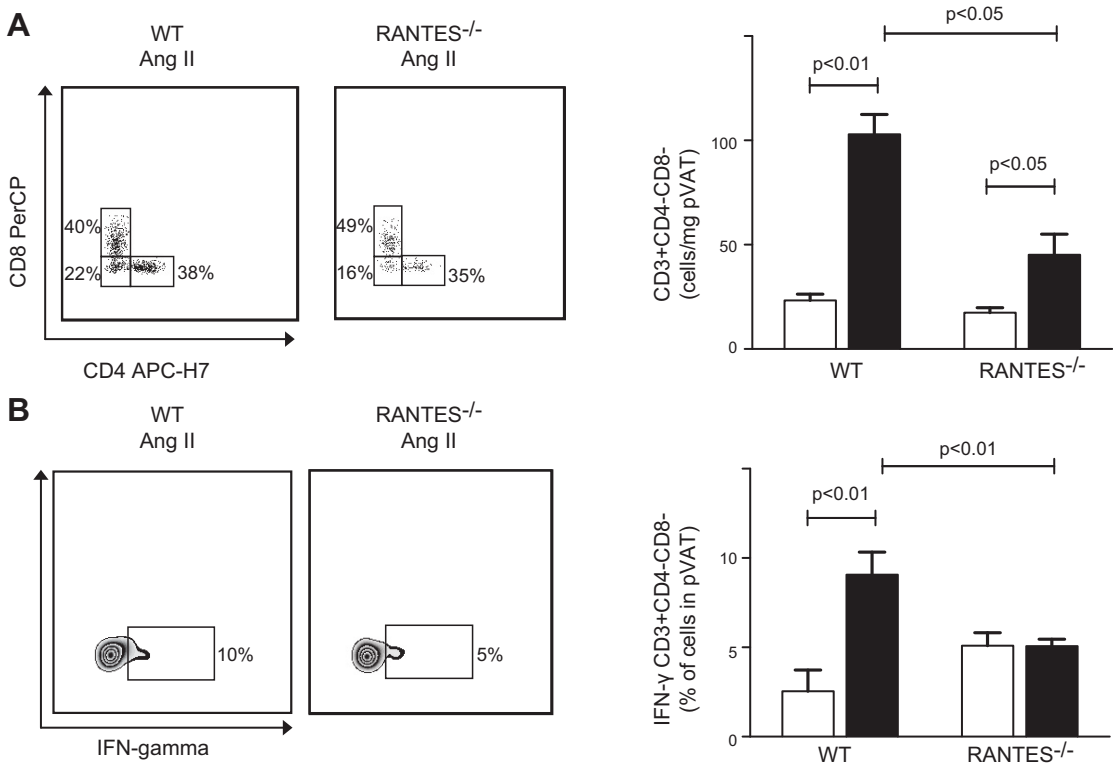

Figure 6. Double-negative $CD3^+CD4^-CD8^-$ T cells are abundantly recruited to vasculature in Ang II–dependent hypertension and contribute to IFN-γ production. *A*) Effect of Ang II–dependent hypertension on mean $CD3^+CD4^-CD8^-$ T-cell content in isolated pVAT in WT and $RANTES^{-/-}$ mice ($n = 5$ each; example of staining at left). *B*) Ang II–dependent changes in IFN-γ-producing $CD3^+CD4^-CD8^-$ T cells in pVAT from WT and $RANTES^{-/-}$ mice ($n = 5$). Data are expressed as means ± SEM.

are operative that govern the entry of inflammatory cells into the pVAT in hypertension. A RANTES-dependent pathway promotes accumulation of macrophages and $CCR5^+$ and IFN-γ-producing T cells into pVAT. Our findings point to reduced recruitment of IFN-γ-producing cells as a mechanism for the protection from endothelial dysfunction and vascular oxidative stress observed in $RANTES^{-/-}$ mice. Indeed the role of IFN-γ-producing T cells has already been linked to cardiac and renal dysfunction in hypertension (27, 28). Our results are in line with the fact that IFN-γ is known to stimulate superoxide production in vascular cells (29) and contribute to endothelial dysfunction, as is also evident in IFN-$\gamma^{-/-}$ mice (13). While our focus was on large vessels as an indication of end-organ damage, future studies will assess whether endothelial dysfunction is also prevented in resistance vessels in the absence of RANTES, although the lack of antihypertensive effect could suggest otherwise.

Moreover, although we have demonstrated direct effects of IFN-γ on endothelial function, future studies should assess how adipokine biology is altered by perivascular inflammation, as this could provide additional pathway for the regulation of vascular dysfunction (7, 30).

We observed that in spite of reduced perivascular infiltration of subsets of T cells and macrophages in $RANTES^{-/-}$ mice, the hypertensive response to Ang II in $RANTES^{-/-}$ mice is similar to that observed in WT mice. This is important because it shows that the endothelial dysfunction that occurs after Ang II treatment is not merely a consequence of increased pressure. Maintenance of elevated blood pressure in the absence of RANTES-dependent inflammation might be associated with RANTES-independent mechanisms. These are complex and may involve various cell types, but also other organs such as kidneys or resistance vessels. In particular, accumulation of T_h17 cells, monocytes, or NK cells has been linked to regulation of blood pressure (12, 13, 31, 32). It may also point to the importance of kidney inflammation in the regulation of blood pressure and indicate that prevention of endothelial dysfunction alone in larger vessels is not sufficient to reduce blood pressure. Moreover, our studies show that resistance vessels' (mesenteric arterioles) proconstrictive properties are not affected by $RANTES^{-/-}$. Taken together with the present data, one can hypothesize that the CCR5/RANTES axis is involved in vascular dysfunction development independent of initial blood pressure increase; the CCR6/IL-17 axis may contribute to blood pressure elevation in hypertension. Moreover, our proof-of-concept studies using met-RANTES show that RANTES is a promising target for the treatment of vascular dysfunction in hypertension.

A cardinal feature of any inflammatory process is the coordinated expression of surface homing markers on leukocytes and ligands for these surface receptors on the endothelium at the affected sites. In the case of T cells, the receptor/ligand interaction is, in some instances, highly specific for the targeted tissue. As an example, T cells bearing the surface marker CCR9 accumulate in the small intestine and interact with the ligand CCL25. In contrast, the interplay of CCR4 with CCL17 attracts inflammatory

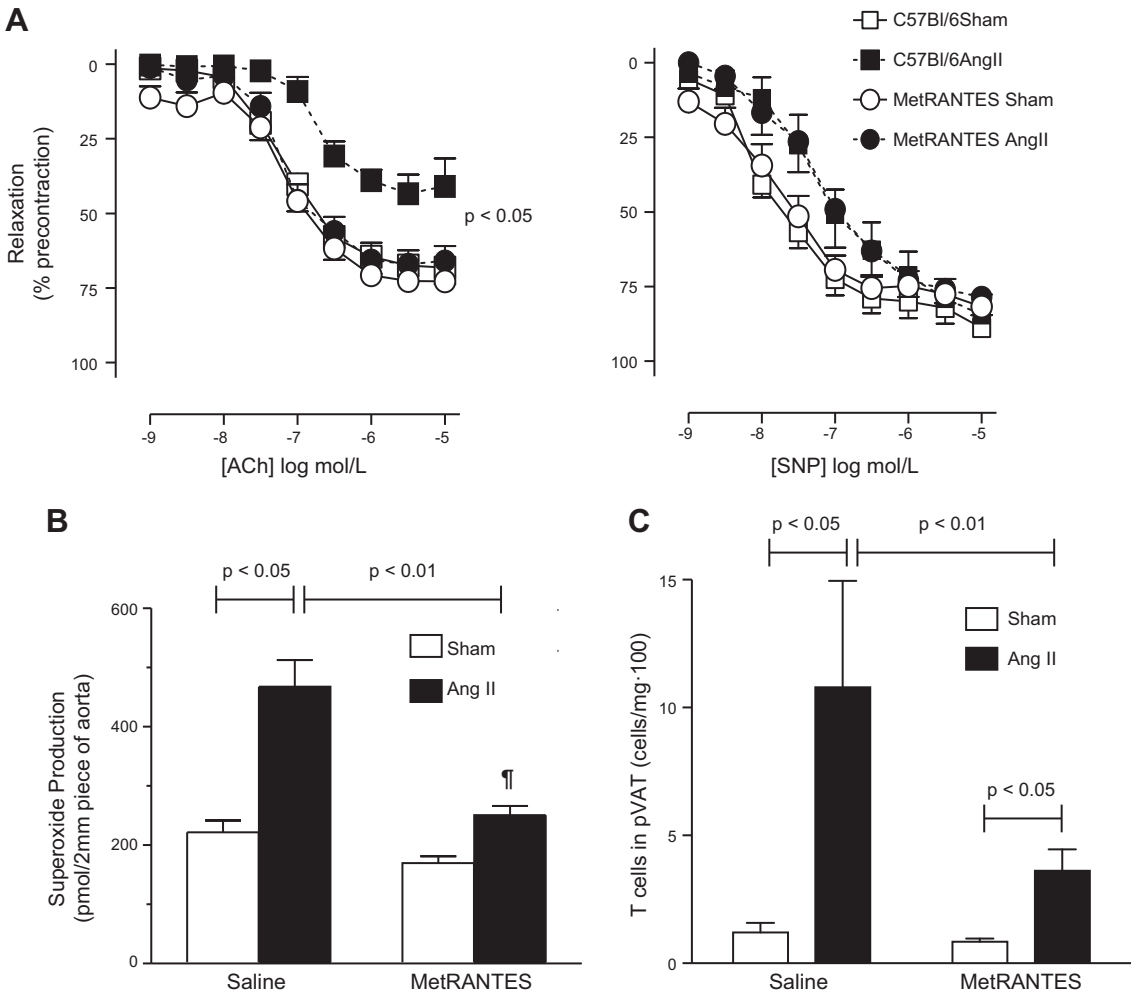

Figure 7. Effects of met-RANTES on vascular function and perivascular T-cell infiltration in Ang II–dependent hypertension. *A*) Effect of Ang II–induced hypertension on endothelium-dependent vasodilatation to ACh in aortas of saline (saline) and met-RANTES-treated (50 mg/kg i.p.) mice (left*;* $n = 5$ for each). Relaxations to sodium nitroprusside as measure of non-endothelium-dependent vasodilatation (right*;* $n = 5$ for each). *B*) Aortic superoxide levels measured by monitoring oxidation of dihydroethidium to 2-hydroxyethidium using HPLC in control and met-RANTES-treated mice infused for 14 d with buffer (sham) or Ang II ($n = 5$). *C*) Effects of met-RANTES on mean T-cell ($CD45^+CD3^+$) infiltration in isolated pVAT (minus aorta) during Ang II–dependent hypertension ($n = 5$).

cells to the skin. Unlike CCL25 and CCL17, RANTES is not specifically localized in one tissue; however, its expression pattern in response to a specific stimulus could provide targeted homing of inflammatory cells. For example, RANTES expression is specifically increased in the lungs in the setting of atopic and nonatopic asthma; in the brain in experimental and clinical forms of encephalitis; and in the synovium in arthritis (33). In the setting of experimental atherosclerosis, RANTES is colocalized with cells in atherosclerotic lesions (20). Recent studies in kidney fibrosis models have indicated a role for RANTES in this process linking it to local T-cell and macrophage infiltration (34). Our finding that Ang II promotes RANTES expression in the perivascular fat might therefore represent an important mechanism for targeting inflammation to this site in hypertension.

As indicated by its name (regulated on activation, normal T-cell expressed and secreted), RANTES was first identified in T cells and was considered to be a T-cell-specific product (35). However, subsequent studies have shown that it can be produced by many cells and contributes to many pathologic processes including cancer, allergy, infection, and atherosclerosis (36). While RANTES can be produced by T cells, in preliminary studies, we found that vascular levels of RANTES mRNA increased to a similar extent in RAG-$1^{-/-}$ and WT mice treated with Ang II. This indicates that T cells are not required for this vascular/AT response and may participate in an effector phase of this reaction. It is conceivable that T cells, once localized to the AT, additionally contribute to tissue RANTES expression in a feed-forward fashion. It is also of interest that in other preliminary studies we found that the increase in circulating $CD44^{high}$ and $CD69^+$ T cells caused by Ang II was similar between RANTES$^{-/-}$ and WT mice, while the vascular homing of these cells is diminished. This indicates that RANTES is not necessary for T-cell activation by the hypertensive stimulus but that it is essential for tissue homing of inflammatory cells in hypertension and can be a valuable treatment target (37).

Prior studies have shown that high-fat feeding of mice causes an accumulation of T cells in visceral AT of male

mice (4, 38). This is accompanied by increases in mRNA and protein levels of RANTES and CCR5 and is dependent on the T_H1 cytokine IFN-γ. Wu *et al.* (4) have shown that subcutaneous fat of obese humans have high levels of T cells, and that these correlate with levels of RANTES and body mass index. Others have shown that effector T cells preferentially home to visceral, but not subcutaneous, AT (39). In this regard, our findings suggest that Ang II infusion shares some characteristics of fat feeding in promoting AT inflammation, with a propensity for targeting the perivascular fat.

The explanation for the preferential effect of hypertension on inflammation in pVAT is unclear. It is possible that this is due to catecholaminergic stimulation in this tissue. More than 4 decades ago, Wirsen (40) showed that AT is highly innervated with sympathetic nerve terminals and proposed that norepinephrine released from periadventitial nerves in vessels might influence adjacent fat. It is of interest that islands of BAT, which are highly innervated, are present in white AT along the aorta and other vessels in mice and humans (41). Although the response of BAT to Ang II is unknown, sympathetic stimulation promotes both thermogenesis and development of BAT. It is also possible that reactive oxygen species or other mediators released by vascular cells diffuse to the adjacent fat cells and promote expression of molecules such as RANTES, leading to the inflammatory response. It has also been reported that stimulation of the AT2 receptor, which we found expressed in pVAT, promotes RANTES expression in glomerular endothelial cells (42). Interestingly, in our own studies, both AT1 and AT2 receptor expression occurred in the pVAT but was not affected by Ang II infusion. It is therefore possible that Ang II promotes pVAT expression of RANTES *via* AT1R or AT2R activation.

Our studies in humans also show a significant inverse relationship between flow-mediated vasodilatation and serum RANTES levels. Likewise, we found the serum levels of vWF, an independent marker of endothelial dysfunction, correlates with serum RANTES. These data suggest that RANTES might also affect endothelial function in humans. These translational results need to be interpreted with caution because the population studied was heterogeneous and exhibited several concomitant factors that may independently affect RANTES levels and vascular pathology. These include type 2 diabetes, obesity, hypercholesterolemia, smoking, and various medications used by patients (Table 1). Our patient population exhibited a high incidence of obesity; Wu *et al.* (4) have shown that AT of humans expresses high levels of RANTES and that this measure correlates with the presence of T cells as estimated by the marker CD3.

In summary, the present study indicates that pVAT represents a novel site of Ang II–induced inflammation and that it responds differently from other fat deposits. Ang II–induced hypertension is associated with a striking increase in T cells and macrophages into pVAT in both RANTES-dependent and -independent fashions. RANTES, through regulation of IFN-γ-producing T cells, seems to affect vascular endothelial function but not the ultimate hypertension caused by Ang II. This pathway could represent a valuable target for prevention of vascular complications of hypertension. The accumulation of inflammatory cells in the perivascular fat in response to Ang II might contribute to the enhancement of atherosclerosis (19) in hypertension. These studies emphasize the complexity of chemokine signaling in AT and the unique role of pVAT in the modulation of vascular disease. FJ

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REFERENCES

1. Omar, A., Chatterjee, T. K., Tang, Y., Hui, D. Y., and Weintraub, N. L. (2014) Proinflammatory phenotype of perivascular adipocytes. *Arterioscler. Thromb. Vasc. Biol.* **34**, 1631–1636
2. Margaritis, M., Antonopoulos, A. S., Digby, J., Lee, R., Reilly, S., Coutinho, P., Shirodaria, C., Sayeed, R., Petrou, M., De Silva, R., Jalilzadeh, S., Demosthenous, M., Bakogiannis, C., Tousoulis, D., Stefanadis, C., Choudhury, R. P., Casadei, B., Channon, K. M., and Antoniades, C. (2013) Interactions between vascular wall and perivascular adipose tissue reveal novel roles for adiponectin in the regulation of endothelial nitric oxide synthase function in human vessels. *Circulation* **127**, 2209–2221
3. Gao, Y. J. (2007) Dual modulation of vascular function by perivascular adipose tissue and its potential correlation with adiposity/lipoatrophy-related vascular dysfunction. *Curr. Pharm. Des.* **13**, 2185–2192
4. Wu, H., Ghosh, S., Perrard, X. D., Feng, L., Garcia, G. E., Perrard, J. L., Sweeney, J. F., Peterson, L. E., Chan, L., Smith, C. W., and Ballantyne, C. M. (2007) T-cell accumulation and regulated on activation, normal T cell expressed and secreted upregulation in adipose tissue in obesity. *Circulation* **115**, 1029–1038
5. Takemori, K., Gao, Y. J., Ding, L., Lu, C., Su, L. Y., An, W. S., Vinson, C., and Lee, R. M. (2007) Elevated blood pressure in transgenic lipoatrophic mice and altered vascular function. *Hypertension* **49**, 365–372
6. Lu, C., Su, L. Y., Lee, R. M., and Gao, Y. J. (2011) Alterations in perivascular adipose tissue structure and function in hypertension. *Eur. J. Pharmacol.* **656**, 68–73
7. Kraus, B. J., Sartoretto, J. L., Polak, P., Hosooka, T., Shiroto, T., Eskurza, I., Lee, S. A., Jiang, H., Michel, T., and Kahn, B. B. (2015) Novel role for retinol-binding protein 4 in the regulation of blood pressure. *FASEB J.* **29**, 3133–3140
8. Guzik, T. J., Hoch, N. E., Brown, K. A., McCann, L. A., Rahman, A., Dikalov, S., Goronzy, J., Weyand, C., and Harrison, D. G. (2007) Role of the T cell in the genesis of angiotensin II induced hypertension and vascular dysfunction. *J. Exp. Med.* **204**, 2449–2460
9. Mattson, D. L., Lund, H., Guo, C., Rudemiller, N., Geurts, A. M., and Jacob, H. (2013) Genetic mutation of recombination activating gene 1 in Dahl salt-sensitive rats attenuates hypertension and renal damage. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **304**, R407–R414
10. Rudemiller, N., Lund, H., Jacob, H. J., Geurts, A. M., and Mattson, D. L.; PhysGen Knockout Program. (2014) CD247 modulates blood pressure by altering T-lymphocyte infiltration in the kidney. *Hypertension* **63**, 559–564
11. Ji, H., Zheng, W., Li, X., Liu, J., Wu, X., Zhang, M. A., Umans, J. G., Hay, M., Speth, R. C., Dunn, S. E., and Sandberg, K. (2014) Sex-specific T-cell regulation of angiotensin II–dependent hypertension. *Hypertension* **64**, 573–582
12. Wenzel, P., Knorr, M., Kossmann, S., Stratmann, J., Hausding, M., Schuhmacher, S., Karbach, S. H., Schwenk, M., Yogev, N., Schulz, E., Oelze, M., Grabbe, S., Jonuleit, H., Becker, C., Daiber, A., Waisman, A., and Münzel, T. (2011) Lysozyme M–positive monocytes mediate angiotensin II–induced arterial hypertension and vascular dysfunction. *Circulation* **124**, 1370–1381
13. Kossmann, S., Schwenk, M., Hausding, M., Karbach, S. H., Schmidgen, M. I., Brandt, M., Knorr, M., Hu, H., Kröller-Schön, S., Schönfelder, T., Grabbe, S., Oelze, M., Daiber, A., Münzel, T., Becker, C., and Wenzel, P. (2013) Angiotensin II–induced vascular dysfunction depends on interferon-γ-driven immune cell

recruitment and mutual activation of monocytes and NK-cells. *Arterioscler. Thromb. Vasc. Biol.* **33**, 1313–1319
14. Barhoumi, T., Kasal, D. A., Li, M. W., Shbat, L., Laurant, P., Neves, M. F., Paradis, P., and Schiffrin, E. L. (2011) T regulatory lymphocytes prevent angiotensin II–induced hypertension and vascular injury. *Hypertension* **57**, 469–476
15. Pons, H., Ferrebuz, A., Quiroz, Y., Romero-Vasquez, F., Parra, G., Johnson, R. J., and Rodriguez-Iturbe, B. (2013) Immune reactivity to heat shock protein 70 expressed in the kidney is cause of salt-sensitive hypertension. *Am. J. Physiol. Renal Physiol.* **304**, F289–F299
16. Zhang, J., Patel, M. B., Griffiths, R., Mao, A., Song, Y. S., Karlovich, N. S., Sparks, M. A., Jin, H., Wu, M., Lin, E. E., and Crowley, S. D. (2014) Tumor necrosis factor-α produced in the kidney contributes to angiotensin II–dependent hypertension. *Hypertension* **64**, 1275–1281
17. Crowley, S. D., Song, Y. S., Sprung, G., Griffiths, R., Sparks, M., Yan, M., Burchette, J. L., Howell, D. N., Lin, E. E., Okeiyi, B., Stegbauer, J., Yang, Y., Tharaux, P. L., and Ruiz, P. (2010) A role for angiotensin II type 1 receptors on bone marrow-derived cells in the pathogenesis of angiotensin II–dependent hypertension. *Hypertension* **55**, 99–108
18. Li, Y., Wu, Y., Zhang, C., Li, P., Cui, W., Hao, J., Ma, X., Yin, Z., and Du, J. (2014) γδT Cell-derived interleukin-17A *via* an interleukin-1β-dependent mechanism mediates cardiac injury and fibrosis in hypertension. *Hypertension* **64**, 305–314
19. Galkina, E., Kadl, A., Sanders, J., Varughese, D., Sarembock, I. J., and Ley, K. (2006) Lymphocyte recruitment into the aortic wall before and during development of atherosclerosis is partially L-selectin dependent. *J. Exp. Med.* **203**, 1273–1282
20. Veillard, N. R., Kwak, B., Pelli, G., Mulhaupt, F., James, R. W., Proudfoot, A. E., and Mach, F. (2004) Antagonism of RANTES receptors reduces atherosclerotic plaque formation in mice. *Circ. Res.* **94**, 253–261
21. Mateo, T., Abu Nabah, Y. N., Abu Taha, M., Mata, M., Cerda-Nicolas, M., Proudfoot, A. E., Stahl, R. A., Issekutz, A. C., Cortijo, J., Morcillo, E. J., Jose, P. J., and Sanz, M. J. (2006) Angiotensin II–induced mononuclear leukocyte interactions with arteriolar and venular endothelium are mediated by the release of different CC chemokines. *J. Immunol.* **176**, 5577–5586
22. Kirabo, A., Fontana, V., de Faria, A. P., Loperena, R., Galindo, C. L., Wu, J., Bikineyeva, A. T., Dikalov, S., Xiao, L., Chen, W., Saleh, M. A., Trott, D. W., Itani, H. A., Vinh, A., Amarnath, V., Amarnath, K., Guzik, T. J., Bernstein, K. E., Shen, X. Z., Shyr, Y., Chen, S. C., Mernaugh, R. L., Laffer, C. L., Elijovich, F., Davies, S. S., Moreno, H., Madhur, M. S., Roberts II, J., and Harrison, D. G. (2014) DC isoketal-modified proteins activate T cells and promote hypertension. *J. Clin. Invest.* **124**, 4642–4656
23. Vandanmagsar, B., Youm, Y. H., Ravussin, A., Galgani, J. E., Stadler, K., Mynatt, R. L., Ravussin, E., Stephens, J. M., and Dixit, V. D. (2011) The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. *Nat. Med.* **17**, 179–188
24. Madhur, M. S., Lob, H. E., McCann, L. A., Iwakura, Y., Blinder, Y., Guzik, T. J., and Harrison, D. G. (2010) Interleukin 17 promotes angiotensin II–induced hypertension and vascular dysfunction. *Hypertension* **55**, 500–507
25. Wolkow, P. P., Kosiniak-Kamysz, W., Osmenda, G., Wilk, G., Bujak-Gizycka, B., Ignacak, A., Kanitkar, M., Walus-Miarka, M., Harrison, D. G., Korbut, R., Malecki, M. T., and Guzik, T. J. (2014) GTP cyclohydrolase I gene polymorphisms are associated with endothelial dysfunction and oxidative stress in patients with type 2 diabetes mellitus. *PLoS One* **9**, e108587
26. Lee, D. L., Sturgis, L. C., Labazi, H., Osborne, J. B., Jr., Fleming, C., Pollock, J. S., Manhiani, M., Imig, J. D., and Brands, M. W. (2006) Angiotensin II hypertension is attenuated in interleukin-6 knockout mice. *Am. J. Physiol. Heart Circ. Physiol.* **290**, H935–H940
27. Saleh, M. A., McMaster, W. G., Wu, J., Norlander, A. E., Funt, S. A., Thabet, S. R., Kirabo, A., Xiao, L., Chen, W., Itani, H. A., Michell, D., Huan, T., Zhang, Y., Takaki, S., Titze, J., Levy, D., Harrison, D. G., and Madhur, M. S. (2015) Lymphocyte adaptor protein LNK deficiency exacerbates hypertension and end-organ inflammation. *J. Clin. Invest.* **125**, 1189–1202
28. Markó, L., Kvakan, H., Park, J. K., Qadri, F., Spallek, B., Binger, K. J., Bowman, E. P., Kleinewietfeld, M., Fokuhl, V., Dechend, R., and Müller, D. N. (2012) Interferon-γ signaling inhibition ameliorates angiotensin II–induced cardiac damage. *Hypertension* **60**, 1430–1436
29. Manea, S. A., Todirita, A., Raicu, M., and Manea, A. (2014) C/EBP transcription factors regulate NADPH oxidase in human aortic smooth muscle cells. *J. Cell. Mol. Med.* **18**, 1467–1477
30. Uemura, Y., Shibata, R., Kanemura, N., Ohashi, K., Kambara, T., Hiramatsu-Ito, M., Enomoto, T., Yuasa, D., Joki, Y., Matsuo, K., Ito, M., Hayakawa, S., Ogawa, H., Murohara, T., and Ouchi, N. (2015) Adipose-derived protein omentin prevents neointimal formation after arterial injury. *FASEB J.* **29**, 141–151
31. De Miguel, C., Rudemiller, N. P., Abais, J. M., and Mattson, D. L. (2015) Inflammation and hypertension: new understandings and potential therapeutic targets. *Curr. Hypertens. Rep.* **17**, 507
32. Schiffrin, E. L. (2014) Immune mechanisms in hypertension and vascular injury. *Clin. Sci.* **126**, 267–274
33. Marques, R. E., Guabiraba, R., Russo, R. C., and Teixeira, M. M. (2013) Targeting CCL5 in inflammation. *Expert Opin. Ther. Targets* **17**, 1439–1460
34. Peng, X., Xiao, Z., Zhang, J., Li, Y., Dong, Y., and Du, J. (2015) IL-17A produced by both γδ T and Th17 cells promotes renal fibrosis *via* RANTES-mediated leukocyte infiltration after renal obstruction. *J. Pathol.* **235**, 79–89
35. Schall, T. J., Jongstra, J., Dyer, B. J., Jorgensen, J., Clayberger, C., Davis, M. M., and Krensky, A. M. (1988) A human T cell–specific molecule is a member of a new gene family. *J. Immunol.* **141**, 1018–1025
36. Levy, J. A. (2009) The unexpected pleiotropic activities of RANTES. *J. Immunol.* **182**, 3945–3946
37. Koenen, R. R., von Hundelshausen, P., Nesmelova, I. V., Zernecke, A., Liehn, E. A., Sarabi, A., Kramp, B. K., Piccinini, A. M., Paludan, S. R., Kowalska, M. A., Kungl, A. J., Hackeng, T. M., Mayo, K. H., and Weber, C. (2009) Disrupting functional interactions between platelet chemokines inhibits atherosclerosis in hyperlipidemic mice. *Nat. Med.* **15**, 97–103
38. Rocha, V. Z., Folco, E. J., Sukhova, G., Shimizu, K., Gotsman, I., Vernon, A. H., and Libby, P. (2008) Interferon-gamma, a Th1 cytokine, regulates fat inflammation: a role for adaptive immunity in obesity. *Circ. Res.* **103**, 467–476
39. Agrewala, J. N., Brown, D. M., Lepak, N. M., Duso, D., Huston, G., and Swain, S. L. (2007) Unique ability of activated $CD4^+$ T cells but not rested effectors to migrate to non-lymphoid sites in the absence of inflammation. *J. Biol. Chem.* **282**, 6106–6115
40. Wirsen, C. (1964) Adrenergic innervation of adipose tissue examined by fluorescence microscopy. *Nature* **202**, 913
41. Frontini, A., and Cinti, S. (2010) Distribution and development of brown adipocytes in the murine and human adipose organ. *Cell Metab.* **11**, 253–256
42. Wolf, G., Ziyadeh, F. N., Thaiss, F., Tomaszewski, J., Caron, R. J., Wenzel, U., Zahner, G., Helmchen, U., and Stahl, R. A. (1997) Angiotensin II stimulates expression of the chemokine RANTES in rat glomerular endothelial cells. Role of the angiotensin type 2 receptor. *J. Clin. Invest.* **100**, 1047–1058

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