Abnormal CD161$^+$ immune cells and retinoic acid receptor–related orphan receptor γt–mediate enhanced IL-17F expression in the setting of genetic hypertension

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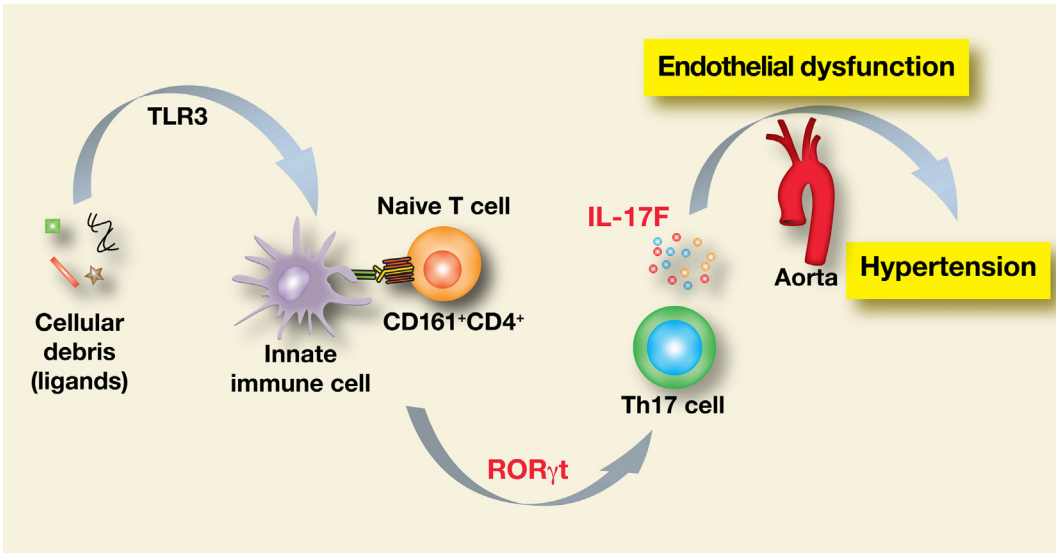
GRAPHICAL ABSTRACT



Background: Hypertension is considered an immunologic disorder. However, the role of the IL-17 family in genetic hypertension in the spontaneously hypertensive rat (SHR) has not been investigated.

Objective: We tested the hypothesis that enhanced T_H17 programming and IL-17 expression in abundant CD161$^+$ immune cells in SHRs represent an abnormal proinflammatory adaptive immune response. Furthermore, we propose that this response is driven by the master regulator retinoic acid receptor–related orphan receptor γt (RORγt) and a nicotinic proinflammatory innate immune response.

Methods: We measured expression of the CD161 surface marker on splenocytes in SHRs and normotensive control Wistar-Kyoto (WKY) rats from birth to adulthood. We compared expression of IL-17A and IL-17F in splenic cells under different conditions. We then determined the functional effect of these cytokines on vascular reactivity. Finally, we tested whether pharmacologic inhibition of RORγt can attenuate hypertension in SHRs.

Results: SHRs exhibited an abnormally large population of CD161$^+$ cells at birth that increased with age, reaching more than 30% of the splenocyte population at 38 weeks. The SHR splenocytes constitutively expressed more RORγt than those of WKY rats and produced more IL-17F on induction. Exposure of WKY rat aortas to IL-17F impaired endothelium-dependent vascular relaxation, whereas IL-17A did not. Moreover, *in vivo*

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inhibition of RORγt by digoxin decreased systolic blood pressure in SHRs.
Conclusions: SHRs have a markedly enhanced potential for RORγt-driven expression of proinflammatory and prohypertensive IL-17F in response to innate immune activation. Increased RORγt and IL-17F levels contribute to SHR hypertension and might be therapeutic targets. (J Allergy Clin Immunol 2017;■■■:■■■-■■■.)

Key words: *Hypertension, innate immune system, Toll-like receptor, retinoic acid receptor–related orphan receptor γt, digoxin, T cells, T_H17, CD161, IL-17, spontaneously hypertensive rat, gene expression*

Abbreviations used
nAChR: Nicotinic acetylcholinergic receptor
PE: Phycoerythrin
Poly-IC: Polyinosinic-polycytidylic acid
RORγt: Retinoic acid receptor–related orphan receptor γt
SBP: Systolic blood pressure
SHR: Spontaneously hypertensive rat
SNP: Sodium nitroprusside
TLR: Toll-like receptor
Treg: Regulatory T
WKY: Wistar-Kyoto

Multiple organ systems involving the kidneys, vasculature, central nervous system, and immune system contribute to the genesis and maintenance of hypertension. The spontaneously hypertensive rat (SHR) is a widely studied rodent model of genetic hypertension that faithfully displays pathologic changes seen in human disease. SHRs show progressive increases in blood pressure, increased sympathetic tone, renal dysfunction, and a dysregulated immune system.[1,2] The role of inflammation and the immune system in hypertension has long been known, but the underlying mechanisms are just beginning to be understood.

SHR immune cells display an inherently enhanced inflammatory response. Cultured splenocytes from prehypertensive SHRs produce greater amounts of proinflammatory cytokines than those from normotensive Wistar-Kyoto (WKY) rats when stimulated by Toll-like receptor (TLR) 7/8 and TLR9 agonists in the presence of angiotensin II or nicotine.[1] In contrast, nicotine suppresses inflammatory cytokine release in WKY rats. In addition, nicotine treatment of cultured splenocytes from SHRs increases the relative abundance of a $CD161^+$ cell population. However, the distribution of CD161 cell-surface markers in the immune system of the SHR and its potential role in hypertension-related inflammation have not been examined.

Originally identified as a rodent homolog of a cell-surface marker for human natural killer cells,[3] the CD161 marker correlates with activation of orphan nuclear receptor retinoic acid receptor–related orphan receptor γt (RORγt), which functions as a master regulator of polarization of IL-17–producing T_H17 cells.[4-6] IL-17A and IL-17F are potent proinflammatory cytokines that drive inflammatory processes in patients with autoimmune and cardiovascular diseases, including hypertension,[7,8] and end-organ damage.[9,10] Angiotensin II–induced hypertension is not sustained in *Il17*$^{-/-}$ mice.[11] Dermal overexpression of IL-17A in psoriasis-like skin disease induces endothelial dysfunction and arterial hypertension.[12] In addition, infusion of IL-17 causes Rho kinase–mediated endothelial dysfunction and hypertension.[13] Antihypertensive treatment with telmisartan and statin in patients with carotid atherosclerosis reduces blood pressure and numbers of IL-17–producing T_H17 cells.[14] Deoxycorticosterone acetate–salt diet–induced organ damage is attenuated by blocking IL-17.[15]

The IL-17 family of cytokines consists of 6 members (IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F). IL-17A (commonly known as IL-17) plays a key role in several autoimmune diseases and has been studied extensively. Relatively little is known about IL-17F, which is the closest homolog of IL-17A and forms a heterodimer with it. IL-17A and IL-17F share a common receptor but are recognized to have nonoverlapping functions.[16] Despite a presumed role of the immune system, inflammation, and T_H17 cells in the setting of hypertension, the effect of IL-17F in a hypertension-related physiologic process has not been demonstrated.

The autonomic nervous system has immunomodulatory effects on expression of proinflammatory cytokines and proliferation of many immune cells.[17] This immunomodulation exerts a tonic inhibition of proinflammatory immune cells by acetylcholine binding to nicotinic acetylcholinergic receptors (nAChR).[18,19] However, the effects of nicotine, an agonist of nAChR, on different cell types can vary depending on the expressed subunit and pharmacology of the receptors.[20-22] T_H17 cells are differentiated from a $CD4^+$ immune cell lineage that expresses nAChR and are subject to the regulatory effects of nicotine.[23-25] The inhibitory effect of nicotine on proinflammatory responses to TLR activation seen in splenocytes of normotensive WKY rats is abrogated in SHR splenocytes and replaced by a more pronounced proinflammatory response.[1]

Here we tested the hypothesis that enhanced T_H17 programming by RORγt transcription factor and IL-17F expression in $CD161^+$ immune cells represent an abnormal proinflammatory adaptive immune response in SHRs. Furthermore, we proposed that this response is driven by the master regulator RORγt and a nicotinic proinflammatory innate immune response.

Our results demonstrate that the CD161 cell-surface marker is widely and profusely expressed on SHR immune cells, including $CD4^+$ and $CD8^+$ T lymphocytes. There are more infiltrating $CD161^+$ cells in SHR kidneys and aortas than in WKY rats. $CD4^+CD161^+$ immune cells constitutively overexpress RORγt and induce IL-17F in response to inflammatory stimulation. Inhibition of RORγt expression with digoxin reduces systolic blood pressure (SBP) and renal infiltration of CD3 cells in kidneys of SHRs. We also report that nicotine, an anti-inflammatory agonist in WKY rats, actually increases $CD4^+CD161^+$ cell numbers in SHRs and potentiates their TLR3-mediated activation and *Il17f* expression. Finally, we identified a significant impairment of cholinergic endothelium-mediated vascular relaxation in response to IL-17F that was not seen with IL-17A.

We conclude that both the innate and adaptive immune systems are inherently dysregulated in SHRs. The dysregulation involves $CD161^+CD4^+$ immune cell RORγt transcription factor, a master regulator of IL-17A and IL-17F expression, that contributes to hypertension. The dysregulation also involves a nicotine-mediated increase in numbers of $CD4^+CD161^+$ splenocytes and expression of IL-17F that disrupts cholinergic endothelial vasorelaxation.

METHODS

Animals

WKY rats and SHRs were obtained from Charles River Laboratory (Wilmington, Mass). Male rats ranging in age from 1 day to 38 weeks were used. All experiments were performed under the guidelines set forth by the University of Iowa Institutional Animal Care and Use Committee and the National Institutes of Health. Rats were infused with saline or nicotine (15 mg/kg body weight) by using miniosmotic pumps (Alzet 2001D; Durect, Cupertino, Calif) for 24 hours.

Flow cytometry

Spleens from WKY rats and SHRs were obtained after exsanguination during anesthesia (isoflurane), by decapitation. Spleen cells were disaggregated in prewarmed HBSS (37°C). After erythrocyte lysis in a hypotonic buffer (155 mmol/L ammonium chloride, 12 mmol/L sodium bicarbonate, and 0.1 mmol/L EDTA), cells were washed with PBS, nonspecific binding was blocked by means of incubation in Fc blocking solution (PBS with 5% FBS and 1% mouse serum), and cells were subjected to antibody labeling with anti-rat fluorescein isothiocyanate–CD161, phycoerythrin (PE)–CD3, peridinin-chlorophyll-protein complex–CD8α, allophycocyanin-CD4, and PE-Cy5–CD45RA. Cells were washed twice in Fc blocking solution and resuspended in the same solution. Flow cytometry was performed with the LSR II flow cytometer (Becton Dickinson, Franklin Lakes, NJ), and data were analyzed with either FlowJo (TreeStar, Ashland, Ore) or BD Aria software. For live-dead cell distinction, a vital fluorescent dye (Hoechst 33258; Thermo Fisher Scientific, Waltham, Mass) was added to the cell samples immediately before flow cytometry.

Cell cultures

Splenocytes from WKY rats and SHRs were plated in culture dishes containing complete RPMI 1640 (RPMI 1640, 10% FBS, 10 mmol/L HEPES, and 1 mmol/L sodium pyruvate supplemented with nonessential amino acids and penicillin/streptomycin). Cells and culture supernatants were collected and stored frozen at −80°C until further use.

Cytokine induction

Polyinosinic-polycytidylic acid (poly-IC; Sigma, St Louis, Mo), the ligand of TLR3, was used for induction of splenocytes at a final concentration of 10 μg/mL added to the cultures, and cells were collected for RNA isolation. The T_H17 polarization program was induced by means of addition of anti-rat CD3 antibody (3 μg/mL; Affymetrix, Santa Clara, Calif), rat TGF-β (5 ng/mL; R & D Biosystems, Minneapolis, Minn), and rat IL-6 (20 ng/mL; R & D Biosystems) for 4 to 7 days. Control cultures were left untreated.

RNA isolation and reverse transcription

RNA was isolated with the Qiagen RNeasy RNA isolation kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol, as described earlier.[26] Equal amounts of RNA (0.5-2 μg for each sample) were used for reverse transcription in 50-μL reactions. Oligo-dT primers were used for first-strand synthesis with SuperScript III (Thermo Fisher Scientific) reverse transcriptase enzyme. In some experiments 2.5 μmol/L of an 18S ribosomal RNA-specific primer (5′-GAGCTGGAATTACCGCGGCT-3′) was added.

Quantitative real-time PCR

Quantitative PCR was performed with either dye intercalation (SYBR green) or the TaqMan method by using reverse transcription reactions as templates. Relative change in gene expression was quantified by using the ΔΔ cycle threshold method with glyceraldehyde-3-phosphate dehydrogenase *(Gapdh)* or 18S ribosomal RNA as loading controls.[27,28]

ELISA

ELISAs for detection of IL-17A and IL-17F were performed on splenocyte culture supernatants with the Rat IL-17A Platinum ELISA kit (Affymetrix) and IL-17F DuoSet ELISA development system (R&D Systems, Minneapolis, Minn), according to the manufacturers' suggested protocols.

Digoxin treatment of SHRs and blood pressure measurements

Prehypertensive male SHRs (5 weeks old) were injected daily subcutaneously with digoxin (10 μg/g body weight in 20-μL volume; Acros Organics, Morris Plains, NJ) for 9 weeks. The dose for the drug was adjusted weekly to keep up with the gain in body weight. Age- and sex-matched control SHRs were injected with vehicle (dimethyl sulfoxide). SBPs were recorded twice every week by using tail cuff plethysmography with the VisiTech 2000 system (VisiTech, Denver, Colo). Weekly SBPs reported were averages of 2 recording sessions each week over a period of 3 months. Statistical analyses of SBPs were performed with a 2-way ANOVA (Prism Software, version 7.0a; GraphPad Software, La Jolla, Calif) on SBP data starting from 7 weeks of age, when the increase in pressure became evident.

Vascular reactivity

Thoracic aortas of adult WKY rats (14-16 weeks old) were used in the study. Vascular tension recordings were performed, as described previously.[29] Briefly, rats were killed by means of CO_2 inhalation, and dissected aortas were placed in ice-cold oxygenated Krebs buffer (118.3 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L $CaCl_2$, 1.2 mmol/L KH_2PO_4, 25 mmol/L $NaHCO_3$, 1.2 mmol/L $MgSO_4$, 11 mmol/L glucose, and 0.0026 mmol/L $CaNa_2EDTA$). Vessels were cleared of connective tissue, cut into 2- to 3-mm rings, and cultured overnight (16-20 hours) in EGM2 medium (Lonza, Walkersville, Md) in the presence or absence of IL-17A or IL-17F. These aortic rings were then suspended between 2 wire stirrups (150 μm) in a 4-chamber myograph system (DMT Instruments, Crystal River, Fla) in Krebs buffer (95% O_2, 5% CO_2, pH 7.4, 37°C). The mechanical force signal was amplified, digitalized, and recorded (PowerLab 8/30; AD Instruments, Bella Vista, Australia). The concentration-effect curves were recorded at the beginning of the optimum resting tone. Endothelium-dependent and independent relaxation were determined by generating dose-response curves to acetylcholine (10^{-9} to 10^{-5} mol/L) and sodium nitroprusside (SNP; 10^{-9} to 10^{-5} mol/L), respectively, of the phenylephrine (PE) (10^{-6} mol/L)–induced precontracted vessel. Vasorelaxation evoked by acetylcholine and SNP was expressed as percentage relaxation, as determined by calculating percentage inhibition of the preconstricted tension.

Tissue and immunohistochemistry

At necroscopy, harvested tissues were fixed in 10% neutral buffered saline (3-5 days), followed by standard processing for paraffin embedding. Tissue sections (4 μm) were either stained with hematoxylin and eosin for histopathological examination or immunostained for CD3 or ED1[30] after heat-induced antigen retrieval (Decloaking Chamber; BioCare Medical, Concord, Calif) by using a rabbit polyclonal antibody (Catalog no. A0452; Dako, Glostrup, Denmark) and counterstaining with Harris hematoxylin. A postexamination masking procedure described previously was used to avoid bias in tissue examination.[31] Random areas of the cortex kidney sections were photographed (×100 magnification; CellSens software, Olympus BX51 microscope; Olympus, Center Valley, Pa) and counted for cellular immunostaining (5 replicates per kidney), and total counts were normalized to unit area (counts of cells per square millimeter).

Statistical analyses

The unpaired *t* test or ANOVA was used for statistical analyses, as shown in respective figures. *P* values of less than .05 were considered statistically significant differences. Results are presented as means ± SEMs.

RESULTS

Age-related increase in $CD161^+$ cell counts in SHRs

Splenocytes from age-matched WKY rats and SHRs ranging from 1 day to 38 weeks were analyzed by using flow cytometry

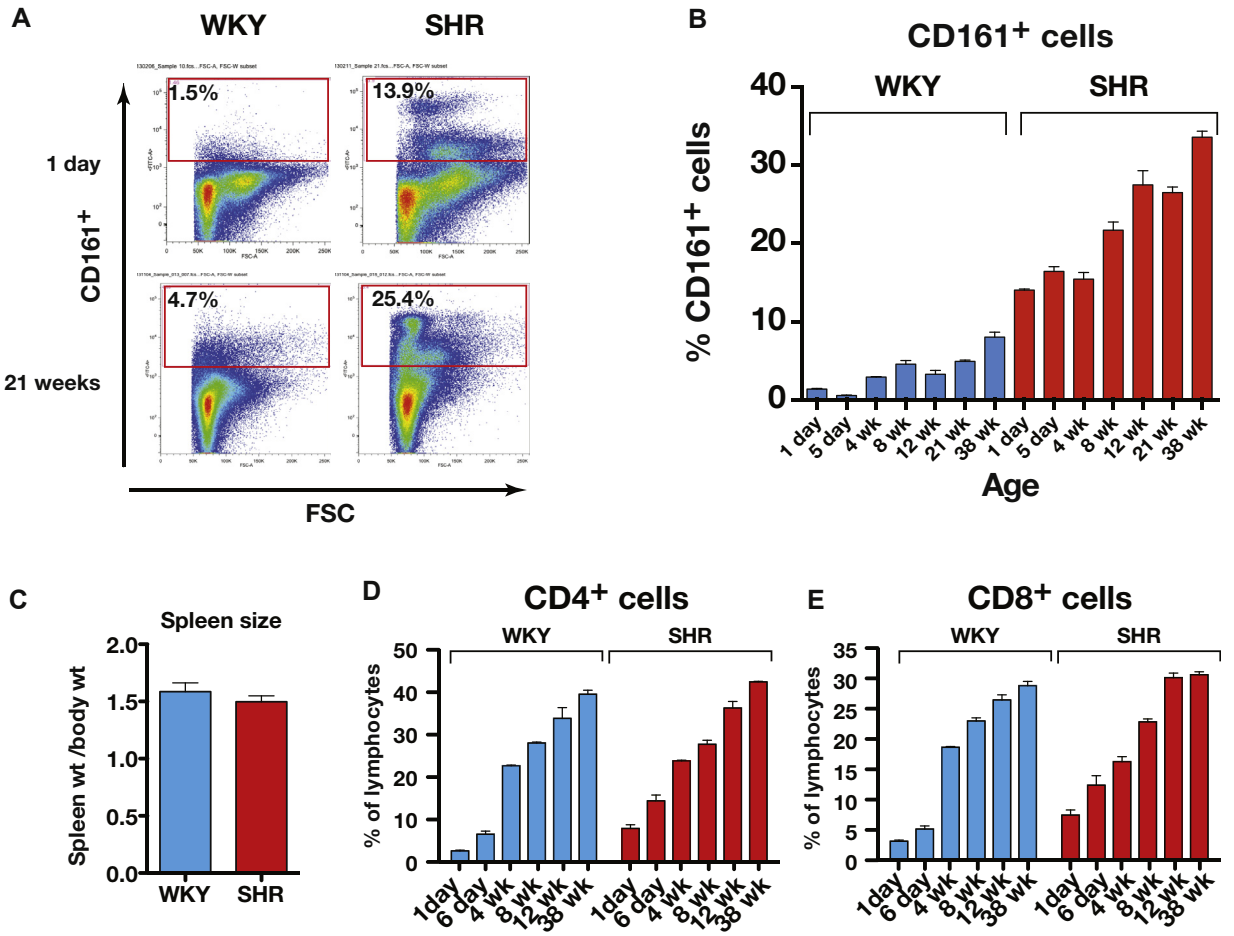

FIG 1. A, Flow cytometry of $CD161^+$ cells in spleens of newborn (1-day-old) and adult (21-week-old) SHRs and WKY rats. **B,** Age-related increase in $CD161^+$ cell counts in SHR spleens (n = 3 rats for each age). **C,** Spleen weight/body weight ratio (grams per kilogram of body weight) of adult WKY rats and SHRs. **D** and **E,** Frequency of $CD4^+$ lymphocytes (Fig 1, *D*) and $CD8^+$ lymphocytes (Fig 1, *E*) in the spleen at different ages in WKY rats and SHRs.

(Fig 1, *A* and *B*). In 1-day-old SHR splenocytes the $CD161^+$ cell population was significantly greater than in WKY rats (1.4% ± 0.1% in WKY rats vs 14.0% ± 0.1% in SHRs). In SHRs the $CD161^+$ cell population progressively increased with age, reaching 33.6% ± 0.8% of the splenic immune cell population at 38 weeks of age. In contrast, in WKY rats $CD161^+$ splenocytes were only 8.0% ± 0.7% by 38 weeks of age. The marked increase in the SHR cell population was not associated with an increase in spleen/body mass ratio of the mature SHRs compared with WKY rats (1.5 ± 0.1 vs 1.6 ± 0.1 mg/g, respectively; Fig 1, *C*). The high abundance of $CD161^+$ cells was not attributable to nonspecific binding of surface marker antibody to dead cells (see Fig E1 in this article's Online Repository at www.jacionline.org). Moreover, the lymphocyte populations of $CD4^+$ and $CD8^+$ cells in both WKY rats and SHRs increased similarly with age (Fig 1, *D* and *E*).

Thus SHRs exhibit an extraordinarily high abundance of $CD161^+$ immune cells in the spleen at birth. Numbers of these cells increase with age to reach surprisingly high levels as hypertension develops. The increase appears to be caused by acquisition of the CD161 cell-surface marker in SHRs rather than proliferation of $CD161^+$ cells.

In addition to the spleen, we also tested the abundance of $CD161^+$ cells in the kidney and aorta. There was greater infiltration of $CD161^+$ cells in kidneys and aortas of SHRs than in WKY rats (see Fig E2 in this article's Online Repository at www.jacionline.org).

$CD161^+$ cell-surface marker is overexpressed on SHR $CD4^+$ and $CD8^+$ T lymphocytes

In spleens of 5-day-old rats, $CD4^+CD161^+$ cells were significantly more abundant in the SHRs than in the WKY rats (0.2% ± 0.02% in WKY rats vs 2.1% ± 0.04% in SHRs, $P < .0001$), as were $CD8^+CD161^+$ cells (0.14% ± 0.00% in WKY rats vs 2.5% ± 0.15% in SHRs, $P < .0001$; Fig 2).

With age, populations of these lymphocytes grew much more abundantly in SHRs, reaching twice the levels seen in WKY rats at 38 weeks. For $CD4^+CD161^+$ cells, values were 8.53% ± 0.62% in WKY rats versus 14.70% ± 0.10% in SHRs, and corresponding values for $CD8^+CD161^+$ cells were 8.76% ± 0.64% in WKY rats versus 16.25% ± 0.15% in SHRs ($P = .0076$). In contrast, the $CD161^+$ marker was not expressed on $CD45RA^+$ B lymphocytes (data not shown). Thus the SHR

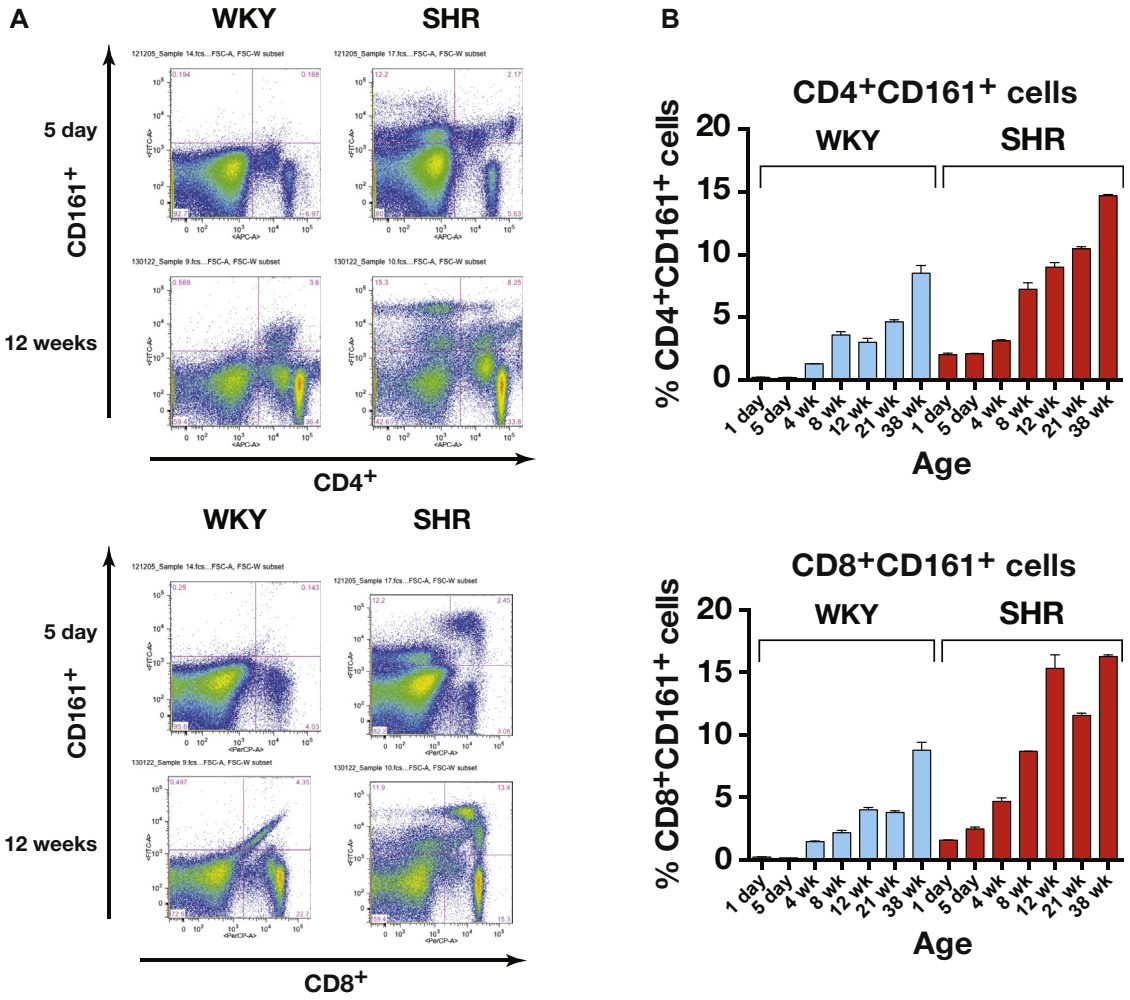

FIG 2. A, Dot plot of flow cytometric results showing greater abundance of $CD4^+CD161^+$ cells, as well as $CD8^+CD161^+$ cells, in young prehypertensive (5-day-old) and adult (12-week-old) SHRs. **B,** SHR immune cells displayed a greater age-related increase in both $CD4^+CD161^+$ and $CD8^+CD161^+$ cell populations (n = 3 rats for each age).

model has a selective and specific abnormality of increased $CD4^+CD161^+$ and $CD8^+CD161^+$ lymphocytes at birth, even before the onset of hypertension, which increases progressively with age, as does hypertension.

$CD161^+$ cells express RORγt transcription factor

The CD161 marker is associated with IL-17–producing T lymphocytes.[4,6] Expression of IL-17 and CD161 surface markers is regulated by the orphan nuclear receptor RORγt (*Rorc* gene), the master regulator transcription factor that determines $CD4^+$ T_H17 and $CD8^+$ T_C17 cell polarization and IL-17A and IL-17F expression.[5,32] At baseline, the expression of *Rorc* in untreated SHR splenocytes was significantly greater than that in untreated WKY rats (approximately 5-fold greater in SHRs, $P = .03$; Fig 3, A). On T_H17 polarization (anti-CD3 antibody, IL-6, and TGF-β), *Rorc* expression in cultured WKY rat splenocytes increased 4.5-fold. However, the large expression of *Rorc* in SHR splenocytes did not change (Fig 3, *A*).

We further investigated which subset of $CD161^+$ cells in SHRs expressed RORγt. Using fluorescence-activated cell sorting of splenocytes from adult SHRs, we separated SHR $CD161^+$ cells into $CD4^+CD161^+$, $CD8^+CD161^+$, and $CD4^-CD8^-CD161^+$ populations (Fig 3, *B*). In addition, as a negative control, we also collected $CD161^-CD45R^+$ cells (Fig 3, *B*). The different populations of $CD161^+$ cells represent specific immune cell populations (Fig 3, *C*). Reverse transcription–quantitative PCR–based analysis showed that *Rorc* RNA was detected in $CD161^+$ cells but not in cells lacking CD161 surface marker ($CD161^-CD45R^+$ cells; Fig 3, *D*). Moreover, the expression of *Rorc* was highest in $CD4^+CD161^+$ cells, followed by $CD4^-CD8^-CD161^+$ cells, and the lowest in $CD8^+CD161^+$ cells (approximately 18-fold difference between $CD4^+CD161^+$ and $CD8^+CD161^+$ cells; Fig 3, *D*). Thus the expression of RORγt transcription factor in SHRs is most abundantly expressed in $CD4^+CD161^+$ cells of the SHR splenocytes.

Enhanced IL-17F expression in SHRs on T_H17 cell induction

Induction of T lymphocytes depends on interaction of the innate (antigen-processing cells) and adaptive (T cells) immune

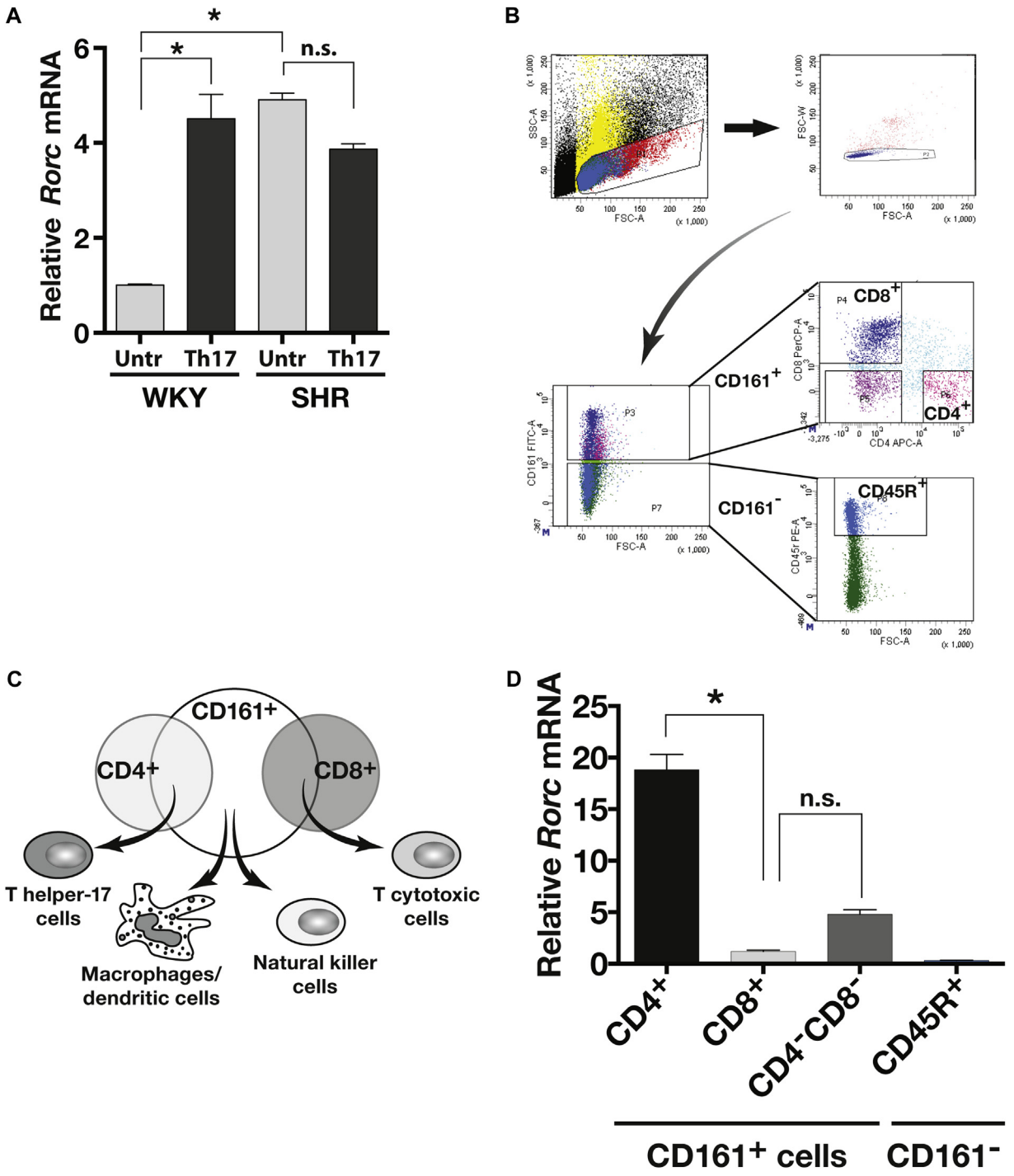

FIG 3. A, Expression of *Rorc* (RORγt) RNA in untreated *(Untr)* and T_H17-polarized WKY rat and SHR splenocytes. **B,** Gating scheme for fluorescence-activated cell sorting (FACS) of SHR splenocytes. The $CD161^-CD45R^+$ subpopulation was the negative control. *FSC,* Forward scatter; *SSC,* side scatter. **C,** Summary diagram of FACS analyses showing the composition of the $CD161^+$ cell population in SHRs. **D,** *Rorc* RNA expression in the FACS-sorted subpopulation of SHR splenocytes. *$P < .05$. *n.s.,* Not significant. N = 3 each.

cells. The master transcription factor RORγt regulates T_H17 differentiation of $CD4^+$ T lymphocytes, leading to expression of IL-17A and IL-17F in these cells (Fig 4, *A*).[5] We stimulated *in vitro* T_H17 polarization in WKY rat and SHR splenic T cells using anti-CD3 agonist antibody (T-cell receptor activation) in the presence of the T_H17-promoting cytokines IL-6 and TGF-β. A significant and similar increase in *Il17a* RNA expression was seen in both WKY rat and SHR splenocytes (3.5-fold in WKY rats vs 3.1-fold in SHRs; Fig 4, *B*). However, the induction of *Il17f* RNA expression was significantly greater in SHR splenocytes (4.7-fold increase) than in WKY rat splenocytes (1.4-fold; Fig 4, *C*). These results suggest that SHR splenocytes

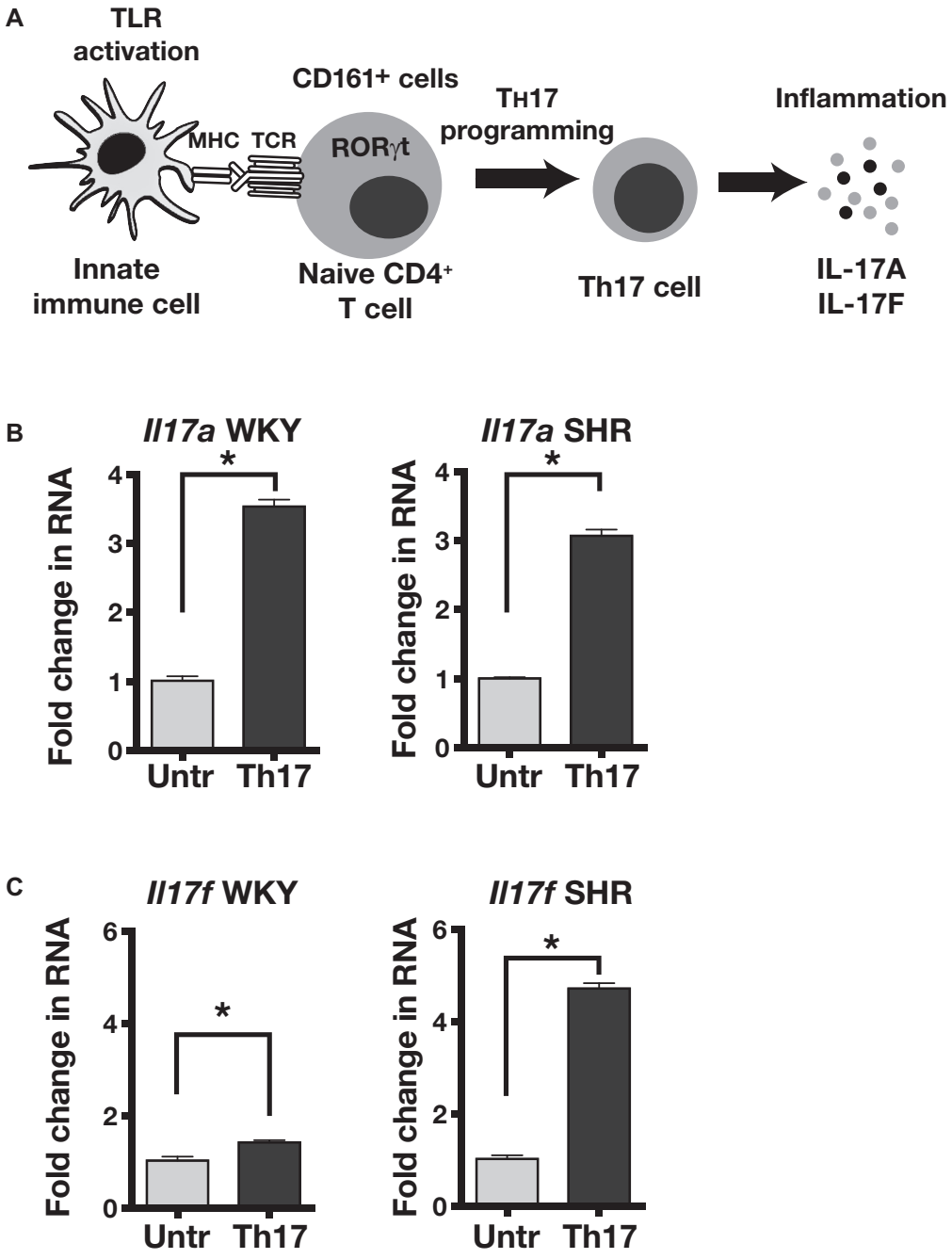

FIG 4. A, Schematic showing the differentiation program of CD161^{+} T cells into IL-17A– and IL-17F–producing T_H17 cells by means of activation of TLRs and the TCR. **B,** *Il17a* RNA expression in untreated *(Untr)* and T_H17-polarized splenocytes from WKY rats and SHRs. **C,** *Il17f* RNA expression in SHR splenocytes on T_H17 polarization. *$P < .05$. N = 3 each.

have greater adaptive immune response through potentially greater T_H17 polarization and expression of proinflammatory IL-17 cytokines.

High expression of IL-17 in SHR splenocytes in response to the TLR3 agonist is RORγt dependent

Innate immune signaling through TLR3 plays a key role in antigen-presenting cell–mediated activation of T cells and their polarization into the T_H17 lineage.[4-6] Therefore we treated splenocytes from adult WKY rats and SHRs with the TLR3 agonist poly-IC and measured expression of *Il17a* and *Il17f* RNA. *Il17a* RNA expression was significantly but modestly induced in splenocytes from both WKY rats (1.7-fold) and SHRs (2.2-fold; Fig 5, *A*). However, expression of *Il17f* RNA was much greater in SHR splenocytes (27-fold) compared with that in WKY rat splenocytes (2.9-fold; Fig 5, *B*). TLR3-mediated increase in *Il17a* and *Il17f* expression in SHR splenocytes was dependent on RORγt. Pretreatment of SHR splenocytes with digoxin, an inhibitor of RORγt,[33] abolished the poly-IC–induced increase in *Il17a* and significantly decreased the increase in *Il17f* expression (Fig 5, *C*).

We also measured IL-17A and IL-17F cytokine levels in the poly-IC–treated culture supernatants of WKY rat and SHR splenocytes (Fig 5, *D*). The results from RNA expression analyses

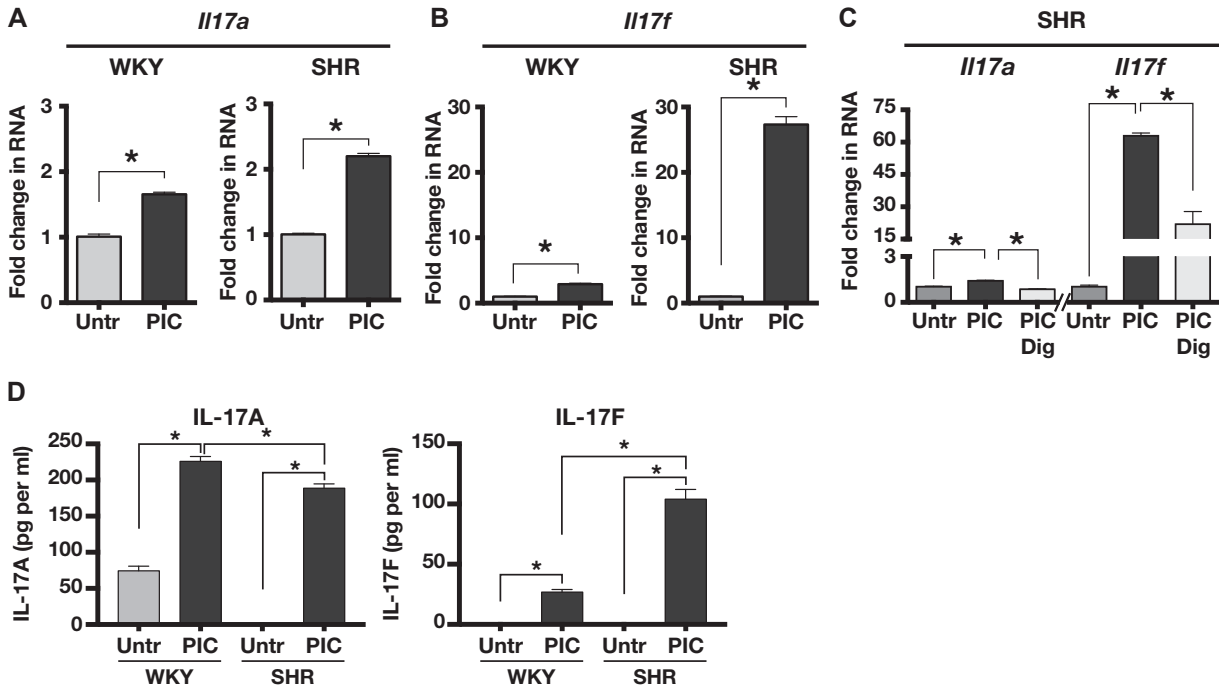

FIG 5. A, Expression of *Il17a* RNA in untreated *(Untr)* and Poly-IC *(PIC)*–treated splenocytes from WKY rats and SHRs. **B,** *Il17f* RNA expression in untreated *(Untr)* versus PIC-treated splenocytes from WKY rats and SHRs. **C,** Effect of the RORγt inhibitor digoxin (2.5 μmol/L) on PIC-induced expression of *Il17a* and *Il17f.* **D,** ELISA-based quantification of IL-17A and IL-17F in culture supernatants of untreated *(Untr)* and PIC-treated splenocytes from WKY rats and SHRs. **P* < .05. N = 3 each.

were paralleled by a similarly increased IL-17A in both WKY rat and SHR culture supernatants (226 pg/mL in WKY rats vs 189 pg/mL in SHRs, $P = .01$). Moreover, there was a 4-fold greater abundance of IL-17F cytokines in SHR culture supernatants than in WKY rats (27 pg/mL in WKY rats vs 104 pg/mL in SHRs, $P = .0008$). These results show that SHRs have an abnormally large population of $CD161^+$ cells that strongly induce proinflammatory IL-17 cytokines in an RORγt-dependent manner on activation of the innate immune system.

Nicotine increases the $CD4^+CD161^+$ immune cell population and its IL-17F response to TLR3 agonist (Poly-IC) in SHRs

Nicotine infusion for 24 hours caused a small but significant decrease in the $CD4^+CD161^+$ lymphocyte population of WKY rat splenocytes (5.6% in saline vs 3.1% in nicotine) but significantly increased the population of $CD4^+CD161^+$ lymphocytes in SHRs (10.3% with saline vs 18.6% with nicotine; Fig 6, *A*). In contrast, the $CD8^+CD161^+$ cell population did not change after nicotine infusion in either WKY rats or SHRs (Fig 6, *B*). Nicotine also has a proinflammatory effect in SHRs.[1] The poly-IC–induced expression of *Il17a* RNA by splenocytes was reduced with nicotine infusion in WKY rats but unchanged in SHRs (Fig 6, *C* and *D*). In contrast, the increased expression of *Il17f* RNA with poly-IC was unchanged by nicotine in WKY rats yet significantly enhanced by nicotine in SHRs. Thus, in contrast to WKY rats, nicotine infusion in SHRs resulted in an increased $CD4^+CD161^+$ cell population. Nicotine also increased the expression of *Il17f* RNA by poly-IC, which is dependent on RORγt in this population of SHRs.

Differential effects of IL-17A and IL-17F on vascular relaxation

We tested endothelium-dependent vascular relaxation in WKY rat aortic rings after treatment with IL-17A or IL-17F. IL-17F treatment impaired acetylcholine-induced, endothelium-dependent vasorelaxation in a dose-dependent manner (Fig 7, *A* and *C*). However, SNP-induced vasorelaxation was not affected in IL-17F–treated aortic rings (Fig 7, *B* and *D*). In contrast, IL-17A treatment did not affect endothelium-dependent vasorelaxation, even at a higher concentration of 1000 ng/mL (Fig 7, *E* and *G*). However, treatment with a higher concentration of IL-17A (1000 ng/mL) shifted the SNP dose-response curve for vasorelaxation to the left (Fig 7, *F* and *H*). Thus IL-17F, but not IL-17A, impairs endothelium-dependent vascular reactivity in the aorta.

Digoxin decreases the spontaneous increase in blood pressure in SHRs

We treated 5-week-old prehypertensive SHRs with daily injections of digoxin (10 μg/g body weight). In control SHRs injected daily with vehicle (dimethyl sulfoxide), SBP increased from 7 weeks of age and continued until the end of the experiment at 14 weeks of age. However, in the prehypertensive SHRs that were injected daily with digoxin, SBP was significantly lower (Fig 8, *A*). We also observed a significant decrease in $CD3^+$ lymphocytes in the kidneys of digoxin-injected SHRs when compared with the control rats (Fig 8, *B* and *C*). However, there was no overt histologic change observed (see Fig E3, *A*, in this article's Online Repository at www.jacionline.org) and no difference in the $ED1^+$

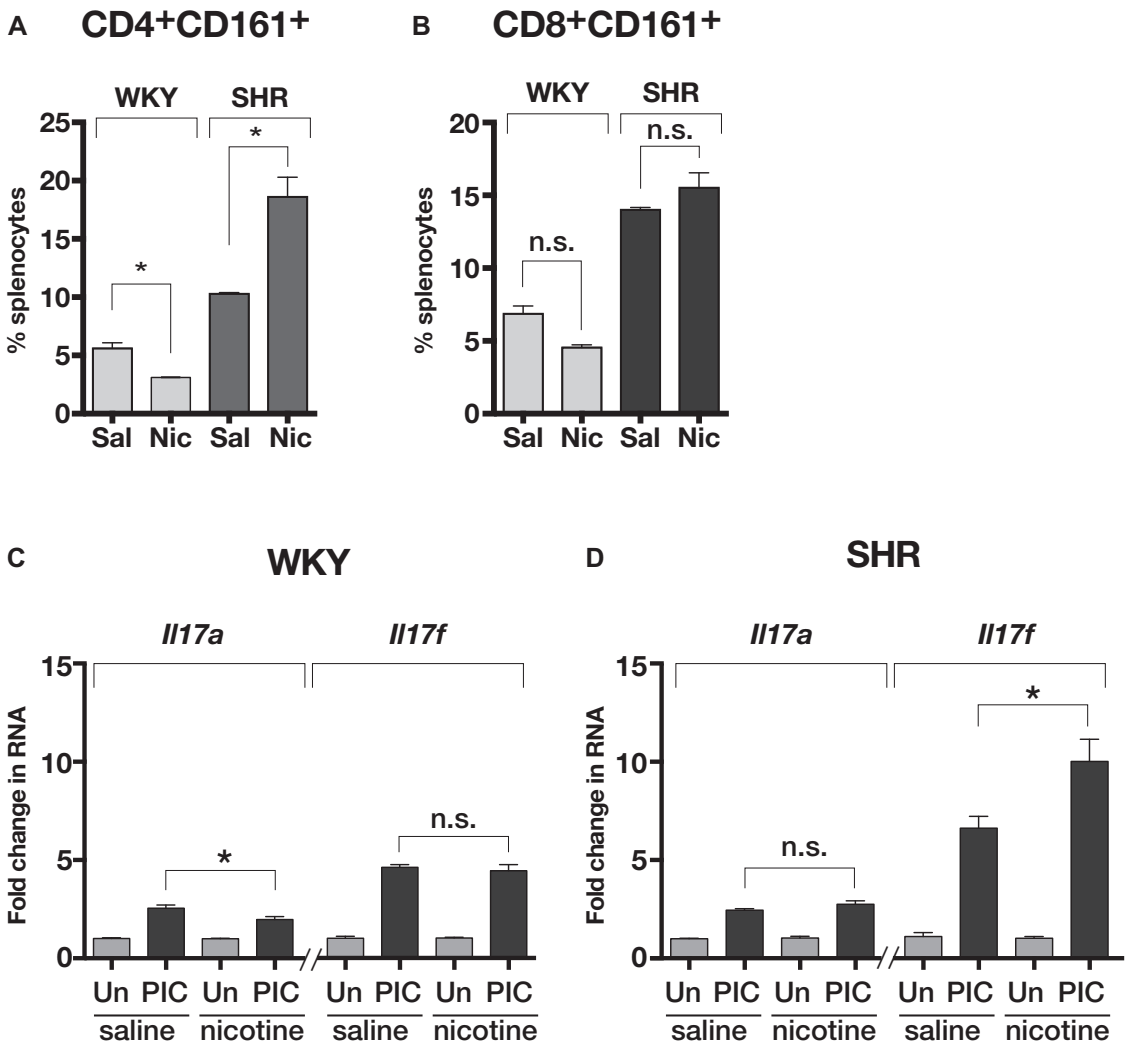

FIG 6. **A,** Change in the $CD4^+CD161^+$ cell population in spleens of nicotine-infused WKY rats and SHRs. **B,** No change in splenocyte population of $CD8^+CD161^+$ cells in either WKY rats or SHRs. **C,** Effect of Poly-IC *(PIC)* treatment on expression of *Il17a* and *Il17f* RNA in saline- or nicotine-infused WKY rat splenocytes. **D,** Effect of PIC treatment on expression of *Il17a* and *Il17f* RNA in saline- or nicotine-infused WKY splenocytes. $*P < .05$. *n.s.*, Not significant. N = 3 each, ANOVA with the Bonferroni *post hoc* test.

macrophage population in the kidneys (see Fig E3, *B* and *C*). Thus RORγt inhibition by digoxin reduced T-lymphocyte infiltration and decreased the increase in SBP.

DISCUSSION

T cells play a significant role in hypertension that has been recognized by several research groups.[7,8,34-36] In SHRs the hypertension is viewed as a genetically determined and environmentally induced pathology. However, despite numerous studies of SHRs, the immunologic and molecular basis of SHR hypertension remains elusive. In this study we have identified a large population of $CD161^+$ innate and adaptive immune cells in SHRs that have enhanced potential for production of the proinflammatory cytokines IL-17A and IL-17F. We show that IL-17F specifically impairs vascular function, and inhibition of RORγt, the master regulator transcription factor for IL-17–producing immune cells, decreases the developing blood pressure in SHRs.

Abundance of $CD161^+$ immune cells

Our results show that normotensive WKY rats have a small population of $CD161^+$ immune cells, whereas SHRs have a much greater abundance of potentially proinflammatory $CD161^+$ immune cells at birth and before the onset of hypertension. These cells dramatically increase with aging and are associated with the progressive increase in arterial pressure. The surface molecule CD161 was originally identified as the human homolog of the NKRP1 glycoproteins expressed on rodent natural killer cells.[3] Later, it was found to be expressed on several different cell types, including $CD4^+$ and $CD8^+$ T cells, as well as on $CD4^-CD8^-$ double-negative T cells.[6] Furthermore, this increase in $CD161^+$ markers is related to their greater potential for production of the proinflammatory cytokines IL-17A and IL-17F. To our knowledge, such expansion of the $CD161^+$ cell-surface marker *in vivo* is unprecedented. Interestingly, the increase in $CD161^+$ cell counts is not from hyperplasia because the spleen size and relative abundance

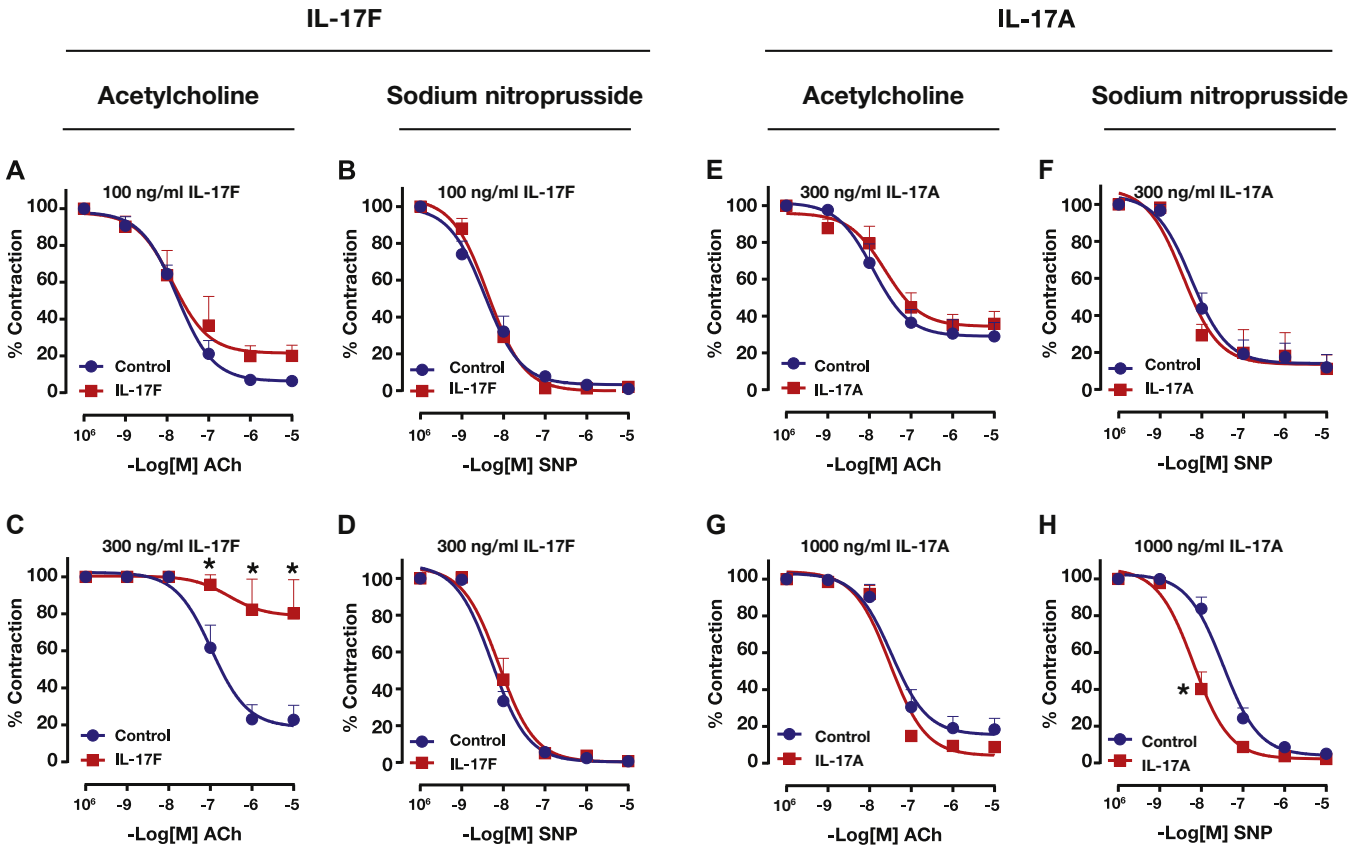

FIG 7. A-D, Effect of IL-17F on WKY rat endothelium-dependent (Fig 7, *A* and *C*) or endothelium-independent (Fig 7, *B* and *D*) vasodilation of aortic rings preconstricted with phenylephrine. **E-G,** Effect of IL-17A on endothelium-dependent (Fig 7, *E* and *G*) and endothelium-independent (Fig 7, *F* and *H*) vasodilation. *Ach*, Acetylcholine. **P* < .05. N = 4 aortic rings per group for IL-17F and N = 3 for IL-17A.

of $CD4^+$ or $CD8^+$ lymphocytes over time in SHRs were similar to that of WKY rats. Instead, a larger population of immune cells in SHRs seemed to have arisen from a dysregulated expression of the $CD161^+$ cell-surface marker.

RORγt overexpression in $CD4^+CD161^+$ T cells

CD161 is considered one of the defining markers of T_H17 cells. This marker is significantly upregulated in $CD4^+$ T_H17 cells that express RORγt and secrete IL-17A and IL-17F.[4,5] In fact, all IL-17–producing cells also express CD161, and a compelling correlation exists between IL-17 production and CD161 expression under the control of the transcription factor RORγt, which might be activated under T_H17 differentiation conditions.[6] Our results show a possible genetic defect in regulation of RORγt in SHRs. Unlike the WKY rat splenocytes that have low basal expression of RORγt but induce its expression under T_H17 polarization conditions, the SHR splenocytes express high basal levels of the RORγt transcription factor. Furthermore, fluorescence-activated cell sorting followed by reverse transcription–quantitative PCR also showed that RORγt expression was highest in the $CD4^+$ cell population, a precursor for the T_H17 lineage.[4] These results are consistent with other studies showing greater constitutive expression of RORγt RNA in $CD4^+CD161^+$ cells[4] and suggested that the higher RORγt transcription factor expression might contribute to greater expression of IL-17 in SHR splenocytes.

RORγt, IL-17, and hypertension

Inhibition of RORγt by digoxin reduced the developmental increase of SBP in SHRs. In this study we demonstrated that SHRs have a genetic predisposition toward generating abundant RORγt-expressing $CD161^+$ cells, which produced IL-17A and IL-17F in an RORγt-dependent manner. IL-17A has been shown to be required for angiotensin II–induced hypertension.[34] Moreover, increased IL-17A is a key inflammatory cytokine in the patients with systemic lupus erythematosus[37] who also have a high prevalence of hypertension.[38] However, a role of IL-17F, as seen in our study, in vascular relaxation has not been demonstrated.

Thus, for the first time, our results implicate RORγt-dependent IL-17F in SHR hypertension and also support the concept of hypertension as an autoimmune disease. Autoimmunity is thought to be at the root of both primary and secondary hypertension.[8,39,40] Both IL-17 and RORγt-expressing $CD161^+$ T_H17 cells have been implicated in autoimmune diseases, including multiple sclerosis, psoriasis, and Crohn disease, as well as in the mouse experimental model of multiple sclerosis–experimental autoimmune encephalomyelitis.[41] We

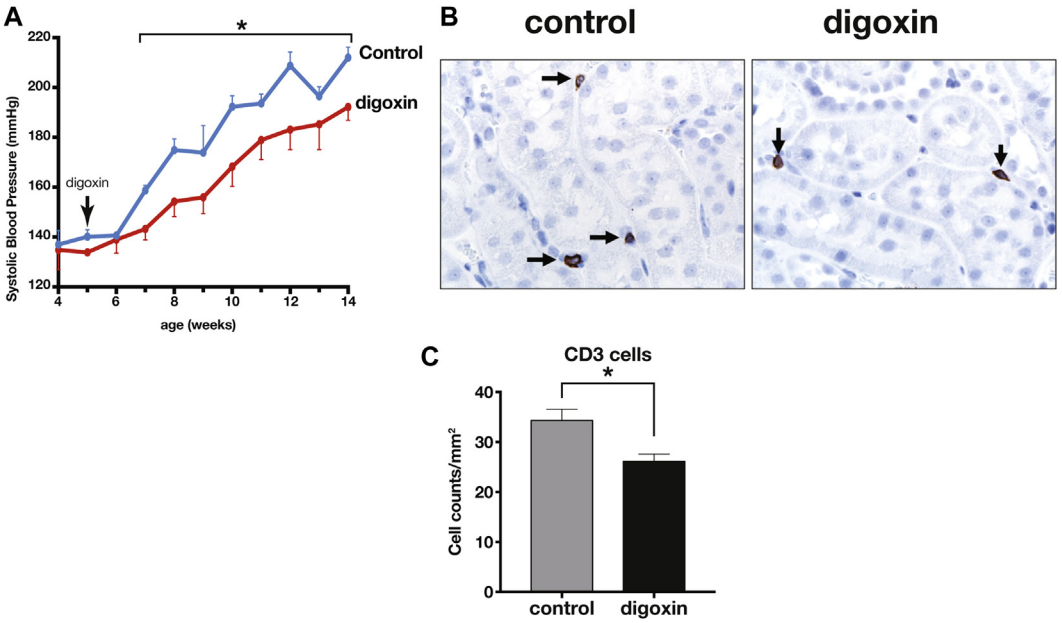

FIG 8. A, Effect of digoxin treatment (10 μg/g body weight) on spontaneously developed SBP in SHRs. Treatment was from age 5 weeks to 13 weeks. N = 4 vehicle-injected (dimethyl sulfoxide) and 6 digoxin-injected rats. **B,** CD3 surface marker immunostaining of T lymphocytes in kidney sections of control and digoxin-injected SHRs. **C,** Summary data of CD3 infiltration in kidneys of control and digoxin-injected SHRs (n = 4 control and 6 digoxin-injected SHRs). *$P < .05$.

also observed a decrease in $CD3^+$ T-lymphocyte counts in the kidneys of digoxin-treated SHRs. Kidneys are a critical organ in hypertension that endure inflammation during hypertension. RORγt has been shown to cause renal inflammation through increased leukocyte counts and cytokine production in a model of glomerulonephritis.[42] Thus inhibition of RORγt is likely to have contributed to decreasing SBP by digoxin. Interestingly, despite greater potential for IL-17 production, there are no reports of skin lesions or experimental autoimmune encephalitis in the SHR.

It is also pertinent to note that hypertension is related to a deficiency of regulatory T (Treg) cells, which are anti-inflammatory.[43-45] Lineage determination of T_H17 and Treg cells is held in a precarious balance. T_H17 cells can convert to Treg cells and *vice versa* or T_H1-type cells. This can occur through a process of plasticity[46] resulting in part from T-bet transcription factor–mediated repression of RORγt,[47] the master regulator transcription factor of T_H17 cells.

IL-17A versus IL-17F

We observed a differential effect of IL-17A and IL-17F on rat aortic vasorelaxation. Despite a similar increase in *Il17a* expression in both WKY rats and SHRs, *Il17f* expression was significantly greater in SHRs. IL-17A and IL-17F belong to the 6-member proinflammatory IL-17 cytokine family and share significant sequence homology. Both IL-17A and IL-17F are secreted as homodimers but can also form IL-17A/IL-17F heterodimers. Importantly, these cytokines have distinct and overlapping roles in the setting of highly pathogenic inflammatory diseases.[16,48,49] Their similar or distinct effects arise from their binding to shared or distinct receptors on target cells.[50,51] However, little is known about either the expression of IL-17F or its effects in rats. We observed a dramatically specific role of IL-17F on vascular function in rats. In our study IL-17F had a profound and specific effect on impairment of endothelium-dependent vascular function, whereas IL-17A had essentially no effect. This IL-17F effect was likely through interference in nitric oxide production by endothelium or by enhancing turnover of nitric oxide. IL-17F did not affect vascular smooth muscle cells because the nitric oxide donor SNP had a similar effect on both control and treated aortic rings. Interestingly, at higher concentrations, IL-17A had an opposite effect to IL-17F on vascular relaxation: it sensitized the aortic vasorelaxation by SNP. We currently do not know the mechanism of this phenomenon, but our results are consistent with the concept that increased inflammatory status is accompanied by reduced nitric oxide production and endothelial function.[52] However, considering that IL-17A and IL-17F share a receptor subunit, our finding is both novel and important because it suggests receptor specificity in the aorta for these cytokines.

The IL-17 receptor complex is heteromeric and consists of IL-17RA and other IL-17 receptors. Both IL-17A and IL-17F bind to the IL-17RA and IL-17RC heterodimeric complex to transduce downstream signaling. The binding affinity of IL-17A and IL-17F are different for IL-17 receptors. For example, mouse IL-17RC preferably binds to IL-17F.[53,54] Although IL-17RA is expressed ubiquitously, IL-17RC expression is low in hematopoietic tissues and high in nonimmune cells, such as the liver and kidney.[55,56] An additional level of complexity is that some members of the IL-17R family are highly spliced at sites in the extracellular domain, potentially giving rise to both agonistic and antagonistic (soluble) forms of the receptors. There are more than 90 splice isoforms of IL-17RC identified in human prostate cancer lines.[57] Moreover, the main responsive cells to IL-17 are epithelial cells, endothelial cells and fibroblasts,[53,56] which is consistent with our results of IL-17F affecting endothelial cells in the rat aorta.

Selective increase in *Il17f* expression in SHRs with TLR3 activation and T_H17 differentiation

IL-17–producing T_H17 cells exclusively originate from a $CD161^+CD4^+$ T-cell precursor.[4] We observed a similar induction of *Il17a* RNA expression in both WKY rats and SHRs but a remarkably greater *Il17f* RNA expression in SHRs. Inhibition of both *Il17a* and *Il17f* RNA expression by RORγt inhibitor digoxin[33] confirmed that expression of these genes was driven by RORγt and hence related to T_H17 differentiation, yet they are known to be regulated differentially.[58-60] Despite their gene location in the same region of the chromosome, their regulatory sequences vary, and their chromatin modifications are different. Thus our results suggest that SHRs can have altered genetic and epigenetic marks or signals that enhance *Il17f* expression.

Antipodal effect of nicotine on SHR $CD4^+CD161^+$ cells

In most hypertensive human subjects and animal models of hypertension, sympathetic nerve activity is exaggerated,[61-63] an effect that can result in increased inflammatory responses. In contrast, the parasympathetic autonomic nervous system, through activation of α7-nAChR, exerts an anti-inflammatory effect on T cells.[64-66] Activation of α7-nAChR expression on the surfaces of $CD4^+$ T cells[67] by nicotine reduces the T_H17 response.[68] Interestingly, $CD4^+$ lymphocytes also express significantly greater α3- and β4-nAChR levels than $CD8^+$ cells.[69] Nicotine promotes an increase in $CD3^+CD4^+$ cell counts through activation of α4-nAChR and regulation of a G protein subunit.[70] Nicotine infusions of 24 hours and 2 weeks were found to induce an increase in renal macrophage/monocyte migration and premature hypertension in SHRs.[30] Thus nicotine can have opposite effects on $CD4^+$ T_H17 cell–mediated inflammation in different contexts.[71] In this study SHR splenocytes responded by increasing their production of proinflammatory cytokines when induced by TLR agonists in the presence of nicotine. In addition, nicotine also increased the $CD161^+$ cell population *in vitro*. Our *in vivo* results are consistent with the observed proliferation of cultured $CD161^+$ cells in SHRs.[1] The underlying molecular mechanism of this opposite effect of nicotine in SHRs is not fully understood. It could result from differential expression of nicotinic cholinergic receptors or through impaired intracellular signaling and deserves further study.

In summary, we have shown that in SHRs onset of hypertension is preceded by an abnormally large population of $CD161^+$ cells that increase with age. Furthermore, a T_H17-like $CD4^+CD161^+$ cell population expresses high levels of proinflammatory IL-17F that can be enhanced further by nicotine. IL-17F impaired endothelium-mediated vasorelaxation in the rat aorta. Moreover, inhibition of RORγt, a transcription factor that regulates IL-17F expression, reduced SBP in SHRs. These findings implicate a role of the dysregulated immune system with increased potential for IL-17F production by RORγt transcription factor in the genetic hypertension seen in SHRs.

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Key messages

- **Inflammation and the immune system play a major role in the onset and maintenance of hypertension.**
- **Our finding demonstrates an inherent abnormality in the immune system of a genetic model of hypertension that potentially produces cytokines involved in several autoimmune diseases.**
- **These findings can have therapeutic potential to ameliorate hypertension and associated end-organ damage by eliminating the responsible cells or neutralizing the proinflammatory cytokines.**

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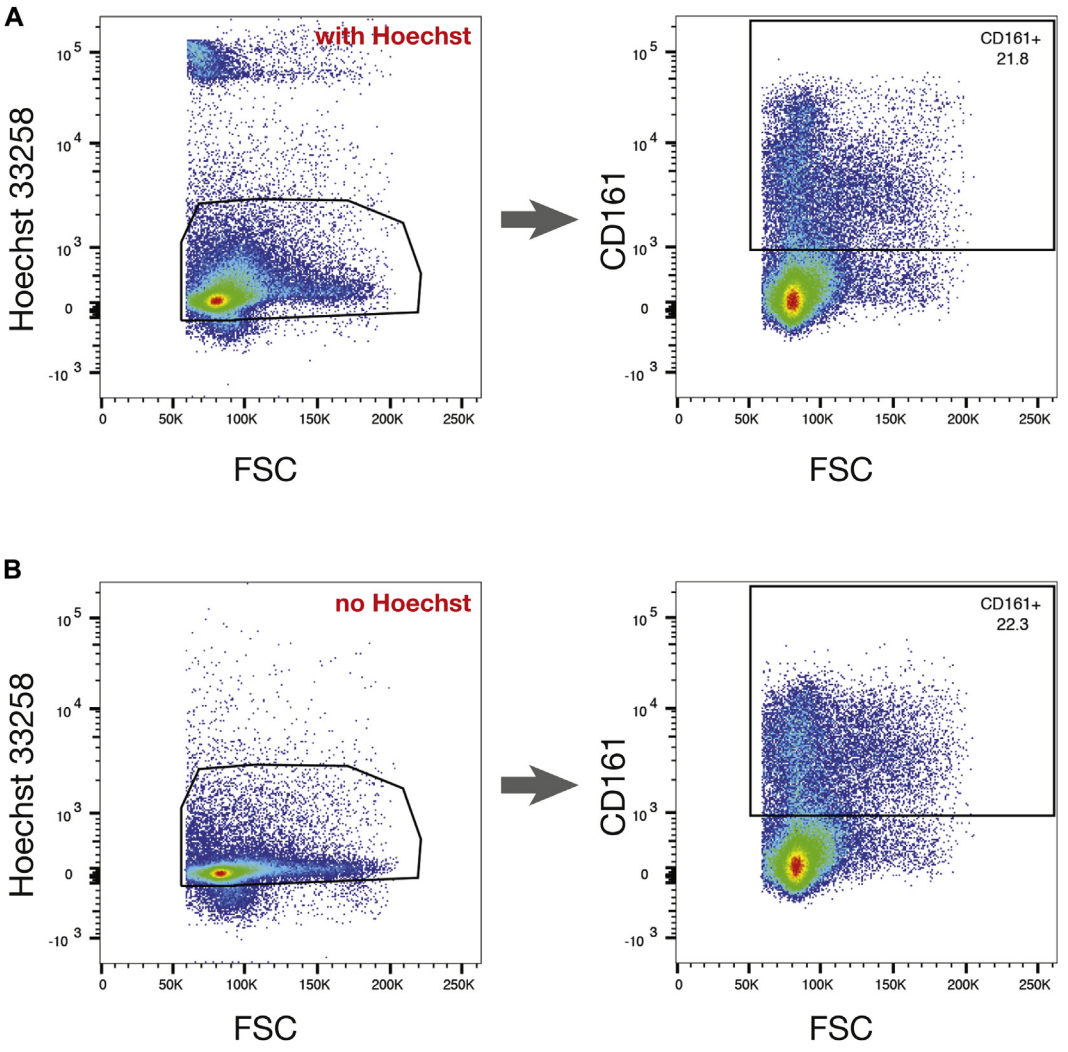

FIG E1. Live-dead cell discrimination of splenocytes from 21-week-old SHRs. Splenocyte aliquots labeled for the cell-surface marker CD161 were subjected to flow cytometry either after mixing the vital dye Hoechst 33258 before flow cytometry **(A)** or without the vital dye **(B)**. The gated live cells or the corresponding gate *(left panel)* were further analyzed for the $CD161^{+}$ population *(right panel)*. *FSC,* Forward scatter.

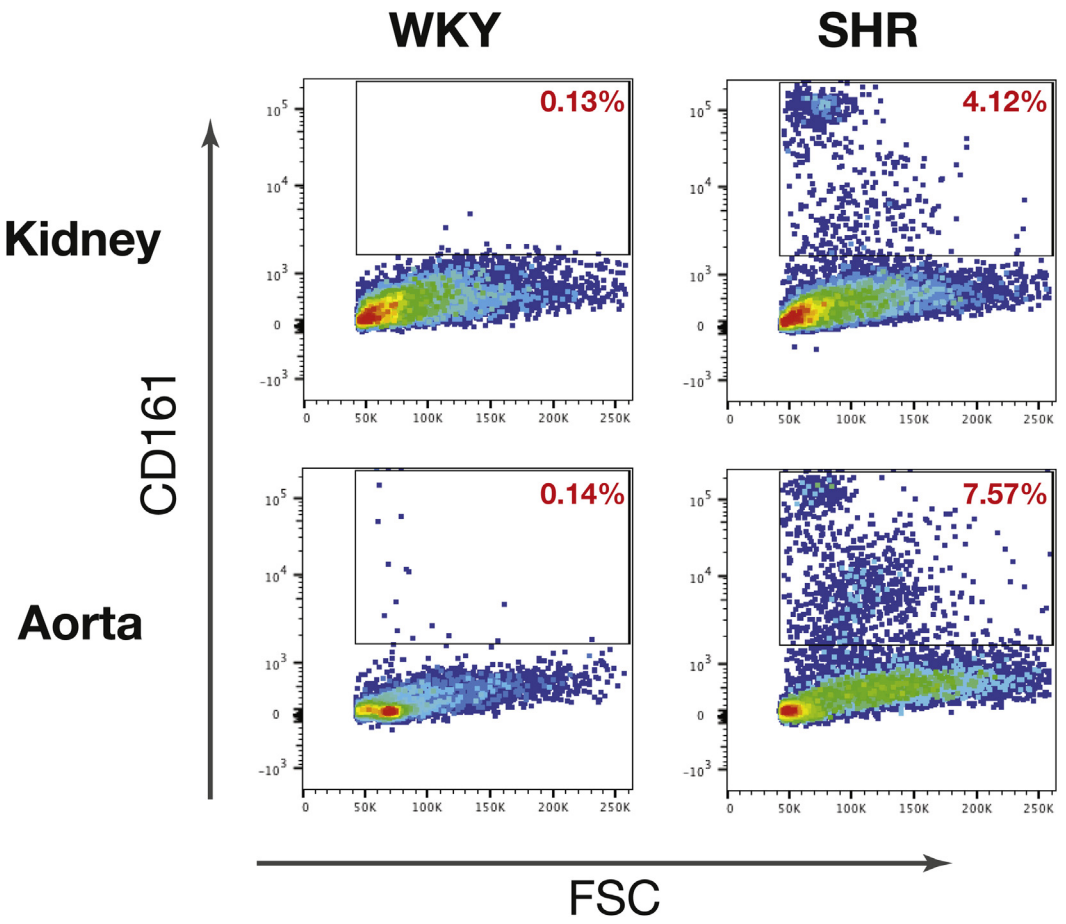

FIG E2. Flow cytometric analysis showing infiltration of CD161^{+} cells in the kidneys and aortas of WKY rats and SHRs. After excision, tissues were minced and suspended in a mixture of 0.5 mg/mL Collagenase Type I and 0.5 mg/mL Collagenase Type-2 (Worthington Biochemical, Lakewood, NJ) to dissociate cells. Collected cells were washed in PBS and labeled with antibody to CD161 (PE-CD161^{+} antibody, BD Biosciences, San Jose, Calif). *FSC*, Forward scatter.

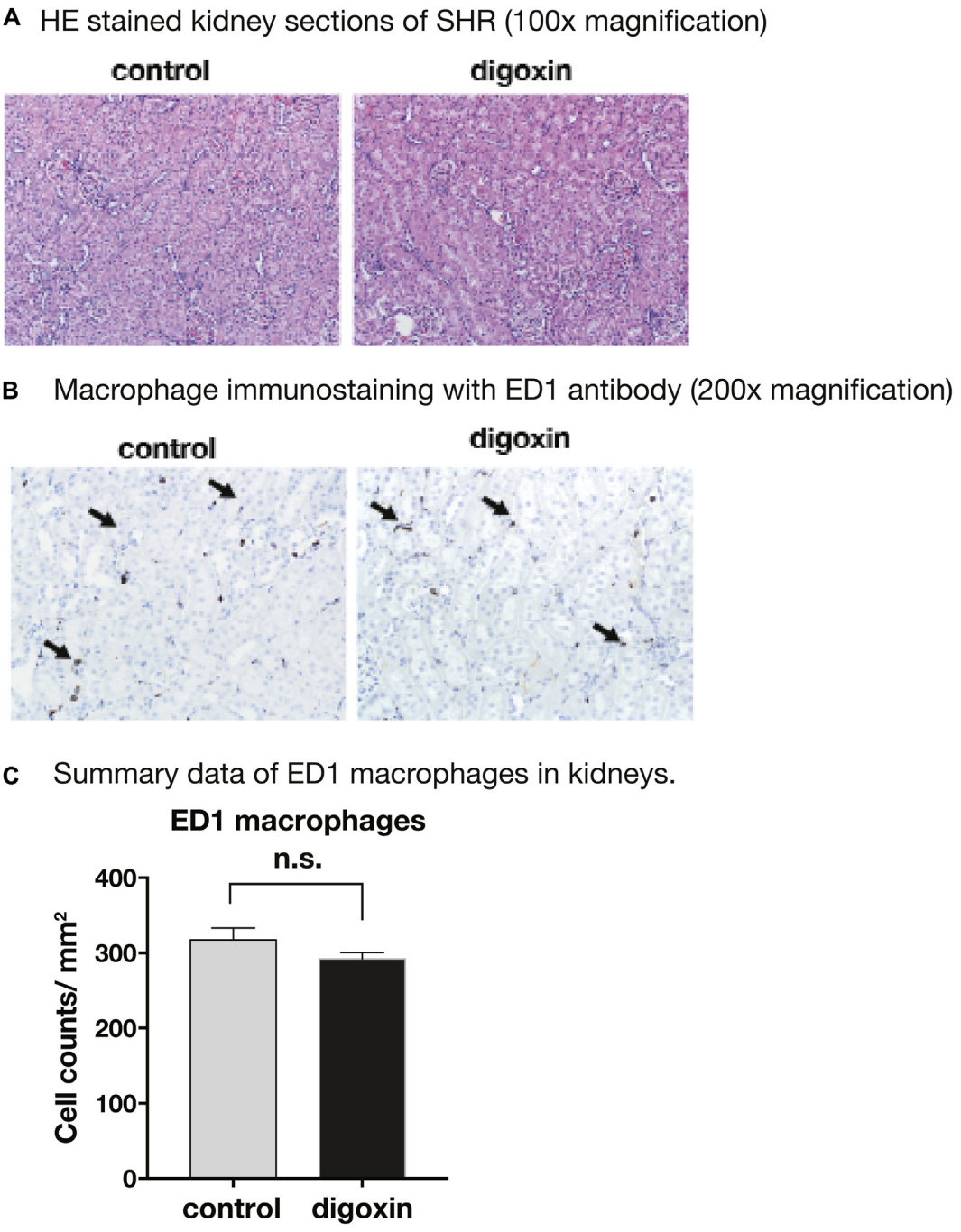

FIG E3. **A,** Hematoxylin and eosin staining of representative sections of kidneys from a control (dimethyl sulfoxide)–treated and digoxin-treated SHR. **B,** Immunostaining for macrophages (ED1 marker) in the kidneys of control- and digoxin-treated SHRs. **C,** Summary data of infiltrated macrophages (ED1 stained) in the kidneys of SHRs. *n.s.*, Not significant.