Induction of Hypertension and Peripheral Inflammation by Reduction of Extracellular Superoxide Dismutase in the Central Nervous System

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Abstract—The circumventricular organs (CVOs) lack a well-formed blood-brain barrier and produce superoxide in response to angiotensin II and other hypertensive stimuli. This increase in central superoxide has been implicated in the regulation of blood pressure. The extracellular superoxide dismutase (SOD3) is highly expressed in cells associated with CVOs and particularly with tanyocytes lining this region. To understand the role of SOD3 in the CVOs in blood pressure regulation, we performed intracerebroventricular injection of an adenovirus encoding Cre-recombinase (5×10⁸ particles per milliliter) in mice with loxP sites flanking the SOD3 coding region (SOD3loxP/loxP mice). An adenovirus encoding red-fluorescent protein was injected as a control. Deletion of CVO SOD3 increased baseline blood pressure modestly and markedly augmented the hypertensive response to low-dose angiotensin II (140 ng/kg per day), whereas intracerebroventricular injection of adenovirus encoding red-fluorescent protein had minimal effects on these parameters. Adenovirus encoding Cre-recombinase–treated mice exhibited increased sympathetic modulation of heart rate and blood pressure variability, increased vascular superoxide production, and T-cell activation as characterized by increased circulating CD69⁺/CD3⁺ cells. Deletion of CVO SOD3 also markedly increased vascular T-cell and leukocyte infiltration caused by angiotensin II. We conclude that SOD3 in the CVO plays a critical role in the regulation of blood pressure, and its loss promotes T-cell activation and vascular inflammation, in part by modulating sympathetic outflow. These findings provide insight into how central signals produce vascular inflammation in response to hypertensive stimuli, such as angiotensin II. (Hypertension. 2010;55:277-283.)

Key Words: superoxide dismutase • circumventricular organ • hypertension • T cell • Cre-Lox

The superoxide dismutases (SODs) are antioxidant enzymes that catalyze dismutation of the superoxide anion (O₂⁻⁻) to hydrogen peroxide and oxygen. There are 3 isozymes of SODs in mammals, including the cytoplasmic Cu/Zn-SOD, the mitochondrial SOD, and the extracellular SOD (SOD3).¹ Considerable evidence supports the view that O₂⁻⁻ plays a critical role in the pathogenesis of hypertension. For example, reducing tissue O₂⁻⁻ by administration of membrane-targeted forms of SOD, SOD mimetics (eg, Tempol), or deletion of the gene encoding for the NADPH oxidase subunit p47phox reduces blood pressure in several experimental models of hypertension.²⁻⁻⁴ In addition, embryonic deletion of SOD3, which would be expected to increase extracellular O₂⁻⁻, augments hypertension in response to angiotensin (Ang) II or deoxycorticosterone acetate-salt challenge.⁵⁻⁻⁶ Although these studies support a role for SOD3 and oxidative stress in the genesis of hypertension, they do not address potential sites or mechanisms by which deletion of SOD3 augments hypertension. Potential sites where this enzyme could modulate blood pressure include the vasculature, the kidney, and the brain. In addition to oxidative stress, recent studies from our laboratory and others have also suggested an important role for peripheral T-lymphocyte activation and vascular inflammation in the development of Ang II–induced hypertension. The relationship between oxidative events in sites such as the central nervous system, T-cell activation, and vascular inflammation remain poorly understood.

The purpose of the present studies was to explore the hypothesis that defects in brain SOD3 might participate in the development of hypertension and immune cell activation evoked by Ang II. This was accomplished by using SOD3loxP/loxP mice with loxP sites flanking the SOD3 coding region enabling targeted deletion of this gene on exposure to Cre-recombinase. By intracerebroventricular (ICV) injection of an adenovirus encoding Cre-recombinase (AdCre), we were able to selectively delete SOD3 in central nervous system (CNS) circumventricular structures and study the role of this protein in the regulation of blood pressure and peripheral immune activation.
Materials and Methods

Mice previously created in our laboratory, with loxP sites flanking the SOD3 coding region, were used in these studies. To delete CNS SOD3, we performed ICV injections of an AdCre and used an adenovirus encoding red fluorescent protein (RFP; AdRFP) as a control. Measurements of blood pressure, T-cell activation, and vascular O$_2^-$ production were performed as described previously. For detailed Material and Methods, please see the online Data Supplement at http://hyper.ahajournals.org.

Results

CNS Expression of AdRFP and AdCre and the Effect on SOD3

To determine the distribution of adenovirus after ICV injections, we analyzed RFP expression by fluorescence at various times after AdRFP injection. We found that RFP was expressed at the highest levels 7 days after AdRFP injection (data not shown). RFP-expressing cells were typically located within \( \approx 50 \) \( \mu \)m of the ventricular wall (Figure S1A, available in the online Data Supplement at http://hyper.ahajournals.org). RFP-labeled cells were most abundant in circumventricular organs (CVOs) and particularly in the subfornical organ (SFO; Figure S1A). Occasional RFP-labeled cells were observed in areas adjacent to the SFO (Figure S1B and S1C) but not in circumventricular tissues far distant from the SFO (Figure S1D and S1E). ICV injection of AdCre also led to expression of Cre-recombinase in the SFO (Figure S1F).

Seven days after ICV injection of AdCre, SOD3 immunostaining was no longer detectable in the SFO or in other cells lining the third ventricle (Figure 1A). In contrast to the results obtained with ICV-AdCre, injection of AdRFP had no effect on SOD3 levels in this region (Figure 1A). Because of these preliminary findings, we began infusion of either Ang II or buffer 2 weeks after ICV injection and used AdRFP as a control.

To confirm that ICV injections of AdCre specifically deleted SOD3 in the CNS, we performed immunostaining and Western analysis for SOD3 in kidneys and aortas of AdRFP and AdCre mice. The results showed no differences in SOD3 expression in these tissues (Figure 1B through Figure 1E). In other experiments we injected an identical volume of AdCre intraperitoneally. Immunostaining and Western analysis indicated that peripherally administered AdCre had no effect on either renal or aortic levels of SOD3 (Figure S2). Moreover, Western blots revealed no expression of Cre-recombinase in...
aortas 7 days after ICV injection of AdCre (data not shown). Taken together, these results indicated that ICV injections of AdCre specifically deleted SOD3 in the CNS, with no detectable action at peripheral sites.

**Effect of CNS SOD3 Deletion on Blood Pressure and Heart Rate**

ICV injection of AdRFP in SOD3loxp/loxp mice did not affect baseline blood pressure measured at 7 to 14 days as determined by either tail-cuff measurements or radiotelemetry monitoring in SOD3loxp/loxp mice (Figure 2A and 2B). Moreover, 2-week infusion of a low dose of Ang II raised blood pressure minimally in these animals. In contrast to the effects of AdRFP, CNS SOD3 deletion with AdCre caused a 21 to 110 mm Hg increase in blood pressure at baseline and markedly augmented the hypertensive response to Ang II as measured using the tail-cuff method (Figure 2A). Radiotelemetry also confirmed these effects of CNS SOD3 deletion (Figure 2B). Two-way ANOVA indicated that the effect of Ang II was significantly greater in mice that received AdCre versus AdRFP (P=0.03). Heart rate was similar in AdCre-injected mice compared with AdRFP-injected mice (Figure S3).

**Effects of CNS SOD3 Deletion on Sympathetic Modulation of Heart Rate and Systolic Pressure**

Power spectral analysis identifies oscillations in heart rate and blood pressure that are modulated by inputs from the renin-Ang system, sympathetic and parasympathetic neurons, and locally released vasoactive factors, such as NO. As an example, sympathetic outflow modulates low-frequency oscillations (0.015 to 0.600 Hz), whereas parasympathetic tone affects both low and high (0.2 to 5.0 Hz) oscillations of heart rate. Thus the ratio of low-frequency:high-frequency heart rate variability reflects sympathetic cardiovascular modulation. This ratio was increased 2-fold after deletion of SOD3 in the CNS (Figure 2C), in keeping with an increase in sympathetic outflow. Likewise, absolute values of low-frequency blood pressure oscillations provide indirect assessment of sympathetic outflow. These low-frequency blood pressure oscillations were 3-fold higher in mice after AdCre as compared with AdRFP injection (Figure 2D). Taken together, these data indicate that SOD3 in the CVO plays an important role in modulating sympathetic outflow.

**Effect of CNS SOD3 Deletion on Reactive Oxygen Species**

Protein nitrotyrosines are markers of endogenous production of reactive oxygen and nitrogen species. Protein nitrotyrosines were not detected after AdRFP injection but were prevalent in cells surrounding the subfornical organ after SOD3 deletion (Figure 3A). These were most obvious in cells immediately adjacent to the third ventricle. Of note, infusion of Ang II did not further increase protein nitrotyrosines in the CVO (Figure 3B).

Figure 2. Effect of SOD3 deletion in circumventricular organs on hemodynamics. Mice received ICV injections of either AdCre or AdRFP, and blood pressure was measured by tail cuff (A) or radiotelemetry (B). Heart rate (C) and systolic blood pressure (D) variability were analyzed using Hemolab software package (LF/HF indicates low frequency/high frequency).
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markedly increased vascular O$_2^-$ production as detected by electron spin resonance and the spin probe CAT1-H. As evident in Figure 3C, CNS SOD3 deletion levels using electron spin resonance and the spin probe CAT1-H (n = 4 to 5). C, Quantification of vascular superoxide production as detected by electron spin resonance. Mice underwent ICV injections of either AdCre or AdRFP and subsequent infusion of angiotensin II as in Figure 2. A, Immunostaining for nitrotyrosine in the SFO and adjacent structures in the third ventricle (3V). B, Quantification of pixel intensities from experiments depicted in A (n = 4-5). C, Quantification of vascular superoxide production as detected by electron spin resonance and the spin probe CAT1-H (n = 6).

To examine this possibility, we examined aortic O$_2^-$ levels using electron spin resonance and the spin probe CAT-1H. As evident in Figure 3C, CNS SOD3 deletion markedly increased vascular O$_2^-$ production as compared with AdRFP-injected mice. Of interest, infusion of low-dose Ang II did not increase vascular O$_2^-$ further in either AdCre- or AdRFP-injected mice.

**Effect of CNS SOD3 Deletion on Peripheral Inflammatory Markers**

Previous studies have shown that T lymphocytes play a key role in the genesis of Ang II–induced hypertension. In these previous experiments, Ang II infusion increased circulating CD44$^{high}$ and CD69$^+$ T cells and promoted vascular accumulation of inflammatory cells, particularly in the perivascular fat. To determine whether CNS SOD3 mediates these effects, we measured markers of peripheral inflammation after deletion of CNS SOD3 with and without peripheral infusion of a low dose of Ang II. Central injection of either AdRFP or AdCre had no effect on the composition of peripheral blood mononuclear cells (Figure 4A). Likewise, ICV injection of AdRFP had no effect on the percentage of CD69$^+$/CD4$^+$ cells either at baseline or in response to Ang II (Figure 4B). In contrast, injection of AdCre increased the percentage of circulating CD69$^+$/CD4$^+$ cells (Figure 4B), even in the absence of Ang II infusion, indicating that deletion of CNS SOD3 by itself is sufficient to stimulate CD4$^+$ cell activation. Angiotensin II caused no further increase in CD69$^+$/CD4$^+$ cells after central SOD3 deletion. In contrast to CD69$^+$/CD4$^+$ cells, the percentage of CD3$^+$CD4$^-$CD8$^-/CD3$^+$ cells was only increased when both Ang II was administered and CNS SOD3 was deleted (Figure 4C).

In aortic tissue, inflammatory CD45$^+$, CD3$^+$, and CD69$^+$ cells were markedly increased by peripheral Ang infusion only when central SOD3 had been deleted by previous ICV injection of AdCre (Figure 4D). Neither Ang infusion in AdRFP-treated mice nor deletion of CNS SOD3 alone increased the prevalence of these inflammatory cells in aortic tissue (Figure 4D). Similar to peripheral blood, CD69$^+$ cells in the aorta were increased by central deletion of SOD3 regardless of whether Ang II was infused (Figure 4E). Confirmatory immunostaining of aortic tissue for CD3$^+$ cells revealed dense, infiltrating accumulations of CD3$^+$ cells in perivascular regions adjacent to the aorta (Figure 4F).

Functional enabling of inflammatory lymphocytes depends on the expression of cell surface adhesion molecules and proinflammatory cytokines. In mice infused for 14 days with Ang II, deletion of CNS SOD3 by previous ICV injection of AdCre significantly increased aortic expression of mRNA for the adhesion molecule intercellular adhesion molecule 1 but not vascular cell adhesion molecule 1 (Figure S4A and S4B). In addition, AdCre treatment combined with Ang II infusion increased mRNA expression for the cytokine interleukin (IL) 17A (Figure S4C) without significantly affecting regulated on activation, normal T expressed and secreted mRNA levels (Figure S4D). Thus, increasing oxidant stress in CNS circumventricular cells by deletion of SOD3 increased peripheral T-cell activation, whereas homing of these cells to vascular tissues depended on both central SOD3 deletion and Ang II infusion.

**Discussion**

In this study, we found that endogenous SOD3 located in circumventricular cells critically modulates blood pressure
and heart rate at baseline and in response to Ang II. Deletion of SOD3 in this region modestly elevated blood pressure at baseline and markedly enhanced the hypertensive response to a low-dose Ang II, which usually has a minimal effect on blood pressure in mice. CVO SOD3 deletion also enhanced \( \text{O}_2^\bullet^- \) production in peripheral vessels and promoted T-cell activation and vascular infiltration. SOD3 in the central nervous system has been implicated in learning and memory\textsuperscript{10,11}; however, our current findings illustrate a previously unknown role for this protein in the central regulation of hemodynamics.

Our current findings support the concept that reactive oxygen species (ROS) in the CNS play an important role in the regulation of blood pressure and the genesis of hypertension. The current results are in accord with those of Zimmerman et al\textsuperscript{12,13} who reported an attenuated pressor response to centrally administered Ang II, as well as prevention of hypertension caused by peripheral Ang II after ICV injections of an adenovirus encoding for intracellular SOD. Taken together, these studies suggest that increasing CNS ROS by deleting SOD augments hypertensive responses to Ang II, whereas decreasing CNS ROS by enhancing SOD action reduces these responses.

Interestingly, Zimmerman et al\textsuperscript{13} have reported that, in contrast to Cu/Zn-SOD, augmenting SOD3 levels by ICV adenoviral delivery had no effect on Ang-induced hypertension. A logical conclusion from their study is that the extracellular SOD and, by inference, extracellular \( \text{O}_2^\bullet^- \) have no role in modulating central signaling. It should be recognized, however, that cells lining the third ventricle express large amounts of SOD3 under normal conditions\textsuperscript{14} and that augmenting these already high levels might have little additional effect on \( \text{O}_2^\bullet^- \) scavenging. Our present study demonstrates that endogenous SOD3 clearly has a role in hemodynamic modulation, both at baseline and in response to Ang II.

The precise location within the CNS where the generation of ROS influences central cardiovascular regulation is not known. Several lines of evidence suggest that CVOs, and the SFO in particular, might be likely sites of action. The CVOs, which are located adjacent to the cerebral ventricles and lack a blood-brain barrier, are the CNS structures most often implicated in the development of hypertension and mediation of the central actions of Ang II. These organs include the organum vasculosum of the lamina terminalis, the area postrema, and the SFO.\textsuperscript{15} Lesions that disrupt the anteroven-tral third ventricle, which includes the organum vasculosum of the lamina terminalis, prevent the development of many forms of experimental hypertension in rodents.\textsuperscript{16,17} In addition, circumventricular cells mediate virtually all of the

![Figure 4](http://hyper.ahajournals.org/)

**Figure 4.** Role of circumventricular SOD3 in T-cell activation and vascular infiltration of inflammatory cells. Mice were treated as in Figure 2, and 14 days after either buffer or Ang II infusion (140 ng/kg per minute), fluorescence-activated cell sorter was used for analysis of peripheral blood and vascular cells. A, Percentage of total peripheral blood mononuclear cells expressing the early activation marker CD69. B, Percentage of total T cells (CD3+) that are double negative for both CD4 and CD8. D, Fluorescence-activated cell sorter analysis of single-cell suspensions of aortic homogenates. E, Total number of CD4+CD69+ cells per aorta. F, Immunostaining for CD3+ cells in aortas and perivascular fat mice treated with either ICV AdRFP or angiotensin II or AdCre and angiotensin II for 2 weeks (PBMC indicates peripheral blood mononuclear cells; n.s. indicates not significant).
central actions of Ang II, including drinking behavior, vasopressin secretion, and sympathetic outflow. In the present studies, ICV administration of adenovirus produced RFP-labeled cells only within 50 to 100 μm of the cerebral ventricles with prominent occurrence of RFP-positive cells and deletion of SOD3 in the SFO. Immunohistochemical studies have identified SOD3-positive cells throughout the CNS, with a particularly concentrated distribution associated with tanycytes abutting the ventral third cerebral ventricle. In addition, Zimmerman et al. reported that peripheral infusions of Ang II produced marked elevation in O$_2^-$ production specifically in the SFO. Collectively, these observations suggest that ROS production in circumventricular structures, perhaps specifically the SFO, could be important for cardiovascular regulation and the development of Ang-induced hypertension. Although we observed a large amount of SOD3 in the vicinity of the SFO, our study does not exclude a role for other circumventricular organs in the modulation of blood pressure or inflammatory cell activation.

Analysis of heart rate and systolic pressure variability provided additional insight into how central SOD3 deletion affected sympathetic cardiovascular regulation. The increased ratio of low-frequency:high-frequency heart rate variability and the absolute increase in systolic pressure variability are compatible with an increase in sympathetic outflow after central SOD3 deletion. These findings are in accord with the above-mentioned role of CVO in modulating sympathetic outflow. In many cells, including cardiac myocytes, vascular smooth muscle cells, and neuronal cells, ROS enhance calcium transients. Calcium sequestration is inhibited and calcium release is enhanced by O$_2^-$ and other ROS. In keeping with this, Ang II activates the NADPH oxidase in neurons and enhances inward calcium flux in these cells in an O$_2^-$-dependent fashion. At first glance, it would seem unlikely that scavenging extracellular O$_2^-$ would affect such intracellular signaling events. Scavenging extracellular O$_2^-$ would reduce the formation of peroxynitrite, which can diffuse inside cells and have myriad effects. For example, peroxynitrite enhances the calcium inward current and mitochondrial calcium release in a variety of cells. Moreover, a recent study has shown that SOD3 deletion leads to partial inactivation of Cu/Zn-SOD in the heart of mice exposed to transaort the constriction, thus leading to an increase in intracellular O$_2^-$ and an alteration of gene expression. Likewise, alterations of the extracellular redox state can have profound effects on intracellular signaling and gene expression, likely via modifying cell surface proteins that convey signals to the cytoplasm. Loss of the extracellular SOD3 could, therefore, have important effects on intracellular signaling events in the CVO. In the present study, we observed increased nitrotyrosine staining after SOD3 in the CVO. Although nitrotyrosines can be formed by other mechanisms, this finding is compatible with increased formation of peroxynitrite resulting from SOD3 loss.

Related to the above, an increase in extracellular O$_2^-$ caused by SOD3 deletion could reduce NO signaling in the CVO via the rapid, diffusion-limited reaction between O$_2^-$ and NO. NO has sympathoinhibitory effects in neurons of the SFO, and its loss could further increase sympathetic outflow, in keeping with the effect of CVO SOD3 deletion on heart rate and blood pressure variability. The precise contribution of NO loss versus excessive O$_2^-$ remains undefined.

An interesting finding in the current study is that deletion of SOD3 in periventricular cells increased the number of circulating T cells expressing the early activation marker CD69 and vascular O$_2^-$ production at baseline and increased the number of circulating double-negative (CD4$^+$CD8$^-$) T cells after low-dose Ang II infusion. These results are in keeping with studies by Ganta et al. indicating that Ang II can promote immune activation via increasing sympathetic nerve firing. The superimposition of Ang II markedly augmented the vascular inflammation after central SOD3 deletion and led to severe hypertension. Consistent with this, we also found that the vascular-level intercellular adhesion molecule 1 mRNA was markedly upregulated by the deletion of SOD3 in the CVO. Our data do not allow for an understanding of whether these phenomena are the cause or consequence of the severe hypertension that occurred on Ang II infusion. Previous studies showing that hypertension is blunted in the absence of T cells or by T-cell suppression support a causative role for inflammation in hypertension.

The abundant expression of IL-17A mRNA in the vessel indicates the presence of CD4$^+$ T-helper 17 cells. T-helper 17 cells develop independently of TH$_1$ or TH$_2$CD4$^+$ cells in response to signals such as IL-23, transforming growth factor-β, and IL-6. It has been recognized recently that IL-17 contributes to a variety of autoimmune diseases, including psoriasis, rheumatoid arthritis, experimental allergic encephalitis, and inflammatory bowel diseases. Interestingly, plasma levels of IL-17 and circulating T-helper 17 cells are elevated in humans with unstable angina and myocardial infarction. The precise role of the double-negative T cells in hypertension is unclear, but in other conditions, double-negative T cells secrete high levels of interferon-γ and promote local inflammation. We also observed an increase in aortic CD45$^+$ cells, which represent not only T cells but also other inflammatory cells, such as macrophages. As evident in Figure 4, approximately one half of the total CD45$^+$ cells were T cells. The presence of these cells further emphasizes the importance of interactions between T cells and macrophages, which often coexist in an inflammatory milieu. Taken together, these data strongly indicate that a central perturbation, such as deletion of SOD3 from periventricular cells, can markedly enhance peripheral inflammatory responses.

**Perspectives**

Previous studies from our laboratory and others have shown that mice with embryonic deletion of SOD3 have normal blood pressure at baseline but demonstrate augmented hypertension in response to either Ang II or deoxycorticosterone acetate-salt challenge. Although these studies show that SOD3 has an important role in blood pressure regulation, they do not demonstrate the specific organs or cells in which O$_2^-$ scavenging is important. Our current results indicate that the CNS and, in particular, the CVO are likely sites where SOD3 modulates blood pressure. Moreover, our finding that deletion of CVO SOD3 enhances the vascular O$_2^-$ level demon-
strates that there is an important interplay between central and peripheral regulation of $O_2^{--}$. It is likely that alterations of central neuronal firing caused by deletion of SOD3 in the CVO enhance neurohumoral stimuli that promote vascular $O_2^{--}$ production and possibly production of ROS in other organs, such as the kidney, leading to the hypertensive state. Finally, this study provides new evidence that central oxidative stress can cause peripheral T-cell activation and vascular inflammation, which further augments hypertension and the target-organ damage caused by this disease.

Sources of Funding
This work was supported by National Institutes of Health grants P01HL58000, R01HL39006, and P01HL58000 and a Veterans' Affairs merit grant. H.E.L. is supported through American Heart Association Postdoctoral fellowship grant 0825345E.

Disclosures
None.

References
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*Hypertension*. 2010;55:277-283; originally published online December 14, 2009; doi: 10.1161/HYPERTENSIONAHA.109.142646

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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AUGEMENTATION OF HYPERTENSION AND PERIPHERAL INFLAMMATION BY REDUCTION OF EXTRACELLULAR SUPEROXIDE DISMUTASE IN THE CENTRAL NERVOUS SYSTEM

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Short Title: Central SOD3 in Hypertension

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Supplemental Methods:

Power Spectral Analysis of Heart rate and blood pressure: Data were extracted with a sampling rate of 500 Hz using the Hemolab Software Suite Version 8.3 (http://www.haraldstauss.com/HemoLab). Artifact free heart rate and systolic pressure beat-to-beat data were resampled at a frequency of 25 Hz and converted from non-equidistant to equidistant time series. From this equidistant data a spectral analysis was performed using the Fast Fourier Transformation technique as previously described. 10, 11

Flow cytometric analysis of inflammatory cells: Aortas were cleared of blood by perfusion with phosphate-buffered saline (PBS), excised, and digested using collagenase type IX (125 U/ml), collagenase type IV (450 U/ml), and hyaluronidase IS (60 U/ml) dissolved in 20 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-PBS buffer containing calcium. The digested tissue was then passed through a 70 mm sterile cell strainer (Falcon, BD), yielding single cell suspensions.

Peripheral blood mononuclear cells (PBMC) were isolated from whole heparinized blood after osmotic lysis of red blood cells. Cells were then centrifuged (800 g), washed twice with PBS and 0.5% BSA (FACS buffer), counted, resuspended in 1% BSA/PBS and stored on ice. Within 30 min, 1x106 cells were stained for 15 min at 4°C with antibodies and washed twice with FACS buffer.

Cell labeling was performed using the following antibodies (all from BD Pharmingen): fluorescein isothiocyanate (FITC) anti-CD45 (30-F11); FITC anti-CD69 (H1.2F3); FITC γ/δ (GL3); FITC CD44 (IM7); PerCP anti-CD45 (30-F11); PerCP CD4 (RM4-5); PerCP anti-CD8 (53–6.7); PE anti-CD4 (GK1.5); PE anti-CD195 (CCR5); PE CD25 (PC61); PE TcR β chain (H57-597); APC anti-CD4 (RM4-5); APC anti-CD4 (GK1.5); APC anti-CD3 (145-2C11). Cells were washed twice with 1% bovine serum albumin-PBS buffer. After immunostaining, cells were resuspended in FACS buffer and analyzed immediately on a LSR-II flow cytometer with DIVA software (Becton Dickinson). For aortic tissue an initial gate was applied to exclude cell debris from further analysis, and CD45 staining was used to identify leukocytes within the aortic cell suspension. In this case, all data were analyzed as total cell number.

Quantitative real-time polymerase-chain-reaction: Aortas were removed immediately after euthanasia, snap-frozen in liquid nitrogen and homogenized. The lysate was cleaned with a QIAshredderTM column and RNA was extracted using the RNeasy Mini Kit (Qiagen, USA) according to the manufacturer’s protocol. RNA with an A260/280-ratio between 1.6 and 2.0 was used for reverse transcription using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems. Quantitative real-time PCR was performed in an ABI 7500
Fast Thermocycler (Applied Bioscience, USA) using SYBR Green (SuperArray Bioscience). Primers for Rantes, ICAM-1, IL-17A, VCAM-1 and GAPDH were obtained from SuperArray Bioscience. Total mRNA copy numbers were normalized to GAPDH.