VASCULAR REMODELING

Chronic 3D Vascular-Immune Interface Established by Coculturing Pressurized Resistance Arteries and Immune Cells

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ABSTRACT: Chronic exposure of the arterial vasculature to high blood pressure recruits immune cells and contributes to the vascular remodeling, dysfunction, and inflammation observed in hypertension. The mechanisms underlying the interaction between vascular and immune cells remain unknown, hampering the development of effective therapies targeting the vascular-immune interface. Overcoming these limitations requires a reliable, physiologically relevant experimental model of vascular-immune interface. By coculturing a 3-dimensional organ culture vascular system with immune cells of interest, we reproduced ex vivo the vascular-immune interface that occurs in hypertension. In the 3-dimensional vascular-immune interface model, CD8 but not CD4 T cells isolated from hypertensive mice increased the contractile properties of resistance arteries in naive mice, indicating that CD8 lymphocytes directly contribute to enhanced peripheral resistance in hypertension. RNA sequencing of CD8 lymphocytes isolated from prehypertensive mice revealed upregulation of gene pathways involved in chemotaxis, response to IFN-γ and other external stimuli, MAPK cascade activation, and positive regulation of intracellular calcium fluxes, as compared with CD4 T cells. Taken together, these results indicate that hypertensive stimuli program CD8 T cells toward a phenotype with promigratory properties that might account for their ability to enhance myogenic tone of resistance arteries when cocultured in the 3-dimensional system. Here, we demonstrate modeling a 3-dimensional organ culture vascular system that recapitulates the in vivo physiological properties of resistance arteries. This platform holds on a substantial translational potential, not only for hypertension but also for other cardiovascular diseases where vascular-immune interfaces are established and relevant for onset and progression of the disease. (*Hypertension*. 2021;78:1648–1661. DOI: 10.1161/HYPERTENSIONAHA.121.17447.) ● Data Supplement

Key Words: arteries ■ blood pressure ■ chemotaxis ■ inflammation ■ phenotype

The myogenic response—also called myogenic tone (MT)—is a hallmark of smooth muscle cells (SMCs) in resistance arteries and arterioles and characterizes ability to counteract blood pressure changes.[1,2] When transmural pressure increases, resistance arteries react by raising the MT through SMCs-mediated vasoconstriction.[3] Conversely, when pressure decreases, resistance vessels respond by lowering MT and vasodilating.[3] The myogenic response process is usually divided into 3 different phases.[4] The first phase, during which MT develops, is characterized by significantly elevated intracellular calcium influx through the L-type voltage-gated calcium channel[5,6] and cellular depolarization and deformation, followed by reduced intraluminal vessel diameter. The second phase, named myogenic reactivity, includes minor changes in membrane potential and intracellular calcium levels, but typically shows an intracellular calcium sensitization.[7,8] This phase includes further constriction in response to elevated intraluminal pressure. The

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Novelty and Significance

What Is New?

- An increased myogenic tone, defined as the intrinsic capability of SMCs of peripheral vasculature to counteract increases in perfusion pressure, is one of the first alterations typically observed in hypertension. Furthermore, the infiltration of T lymphocytes activated by hypertensive stimuli is a process associated with increased myogenic tone. Classically, the vascular function was studied in pressure myographs permitting to reproduce the physiology of arterial vasculature and to study their contractile and vasodilating properties, but only for few hours.

What Is Relevant?

- Here, we established a long-term culture system that preserves resistance arteries under pressurized physiological conditions, which keep structural and functional properties unaltered for several days. The system was also optimized to coculture immune cells and study their direct effects on the vasculature.
- We established a 3-dimensional organ culture system for resistance arteries, which maintains vessels viable and functional for 3 days.
- We optimized the system to coculture freshly isolated, purified, and enriched T lymphocytes of interest for 3 days.
- We found that CD8 T cells of hypertensive mice, but not CD4 T cells, directly affect vascular function when

cocultured with naive arteries, enhancing the myogenic tone of resistance arteries to levels typically observed in vessels isolated from hypertensive mice.
- We demonstrate that AngII, in a prehypertensive phase, programs CD8 T cells toward an effector and migratory phenotype.
- Objectives were (1) to expand the development a 3-dimensional organ culture system reproducing a vascular-immune interface and (2) to propose a tool with a high translational potential, for studying the direct vascular-immune interactions in the onset and progression of cardiovascular diseases.

Summary

Here, we propose a chronic 3-dimensional organ culture system obtained by coculturing resistance arteries with immune cells, allowing to reproduce an ex vivo model of the vascular-immune interface established in hypertension. We observed that CD8 but not CD4 T cells isolated from hypertensive mice increase the myogenic tone of resistance arteries in naive mice. The phenotype of CD8 T cells highlighted by our RNAseq analysis, showing a promigratory and effector gene expression pattern, might account for their direct ability to interfere with vascular mechanisms regulating blood pressure levels.

Nonstandard Abbreviations and Acronyms

AngII	angiotensin II
MT	myogenic tone
SMC	smooth muscle cell

last phase, termed dilatative, is defined by complete loss of tone as well as vasodilation in response to high transmural pressure.[8–10]

Interestingly, increased MT is one of the earliest peripheral vasculature changes that might account for the enhanced total peripheral resistance typically observed in hypertension.[11] Extensive research shows that both animal models of hypertension and patients with high blood pressure have higher peripheral vascular resistance that contributes to chronic hypertensive status.[12–15] Numerous studies have analyzed the link between immune system and development of hypertension, highlighting that activated T cells infiltrate the peripheral vasculature, where they affect blood pressure-regulating mechanisms.[16–18] Whether infiltrating T cells can directly modulate

vascular function is still unknown. One of the difficulties in exploring the molecular mechanisms regulating vascular-immune interaction is the complexity of the in vivo vascular milieu, where neurohormonal substances, nervous counterregulatory mechanisms, and many other factors can affect vascular function. Isolated vessel segments' myogenic response to changes in transmural pressure can be studied using the pressure myograph, an experimental system that measures changes in vascular diameter to evaluate vessel responsiveness to increased intraluminal pressure. This ex vivo technique can precisely determine vascular structural characteristics and assess vessel functionality via evaluating both MT and responses to specific vasoconstricting or vasodilating stimuli.[19] However, the ex vivo pressure myograph system does not reproduce the complexity of chronic vascular-immune interactions. The purpose of this study was to establish a long-term culture system that maintains vessels under pressurized physiological conditions, preserving structural and functional properties, for several days. The system was also optimized to coculture immune cells of interest and study their direct effects on the vasculature.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Animals

All animal handling and experimental procedures were performed according to European Community guidelines (EC Council Directive 2010/63) and Italian legislation on animal experimentation (Decreto Legislativo D.Lgs 26/2014). C57Bl/6J (house in standard conditions of temperature (21 ± 2 °C) and humidity ($50\%\pm10\%$), with a 12-hour light/dark cycle, sawdust as bedding, standard pellet food and tap water ad libitum.

Angiotensin II Infusion and Blood Pressure Measurement

Mice anesthetized with isoflurane were subcutaneously implanted with osmotic minipumps infusing AngII (angiotensin II; 0.5 mg/kg per day; Sigma-Aldrich) or vehicle (NaCl 0.9%). Blood pressure was measured by tail-cuff plethysmography (BP-2000 Series II, Visitech Systems), as previously described,[20] and mice were culled as indicated for each experimental setting.

Surgical Procedure and Mounting Vessels on the Pressure Myograph

The third branch of mesenteric artery was excised from mice culled by cervical dislocation and cannulated for mounting on the culture myograph system—204CM (DMT, Danish Myo Technology). After mounting, vessels were allowed a 30-minute equilibration period under a static pressure of 60 mmHg to ensure an appropriate axial stretch.[21]

The mesenteric artery branch was maintained in the culture system up to 4 days at 37 °C, 60 mmHg and with a 95% O_2 and 5% CO_2 gas mixture. The vessel was perfused with oxygenated inflow medium, bubbled with the gas mixture, and surrounded by superflow medium comprising complete Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% dialyzed fetal bovine serum (FBS) to remove small contractile factors that could affect our results[22] and to improve endothelium-dependent relaxation.[23]

After equilibration, vessels were transilluminated under a microscope connected to a computerized system to continuously record vessel walls and lumen measures. Vessel functionality was evaluated by testing potassium physiological salt solution (KPSS) physiological salt solution, norepinephrine (NE, 2 µmol/L) and acetylcholine (ACh, 10 µmol/L) responses. After mounting, equilibration and testing procedures, only vessels that constricted in response to NE and KPSS (minimum response >80%) and dilated in response to ACh (minimum response >75%) were prepared to receive T cells and perform the coculture.

For acute vascular function studies, third-order branch of mesenteric artery was excised from mice culled by cervical dislocation and placed in PSS before mounting in the vessel chamber of a pressure myograph (110P System, DMT Danish Myo Technology). After mounting procedures, vessels were allowed a 30-minute equilibration period under a static pressure of 60 mmHg, before studying MT, according to previously published protocols.[20]

T Cell Isolation and Coculturing Protocol

Anesthetized mice were culled by cervical dislocation to isolate the spleens and prepare single-cell suspensions of splenic leukocytes. The enrichment fractions of CD8 T lymphocytes and of CD4 T lymphocytes from total splenic leukocytes were obtained using the negative selection kits Mouse CD8 T Lymphocyte Enrichment Set (BD IMag, 558471) and Mouse CD4 T Lymphocyte Enrichment Set (BD IMag, 558131), respectively, as indicated by the manufacturer.

To coculture the vessels, 2.5×10^5 CD4 or CD8 T lymphocytes were resuspended in a final volume of 2 mL of cell culture medium and infused into the vessel chamber. A second infusion of the same number of T lymphocytes was performed after 2 hours. At the end of the incubation time (2 hours after the first and the second infusion), cells present in the outflow were counted.

To expose vessels to lymphocytes from the intraluminal side, the same experimental setup used for the incubation from the extraluminal side was applied. The number of cells necessary for the infusion was calculated at the same density used in the superflow (1.25×10^5 cells/mL), and cells were infused twice through the inflow circuit.

Flow Cytometry

To assess the purity of CD8 and CD4 T cell preparations, 1×10^5 purified cell preparations (CD4 or CD8 T lymphocytes) were resuspended in IMag buffer and preincubated with anti-CD16/32 Fc receptor (BD Pharmingen, 553141; 1:100) and then incubated with a combination of fluorochrome-conjugated monoclonal antibodies for CD45 (BD Horizon, 564279; 1:100), CD11b (BD Horizon, 612800; 1:100), CD11c (BD Horizon, 562782; 1:100), CD3 (BD Pharmingen, 553061; 1:100), CD4 (BD Horizon, 563747; 1:100), and CD8 (BD Pharmingen, 553032; 1:100), and the live/dead marker 7-AAD (BD Pharmingen, 559925). Cells were analyzed with FACS Celesta (BD Bioscience) equipped with FACSDiva Software, and the data obtained were analyzed with FlowJo V10 Software.

Myogenic Tone

MT was obtained by measuring vessel inner diameters in the presence of DMEM-dialyzed FBS free or PSS-Ca^{2+} free. These measurements were taken while intravascular (transmural) pressure progressively rose from 0 to 125 mmHg, by steps of 25 mmHg (10 minute per step). The percentage of myogenic tone (% MT) was calculated as previously described.[20]

RNA Sequencing

Total RNA was obtained by phenol chloroform extraction of samples lysed in Trizol (Life Technologies, 15596018).

Next-generation sequencing experiments were performed by Labospace S.R.L. (Milano, Italy), the raw reads of the experiment were submitted to NCBI-GEO, accession number GSE181198.

R was used to create a matrix of all genes expressed in all samples with the corresponding read-counts, and the Bioconductor package DESeq2 was used to normalize the data, using the median of ratio, to perform the differential expression analysis.[24] Counts were divided by sample-specific size factors
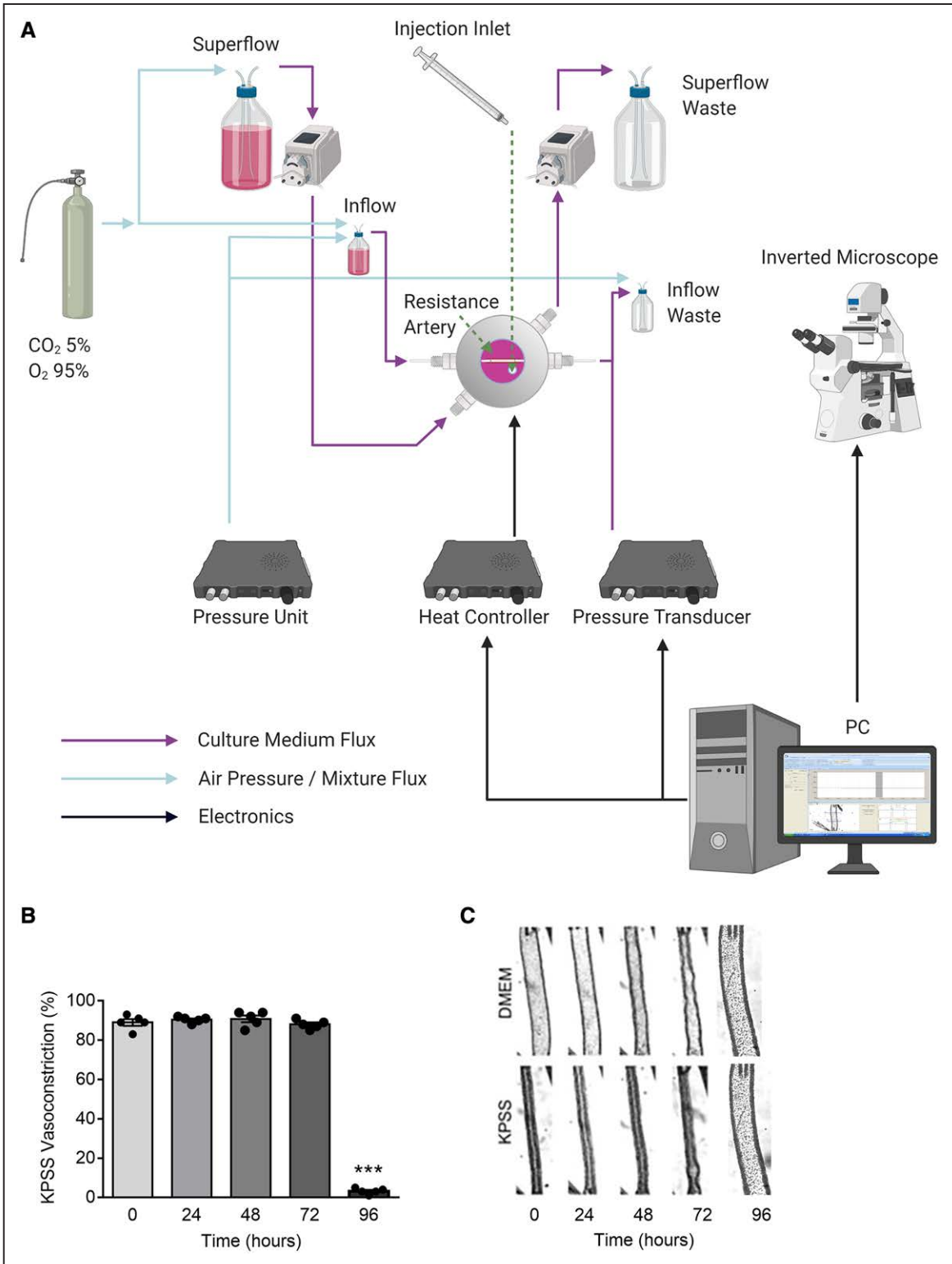
Figure 1. Establishing a 3-dimensional (3D) vascular organ culture system.
Schematics showing the fluidic and electronics composing the system (**A**). Vasoconstriction to potassium physiological salt solution KPSS infusion in the chamber was measured in vessels at baseline, 24, 48, 72, and 96 h in the 3D vascular organ culture system. Vessel viability and functionality were comparable at any time point up to 72 h, while they were completely absent at 96 h (n=5; 0 vs 24 h: t=0.8009, P=0.8964; 0 vs 48 h: t=1.03, P=0.7804; 0 vs 72 h: t=0.572, P=0.9670; 0 vs 96 h: t=49.08, P<0.0001) (**B** and **C**). One-way ANOVA with Tukey post hoc correction. Created with www.BioRender.com.

determined by median ratio of gene counts relative to geometric mean per gene. Visualization of the results were performed by ClustVis, VolcaNoseR and Morpheus (Morpheus. https://software.broadinstitute.org/morpheus).[25,26] Gene enrichment analyses were obtained by EnrichR[27] using default parameters with the input set of upregulated genes in CD8 cells.

Statistical Analysis

Based on preliminary data and previous studies assessing the effect of hypertension on mesenteric arteries myogenic tone, we hypothesized a predicted difference in myogenic tone of 13% and used this parameter to calculate the power of the study. Our analysis estimated to perform n=5 experiments for each treatment to achieve an alpha of 0.05 and a beta power of 0.80.

Data are presented as mean±SEM. We performed the Shapiro-Wilk test to assess normality. Normal distributions were analyzed using parametric tests (Student t test, 1-way ANOVA with Tukey correction for multiple comparisons, or 2-way ANOVA with Sidak correction for multiple comparisons), according to the experimental design. Data with non-normal distribution were analyzed by Mann-Whitney U test or Kruskal-Wallis Test with Dunn correction for multiple comparisons. Analyses were performed using GraphPad software PRISM5 (GraphPad Software, Inc), and $P<0.05$ was considered significant. Calculated P are specified in figure legends.

RESULTS

Establishing a 3D Vascular Organ Culture System

Resistance arteries of hypertensive mice have been shown to display enhanced MT.[11,20] Also, several works have demonstrated that T cells infiltrate the vasculature of hypertensive mice, contributing to their typically elevated peripheral resistances.[16,17,28] Our previous work clarified that, in response to hypertensive stimuli like AngII, T cells migrate to the vasculature of high blood pressure target organs, like peripheral resistances and kidneys, even before hypertension is established, that is, after only 3 days when mice are still in a prehypertensive phase.[29,30] Thus, it is conceivable that T cells establish a vascular-immune interface where they participate in altering mechanisms that regulate blood pressure, thereby contributing to the onset of hypertension. With the aim of reproducing a 3-dimensional (3D) vascular-immune interface, we first set up a vascular organ culture system that could maintain unaltered vascular properties for at least 3 days, a time point that corresponds to the earliest infiltration of T cells in peripheral vasculature during AngII-induced hypertension.

To this aim, the culture system (Figure 1A) was fixed at 37 °C and 60 mm Hg. A gas mixture of 95% O_2 and 5% CO_2 provided oxygenation. The vessel was perfused with oxygenated inflow medium and surrounded by superflow complete DMEM.

Vessel viability was evaluated by daily KPSS stimulations until day 4 of organ culture, as shown in Figure 1B and 1C. During the 4-day culture period, the contraction to KPSS was well preserved with no loss in K+-mediated responses only up to the third day. At day 4, vessels were unresponsive, suggesting that viability was impaired (Figure 1B and 1C). To investigate whether the lack of functional responses after 3 days from vessel mounting could be related to loss of viable SMCs, we analyzed cell death/apoptosis by TUNEL assay at the end of each day of incubation. As shown in Figure S1 in the Data Supplement, organ culture conditions induced significant SMCs apoptosis at 96 hours. Also, at the end of the third day, the KPSS response observed in the 3D organ culture system was comparable to the response of a freshly isolated artery mounted on an acute pressure myograph (Figure 2A). This organ culture system effectively maintained a mesenteric artery for 3 days, thereby allowing us to functionally assess resistance arteries in a 3D vascular-immune interface.

Hence, vascular function studies were executed at 1, 2, and 3 days of organ culture. Given the relevance of α1-adrenergic receptors in the adrenergic sympathetic and humoral regulation of vascular tone,[31] we used acute NE stimulation to verify the integrity of our system. Although a slight—not significant—reduction in NE-induced vasoconstriction was noted after 3 days in organ culture conditions, the response of resistance arteries was comparable to that of a freshly isolated one (Figure 2B), thus demonstrating that vessels remained responsive to agonist-mediated contraction. At the end of the protocol (3 days later), we tested ACh-dependent vasorelaxation to determine whether the chronic culturing conditions affected endothelial function. As shown in Figure 2C, the endothelium-dependent vasodilation induced by ACh was not altered by 3 days of organ culture conditions.

MT was analyzed at every time point (1, 2, and 3 days of organ culture) and compared with the MT of a freshly isolated vessel (Figure 2D). Our results showed that chronic and acute conditions generated comparable and nonsignificantly different percentage of myogenic response measurements. Also, the analysis of active and passive vessel contractile responses to the increasing perfusion pressure showed comparable results in the 2 experimental settings (Figure 2E and 2F).

AngII Induces Early increases of Both MT in Resistance Arteries and T Cell Infiltration Into the Vasculature of Hypertension Target Organs

C57Bl/6J mice were implanted with osmotic minipumps to deliver 0.5 mg/kg per day of AngII or vehicle as control. Blood pressure was monitored for the following 3 days to confirm prehypertensive status (systolic blood pressure: vehicle, 109.8±0.7 mm Hg versus AngII, 115.2±2.3 mm Hg, $P=0.053$; DBP: vehicle, 50±1.3
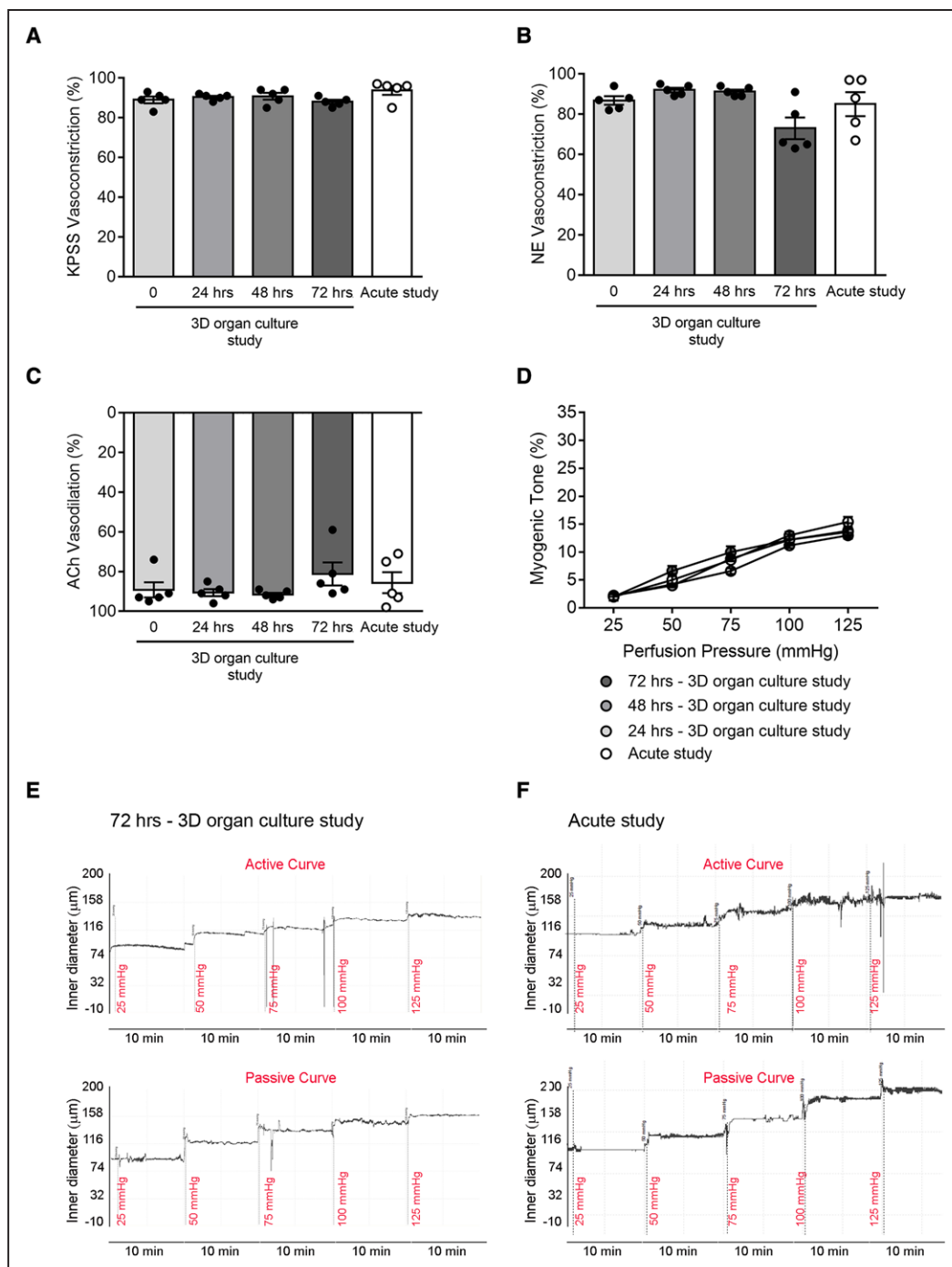
Figure 2. Functional assessment of resistance arteries in the 3-dimensional (3D) vascular organ culture system.
To ensure that vascular function parameters were unaltered by 3 d of organ culture, we compared the organ culture vascular function responses every 24 h to those obtained in parallel acute myograph studies. Vasoconstriction to KPSS (n=5; 3D organ culture system 0 h vs acute study: $P=0.6022$; 3D organ culture system 24 h vs acute study: $P>0.9999$; 3D organ culture system 48 h vs acute study: $P>0.9999$; 3D organ culture system 72 h vs acute study: $P=0.1150$), Kruskal-Wallis test with Dunn post hoc correction (**A**), and to NE (n=5; 3D organ culture system 0 h vs acute study: $t=0.4767$, $P=0.9970$; 3D organ culture system 24 h vs acute study: $t=1.854$, $P=0.6878$; 3D organ culture system 48 h vs acute study: $t=1.642$, $P=0.7726$; 3D organ culture system 72 h vs acute study: $t=3.178$, $P=0.2033$), 1-way ANOVA with Tukey post hoc correction (**B**). Vasodilation to acetylcholine (ACh) was evaluated at 0, 24, 48, and 72 h and compared with that observed in acute studies (n=5; 3D organ culture system 0 h vs acute study: $P>0.9999$; 3D organ culture system 24 h vs acute study: $P>0.9999$; 3D organ culture system 48 h vs acute study: $P>0.9999$; 3D organ culture system 72 h vs acute study: $P>0.9999$). **C**, Kruskal-Wallis test with Dunn post hoc correction. Finally, we tested MT of vessels in the 3D organ culture system (at 24, 48, and 72 h), which showed a response comparable over time to that obtained in the acute studies with pressure myograph (n=5; max difference 3D organ culture system 24 h vs acute study [at 125 mm Hg of intraluminal pressure]: $t=2.41$, $P=0.0538$). **D**, Two-way ANOVA with Sidak post hoc correction. Representative recordings of active (complete DMEM) and passive (calcium free) internal diameters of resistance arteries at increasing perfusion pressures in the 3D organ culture system (**E**) or in the acute setting (**F**), respectively.

mm Hg versus AngII, 54.4±2.1 mm Hg, *P*=0.114; n=5 per group) before animals were culled for evaluation of resistance arteries' MT and T cell infiltration into target organs' vasculature. As shown in Figure S2A, resistance arteries from prehypertensive mice display significantly higher MT compared with those from vehicle-infused control mice. Kidneys isolated from the same animals were processed for immunohistochemistry to assess the presence of infiltrating T cells. As shown in Figure S2B through S2E, CD4 and CD8 T lymphocytes infiltrate the perivascular renal tissues of prehypertensive mice. The same tissues isolated from vehicle-infused control mice lacked T cells (Figure S2C through S2E).

Establishing a 3D Vascular-Immune Interface: CD8 T Cells From Hypertensive Mice Enhance MT of Resistance Arteries

To test the direct effect of immune cells infiltrating the vasculature of tissues and organs targeted by high blood pressure, we developed a protocol to reproduce a 3D vascular-immune interface by coculturing resistance arteries, as described above, in the presence of immune cells of interest. As we and others observed the presence of both CD4 and CD8 T cells in hypertension target organs, we performed a chronic vascular organ culture with either CD4 or CD8 T cells isolated and enriched from the spleens of prehypertensive mice or vehicle-infused controls (Figure 3A). Purity of CD4 and CD8 T cell preparations, analyzed by flow cytometry according to the gating strategy shown in Figure 3B, was assessed as >90% (Figure 3C through 3F).

CD4 T cells were infused into the vessel chamber through the superflow medium and then allowed to coincubate. After establishing the 3D vascular-immune interface, we monitored the organ coculture system for 3 days, then analyzed function. To compare the effect of CD4 T cells isolated from AngII-infused prehypertensive mice to those isolated from vehicle-infused control animals, we prepared 2 parallel systems with resistance arteries from 2 C57Bl/6J mice supplemented by the same superflow medium. Coculturing CD4 T cells from AngII mice did not alter vasoconstriction in response to either KPSS (Figure 4A) or NE (Figure 4B), vasodilation to ACh (Figure 4C), nor the MT of resistance arteries, as compared with the vascular function of arteries incubated with CD4 T cells from vehicle-infused mice (Figure 4D). Interestingly, while using the same protocol to coculture CD8 T cells isolated from AngII-infused similarly resulted in unaltered KPSS- and NE-induced contraction and ACh-induced vasodilation (Figure 5A through 5C), CD8 T cells from AngII mice significantly enhanced the MT of resistance arteries (Figure 5D). No effect on SMCs viability was observed after coculturing with neither CD4 (Figure S3A and S3B) nor CD8 T cells (Figure S3C and S3D).

While T cells typically extraluminally accumulate in perivascular fat tissues, the primary source of lymphocytes would be from the circulation. To test the hypothesis that circulating cells colonize the perivascular tissues by infiltrating from the intraluminal side, we evaluated the effect of intraluminal exposure of resistance arteries to CD8 T cells isolated from prehypertensive mice (Figure S4A). Interesting to notice, CD8 T cells administered in the inflow did not affect vascular function, which remained comparable in vessels receiving CD8 T cells from vehicle or AngII mice (Figure S4B through S4E). Taken together, these data suggest the CD8 T cells from AngII mice acquire the ability to infiltrate and colonize the perivascular tissue of resistance arteries.

In Vivo AngII Programs CD8 T Cells to Promote Their Response to External Stimuli and Activate Cell Migration

To explore networks of genes that might determine the differential effects induced by CD8 and CD4 T cells from hypertensive mice, we profiled both T cell subtypes from AngII prehypertensive mice by bulk RNA-seq. As shown in Figure 6A and 6B, we identified 342 genes that were differentially expressed (342 with p-false discovery rate <0.05; 108 decreased and 169 increased in CD8 AngII versus CD4 AngII; fold-change >1.5, p-false discovery rate <0.05), with a distinct transcriptional signature of CD8 T cells. CD8 T cells of AngII mice reduced the expression of genes involved in repression of cytotoxic effector genes perforin, Granzyme B, and Interferon (IFN)-γ, while increasing the expression of genes related to cell maturation into effector and resident memory cells and to cell migration (Figure 6C). Notably, CD8 T cells from hypertensive mice, as compared with CD4 counterpart, were characterized by activation of pathways related to mitogen-activated protein kinase MAPK cascade (with a positive regulation of ERK1 and ERK2), positive regulation of intracellular calcium fluxes, response to IFN-γ and other external stimuli, and lymphocyte chemotaxis (Figure 6D).

In line with this, we have also found that CD8 T cells isolated from hypertensive mice had a significantly lower rate of outflow from the chamber after the incubation time, compared with CD8 T cells from vehicle controls or CD4 T cells from both AngII and vehicle animals (Figure S5A and S5B). Conversely, CD8 T cells injected in the intraluminal side of the organ coculture system showed a high rate of outflow (Figure S5C), suggesting that retainment in the vasculature depends not only on the immune cell itself but also on the specific vascular cell type (endothelium or SMCs). This result was suggestive of an increased capability of activated CD8 T cells to be attracted and infiltrate the perivascular tissues and is in accordance with the effect of enhancing the myogenic tone, which was assessed 72 hours later. In fact, resistance arteries collected at the end of the vascular
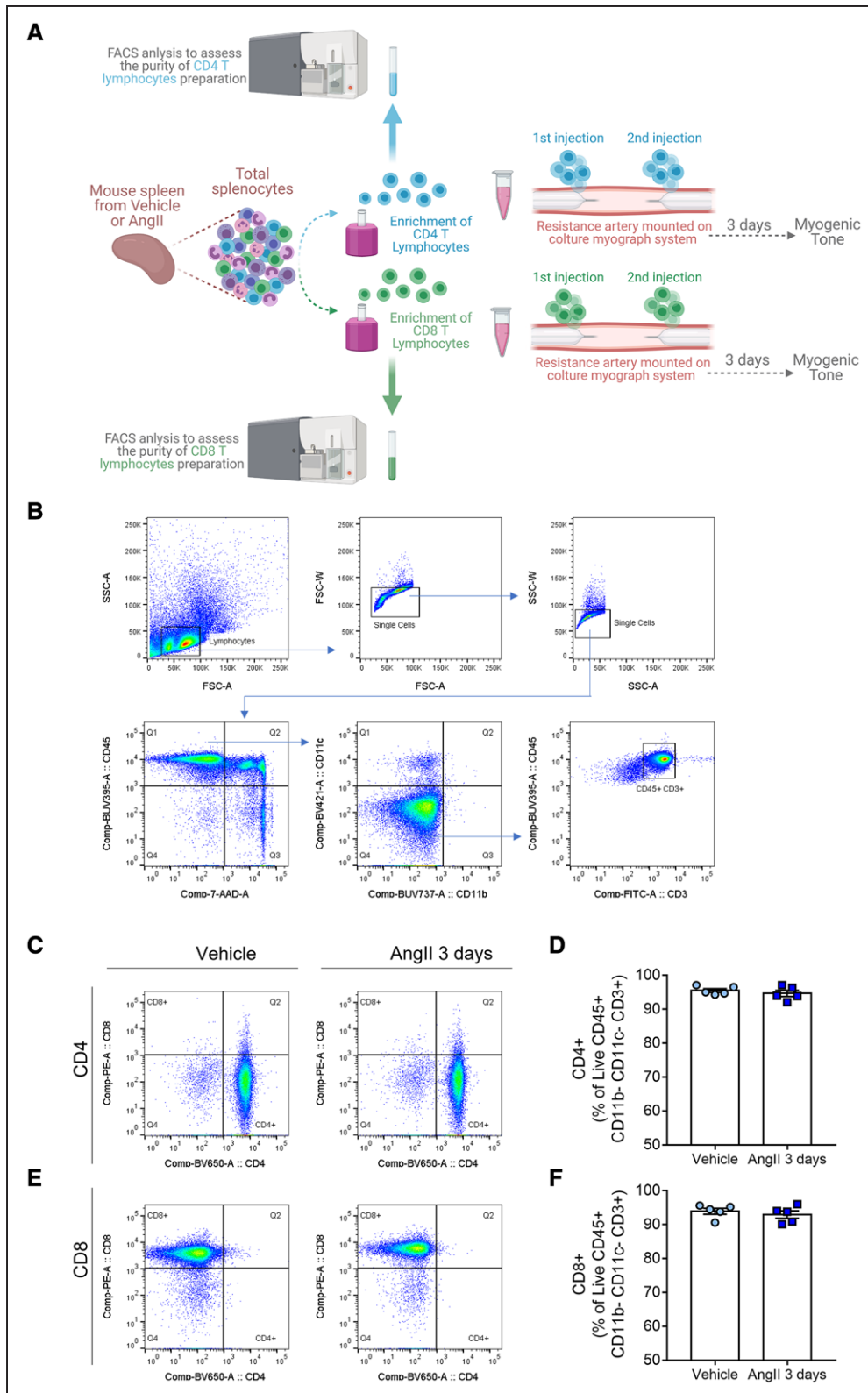
Figure 3. Protocol for T cell isolation, purification, and establishment of the vascular-immune interface.
Schematics of the experimental design and flow chart for preparation of cells and establishment of the 3-dimensional (3D) vascular-immune interface (**A**). Gating strategy for assessing the purity of T cell enrichment procedure (**B**) for either CD4 T cells (n=5 Veh vs 5 AngII: t=0.7992, *P*=0.4473) (**C** and **D**) or CD8 T cells (n=5 Veh vs 5 AngII: t=0.6993, *P*=0.5042) (**E** and **F**), Student *t* test, isolated from the spleen of AngII-infused mice or relative control mice infused with vehicle alone. Created with www.BioRender.com.
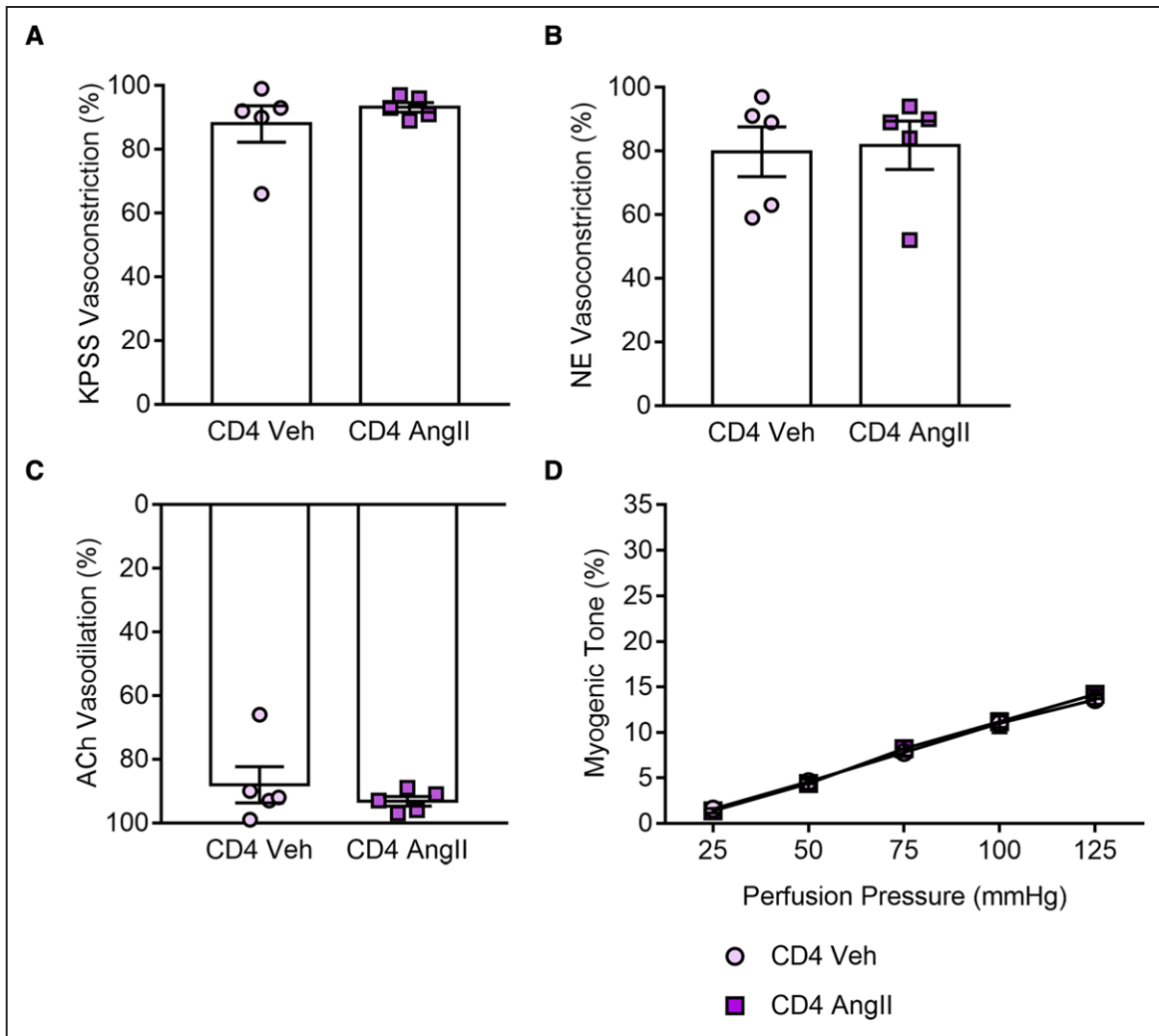
Figure 4. CD4 T cells do not affect vascular function and myogenic tone of resistance arteries in the 3-dimensional (3D) organ culture vascular-immune interface.
Naive mesenteric resistance arteries were cocultured either with CD4 T cells obtained from AngII (angiotensin II) infused mice or from their respective controls infused with vehicle alone. Vascular function was unaltered to potassium physiological salt solution (KPSS) and norepinephrine (NE) contractile agents (KPSS: n=5 CD4 Veh vs n=5 CD4 AngII: t=0.8822, P=0.4034) (A), (NE: n=5 CD4 Veh vs n=5 CD4 AngII: t=0.1833, P=0.8591) (B), and to vasodilating substances (acetylcholine [ACh]: n=5 CD4 Veh vs n=5 CD4 AngII, P=0.6003). C, Also, myogenic tone to increasing perfusion pressures was comparable in MRA cocultured with CD4 T cells from AngII or from vehicle mice, at any pressure step (n=5 CD4 Veh vs n=5 CD4 AngII; 25 mmHg: t=0.237, P=0.9998; 50 mmHg: t=0.237, P=0.9998; 75 mmHg: t=0.474, P=0.9938; 100 mmHg: t=0.237, P=0.9998; 125 mmHg: t=0.7111, P=0.9624). D, Student t test for KPSS, NE, ACh, 2-way ANOVA with Sidak post hoc correction for MT%.

function study were analyzed by immunohistochemistry to detect the presence of lymphocytes used in the coculture system. CD8 T cells from AngII mice, but not CD8 from vehicle or CD4 from both AngII and control mice, were found in the perivascular tissue of mesenteric arteries (Figure S6A and S6B). In agreement with the functional data, the same effect was not observed when CD8 T cells from hypertensive mice were administered from the intraluminal side (Figure S6C).

DISCUSSION

While other groups have attempted to establish vascular organ culture systems,[32] the complexity of vascular physiology has not previously been reproduced, nevertheless

conserved for several days. Preserving physiological properties under pressurized conditions was the most challenging condition to mimic. Adding immune cells to the 3D organ culture system created another level of complexity, as we needed to find the appropriate culture medium for both vessels and immune cells. As we here present, we have devised a 3D organ coculture system that effectively reproduces the vascular-immune interface occurring when activated lymphocytes infiltrate the vasculature of hypertension target organs. After establishing long-term organ culture for resistance arteries, so that vessels were maintained in physiological conditions that preserved their functional properties, we introduced enriched lymphocytes from the spleens of prehypertensive mice. To determine at the functional level that
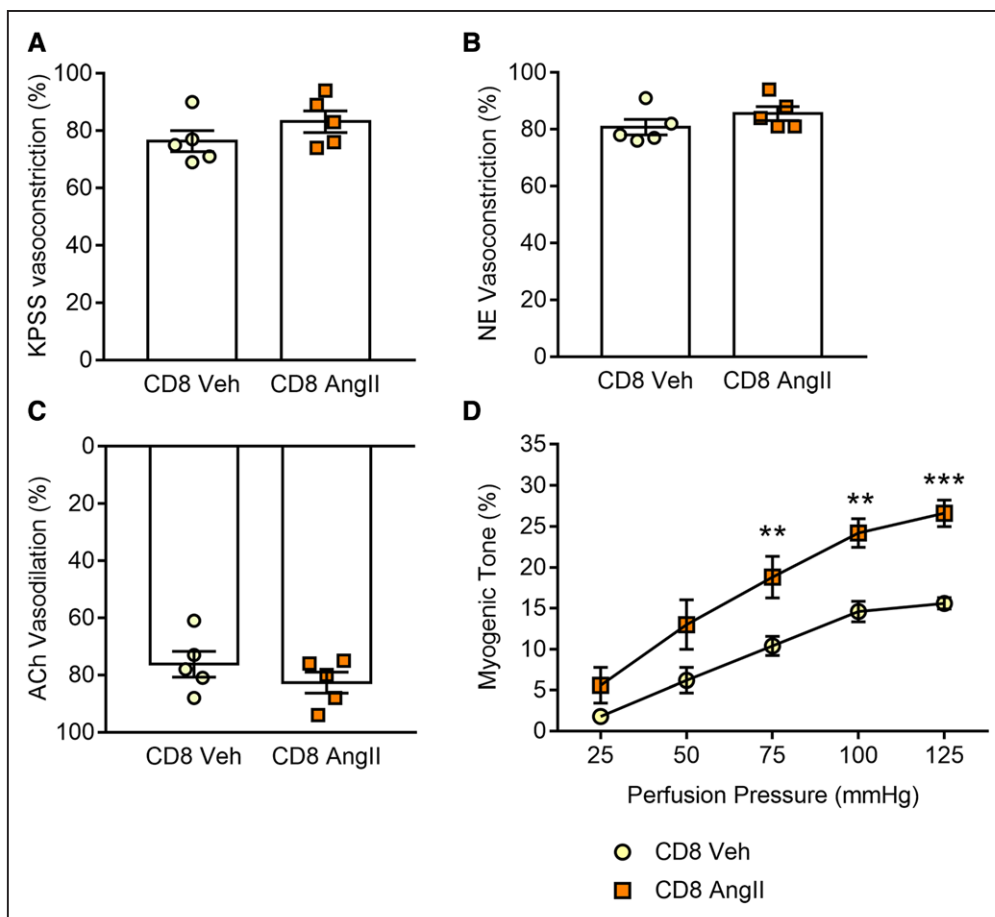
Figure 5. CD8 T cells increase the myogenic tone of resistance arteries in the 3-dimensional (3D) organ culture vascular-immune interface.
Naive mesenteric resistance arteries were cocultured with CD8 T cells purified and enriched from AngII (angiotensin II) infused mice. Parallel arteries were cocultured with CD8 T cells isolated from vehicle-infused control mice. While vascular function to potassium physiological salt solution (KPSS) and norepinephrine (NE) contractile agonists was unaltered (KPSS: n=5 CD8 Veh vs n=5 CD8 AngII: t=1.287, P=0.2340) (**A**), (NE: n=5 CD8 Veh vs n=5 CD8 AngII: t=1.302, P=0.2293) (**B**), as vasodilation to ACh was (ACh: n=5 CD8 Veh vs n=5 CD8 AngII: t=1.102, P=0.3023) (**C**), the myogenic tone was significantly increased by the presence of CD8 T cells enriched from AngII-infused mice (n=5 CD8 Veh vs n=5 CD8 AngII; 25 mm Hg: t=1.505, P=0.5298; 50 mm Hg: t=2.694, P=0.0503; 75 mm Hg: t=3.328, P=0.0094; 100 mm Hg: t=3.803, P=0.0024; 125 mm Hg: t=4.358, P=0.0004) (**D**) Student t test for KPSS, NE, ACh, 2-way ANOVA with Sidak post hoc correction for MT%.

a coculture 3D vascular-immune interface had been successfully established, we performed vascular reactivity studies at the end of the coculturing protocol. Our system will expand our understanding of the molecular and cellular interactions between immune and vascular cells in steady state and during disease. Our 3D organ coculture system has been conceived and developed to model the vascular-immune interface established at key vascular sites during hypertension. However, the current model could be applied to several physiological and disease contexts where vascular-immune interfaces are known to be involved.

As intended, our 3D organ coculture vascular-immune interface advanced our knowledge about immune cells recruited during hypertensive challenges. In fact, while the idea that immunity is involved in hypertension is increasingly accepted, how immune cells influence mechanisms directly involved in regulating blood pressure is

less clear. In vivo models of hypertension have provided substantial data demonstrating that immune cells are recruited to tissues and organs that are typically involved in regulating blood pressure and/or are targets of hypertension-induced damage, like the peripheral vasculature and kidneys.[16,17,28] At the same time, the complexity of the in vivo milieu, where immune cells and vasculature are directly and indirectly influenced by neurohormonal circulating substances and by the innervation provided by the autonomic nervous system, could not elucidate whether immune cells directly modify the function of resistance arteries, which are known to play a crucial role in homeostatic and altered blood pressure levels. In recent decades, the conventional pressure myograph system for acute vascular function studies proved an invaluable tool for dissecting the molecular bases of resistance arteries' functions in homeostasis and during hypertension.[8,9,33,34] Yet, the discovery that the immune
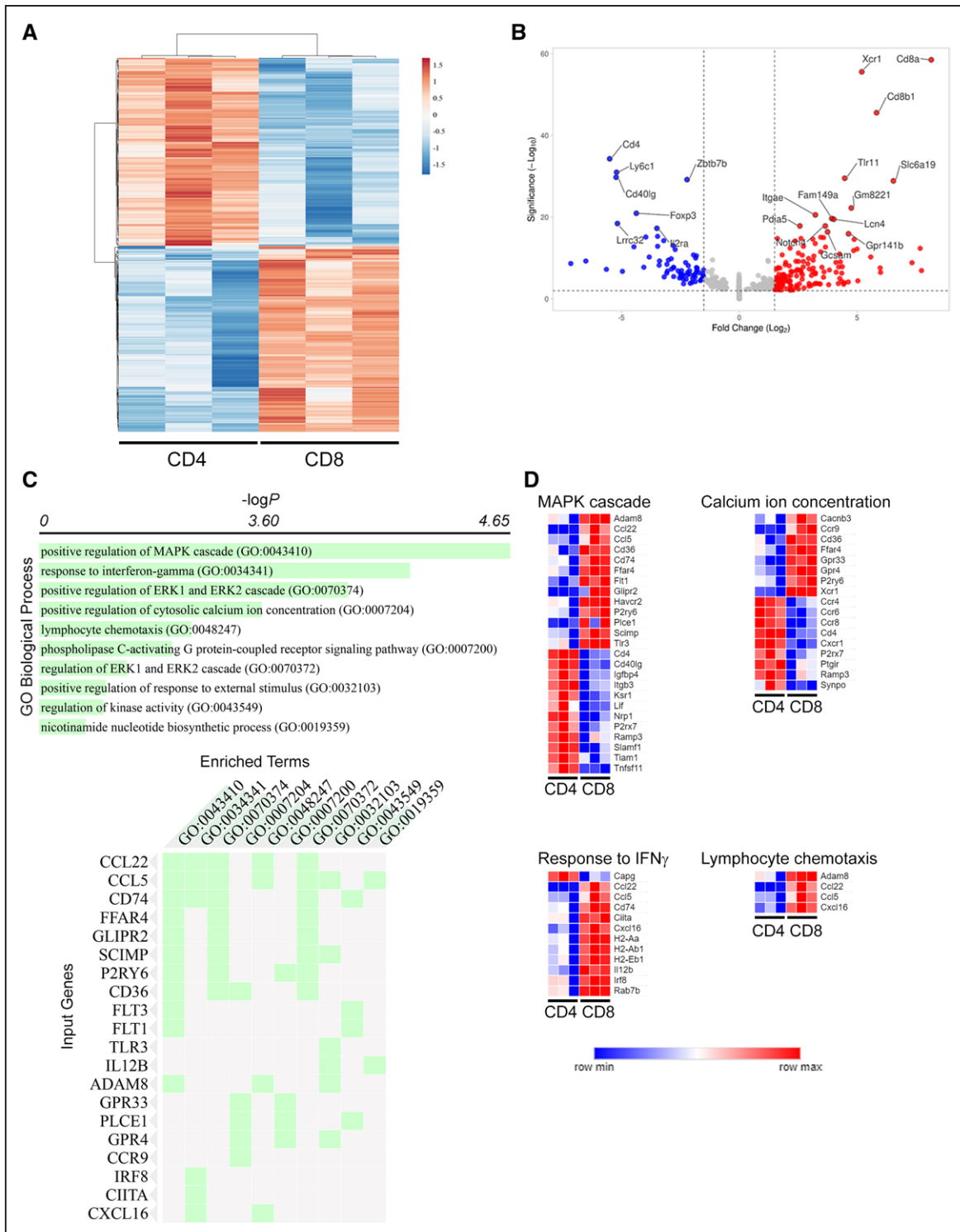
VASCULAR REMODELING

Figure 6. In vivo AngII (angiotensin II) programs CD8 T cells toward a migratory phenotype, responsive to external stimuli.
A, Heat map of key differentially regulated genes in CD8 T cells from prehypertensive mice, as compared with CD4 T cells (n=3 mice).
B, Volcano plot of differentially regulated genes (fold-change >1.5, p-FDR<0.05). **C**, Gene ontology (GO) biological processes analysis of differentially upregulated genes in CD8 vs CD4 T cells from prehypertensive mice. Clustergram of the most characterizing upregulated genes (vertical axis) for each significantly enriched ontology (horizontal axis). **D**, Heat maps of key differentially regulated genes in CD8 T cells from prehypertensive mice compared with CD4 T cells (n=3 mice) grouped by significantly enriched ontologies of interest identified by CD8 upregulated genes. The biological processes of response to IFN-γ and lymphocyte chemotaxis are associated uniquely to genes upregulated by CD8 T cells.

system influences hypertension[12] indicated additional complex interplay with the classical components of the resistance arteries, that is, SMCs and endothelial cells. New systems would be necessary to study such interactions. Only a 3D organ culture system that chronically cocultures immune cells and resistance arteries could reproduce the complex cell-cell interactions that occur in vivo and, at the same time, remain free from neurohormonal or neural influences. Having designed just such a system, we have showed that CD8, but not CD4, T cells isolated and purified from prehypertensive mice significantly increased the MT of a naive resistance artery, setting its contractile response to the level of arteries isolated from hypertensive mice. This result suggests that once in contact with the resistance vasculature—a process that occurs during hypertensive challenges in vivo—CD8 T cells can directly modulate the SMCs apparatus responsible for establishing MT in response to rising perfusion pressure. Also, this finding aligns well with the in vivo observation that CD8-deficient, but not CD4-deficient, mice are protected from hypertension.[35] How CD8 T cells activated by hypertensive stimuli exert this function on resistance arteries remains to be elucidated. To further elaborate on the mechanisms regulating the vascular-immune interface established between CD8 T cells and resistance arteries, we also tested whether the same cell preparation administered from the intraluminal side would have the same effect. Interestingly, CD8 T cells from AngII prehypertensive mice were unable to affect the MT when intraluminally incubated, thus suggesting that CD8 T cells might establish a direct interaction with the SMCs.

Our RNAseq analysis of CD8 and CD4 T cells isolated from prehypertensive mice identified clusters of differentially expressed genes consistent with the functional data. CD8 T cells were characterized by the activation of GO biological processes related to the MAPK cascade, to the positive regulation of intracellular calcium fluxes, to the response to IFN-γ and other external stimuli, and to lymphocyte chemotaxis. Studies have shown that the Erk MAPK pathway plays a critical role in proliferation and survival decisions fate decisions within CD8 T cells.[36] Also, it has been shown that IFN-γ exerts direct effects in regulating the expansion, contraction, and memory phases of the polyclonal CD8 T cell responses.[37] The activation of pathways related to positive regulation of cytosolic calcium ion concentration suggests that AngII activates a process of differentiation of naive CD8 T cells into fully functional cytolytic effector cells.[38] CD8 T cells have evolved as one of the most motile mammalian cell types and the process of activation induces a switch to infiltration of nonlymphoid tissues.[39] The activation of processes related to lymphocyte chemotaxis indicates that AngII promotes a migratory behavior of CD8 T cells that might be necessary to infiltrate and colonize target vasculature of peripheral organs. One of the differentially expressed genes emerging from

this GO analysis for biological processes related to chemotaxis and migration is Ccl5/RANTES, which has been previously shown to be involved in hypertension.[40,41] While a study showed that Ccl5/RANTES knockout protected against T lymphocyte infiltration and vascular dysfunction upon AngII,[40] another work demonstrated that Ccl5 inhibition aggravated renal dysfunction in AngII-induced hypertension,[41] thus suggesting that additional studies will be necessary to clarify the role of the Ccl5/RANTES at the vascular-immune interface. Other chemokines and chemokine receptors emerged as differentially regulated between CD4 and CD8 T cells subjected to hypertensive stimuli (ie, Ccr9, Ccr4, Ccr6, Ccr8, Cx3Cr1, Ccl22, CxCl16). Future studies exploiting this 3D organ culture system reproducing a vascular-immune interface will be instrumental to unravel the role of ligands/receptors in the process of CD8 T cell recruitment at vascular district and their consequent effect on vascular function.

Clinically, the onset of hypertension typically manifests earlier in men than in women, and a parallel sex difference is observed in experimental models of hypertension.[42] It is important to notice that all our experiments were performed in resistance arteries isolated from male mice, since it has been shown that the resistance arteries of female mice develop a significantly lower myogenic tone as compared with male mice arteries.[43] Based on these considerations, arteries dissected out from male and female mice cannot be studied as a homogenous group, requiring a specific analysis of sex-dependent function. Interestingly, also some immune system responses associated with hypertension have shown a dependence on sex.[42,44] Thus, the new organ coculture system for immune cells and vessels could be particularly relevant to elucidate with future experiments the mechanisms that underlie this robust sex difference in susceptibility to hypertension.

PERSPECTIVES

There are several ways to further improve and exploit our model. First, the 3D vascular-immune organ culture system can test the effects of not only specific CD8 T cell subpopulations but also any other immune cell of interest. As a specific example, CD8 T cells recruited to hypertension target organs are characterized by the acquisition of effector functions (CD8 T_{eff}) that might in turn be responsible for effects on vasculature.[16,29,30] Testing their direct effects on resistance arteries would help identify novel cellular and molecular mechanisms that could be targeted therapeutically to limit hypertension-induced target organ damage. Interestingly, CD4 T cell subtypes with immunomodulatory functions—T regulatory cells (T_{reg})—can improve the vascular dysfunction observed in hypertension.[45] Testing whether T_{reg} can directly counteract increased

MT in resistance arteries isolated from hypertensive mice could reveal therapeutical opportunities. In addition, in perspective, this system could be potentially adapted to study the effect on vascular function of immune cells of interest isolated from hypertensive patients. Moreover, we anticipate that, going forward, our resistance artery and immune cell coculture system will help answer these and many other questions posed by researchers in multiple disciplines.

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