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The Requirement of CD8$^+$ T Cells To Initiate and Augment Acute Cardiac Inflammatory Response to High Blood Pressure

Feifei Ma,†,‡ Jin Feng,†,‡ Chao Zhang,†,§ Yulin Li,† Guanning Qi,† Huihua Li,† Yuzhang Wu,§ Yangxin Fu,§ Yang Zhao,† Hairong Chen,† Jie Du,* and Hong Tang†,‡

Macrophage infiltration and activation in myocardium are hallmarks of acute cardiac inflammatory response to high blood pressure. However, the underlying mechanisms remain elusive. In this article, we report that CD8$^+$ T cells are required for cardiac recruitment and activation of macrophages. First, mice with CD8 gene-targeted (CD8 knockout) or CD8$^+$ T cells depleted by Ab showed significantly reduced cardiac inflammatory response to the elevation of blood pressure after angiotensin II (Ang II) infusion, whereas CD8 knockout mice reconstituted with CD8$^+$ T cells restored the sensitivity to Ang II. More importantly, CD8$^+$ T cells were required for macrophage infiltration in myocardium and subsequent activation to express proinflammatory cytokines and chemokines. Furthermore, macrophage activation required direct contact with activated CD8$^+$ T cells, but with TCR dispensable. TCR-independent activation of macrophages was further confirmed in MHC class I–restricted OVA-specific TCR transgenic mice, which showed a CD8$^+$ T cell activation and cardiac proinflammatory response to Ang II similar to that of wild-type mice. Finally, only myocardium-infiltrated, but not peripheral, CD8$^+$ T cells were specifically activated by Ang II, possibly by the cardiac IFN-$\gamma$ that drove IFN-$\gamma$R$^+$ CD8$^+$ T cell infiltration and activation. Thus, this work identified a TCR-independent innate nature of CD8$^+$ T cells that was critical in initiating the sterile immune response to acute elevation of blood pressure. The Journal of Immunology, 2014, 192: 3365–3373.

Persistent high blood pressure load usually leads to interstitial or nonadaptive cardiac fibrosis, which can cause left ventricular hypertrophy and diastolic/systolic dysfunction (1–3). Studies in human (4) and animal models (5, 6) have suggested that deregulated inflammation contributes to cardiac fibrosis, remodeling, and even heart failure. In this process, macrophage accumulation and activation in myocardium is a critical step in initiating such a proinflammatory cascade (7, 8). However, the underlying mechanism of how macrophages are recruited to and activated in the lesion remains elusive. Recently, T cells have been revealed as a critical modulator in the onset of hypertensive perivascular injury (9).

The role of different Th cells may vary, with Th17 cells promoting hypertension and vascular dysfunction (10), and regulatory T cells (CD4$^+$CD25$^+$Foxp3$^+$) ablatting hypertensive vascular injury (11). The presence of CD8$^+$ T cells has been documented in the perivascular adipose tissue of aorta (9), in areas of myocardial infarction (12), and in areas affected by chronic chagasic myocarditis caused by parasite infection (13). However, the specific role of CD8$^+$ T cells in the cardiac inflammatory response to transient high blood pressure load, in particular, has not been fully understood.

In this work, we have demonstrated that CD8$^+$ T cells were sufficient and necessary to determine the cardiac proinflammatory response to angiotensin II (Ang II)–induced acute high blood pressure load. Furthermore, myocardial infiltration and activation of CD8$^+$ T cells depended on IFN-$\gamma$R, and were required for the subsequent recruitment and activation of macrophages upon Ang II infusion. Moreover, CD8$^+$ T cells activated macrophages in a contact-dependent, but TCR-independent, manner. This study thus further suggested an interesting innate cell-like function of CD8$^+$ T cells that essentially involved a proinflammatory response to the myocardial tissue stress imposed by acute elevation of blood pressure.

Materials and Methods

Mice and treatment

B6.129S2–Cd8a$^{tm1Mol}$J [CD8 knockout (KO)], B6.129S7–Ifng$^{tm1Tyt}$J (IFN-$\gamma$ KO), B6.129S7–Ifngr1$^{tm1Asian}$J (IFN-$\gamma$R KO), and B6.129S4–Ccl2$^{tm1Jrb}$J (MCP-1 KO) mice and wild-type (WT) littersmates were purchased from The Jackson Laboratory. MHC class I–restricted OVA-specific TCR transgenic (OT-I) mice were kindly provided by Dr. J. Chen (Massachusetts Institute of Technology, Cambridge, MA). Mice were maintained under specific pathogen–free conditions. For the CD8$^+$ T or $\gamma$6T depletion experiment, mice were injected i.p. with 150 $\mu$g Abs (CD8, TIB210; $\gamma$6T, UC7-13D5) 1 d prior or 3 d after Ang II infusion. For macrophage depletion, mice were injected i.p. with 200 $\mu$l clodronate liposomes 2 d prior and 3 d after Ang II infusion, as described previously.
Blood pressure was increased transiently by s.c. infusion with Ang II (1500 ng/kg/min, Sigma-Aldrich) for 7 d (0.5 μl/h) or 14 d (0.25 μl/h) via osmotic mini-pumps (Alzet 1007D; DURECT), as previously described (15). Control animals received saline infusion only. Mice were euthanized at the indicated time point for experimental analysis. Animal experiments were performed in accordance with institutional and National Institutes of Health guidelines and were approved by the Animal Care and Use committees at Capital Medical University and Institute of Biophysics, Chinese Academy of Sciences.

**Histological and immunohistochemical analyses**

Paraffin-embedded hearts were sectioned at 200-µm intervals from base to apex, and stained with Masson’s trichrome. Ventricles were shown (×40 magnification), and the percentage of fibrotic areas in the entire slice area was calculated with the software provided (Nikon ECLIPSE 80i/90i). Representative fibrotic papillary muscle masses were boxed and shown at ×200 magnitude. Immunohistochemical analyses of fibrotic lesions were performed with an avidin-biotinylated peroxidase complex kit (Zhongshan GoldenBridge). The conditions for each primary Ab were as follows: collagen I (1:800 dilution; Abcam), CD31 and α-smooth muscle actin (both 1:200 dilution; Abcam), IFN-γ, MCP-1, TGF-β, and Mac-2 (1:300 dilution; Santa Cruz Biotechnology). The statistical results were averaged from 20 random view fields of each section (×400 magnitude) under ECLIPSE 80i/90i. For immunofluorescence staining, the primary Abs were Alexa Fluor 568–conjugated donkey anti-rabbit (1:1000; Invitrogen). FITC-conjugated goat anti-Armenian hamster (1:500; Abcam) and Rhodamine-conjugated F4/80 (1:200 dilution; BD Pharmingen), rabbit anti-mouse Mac-2 (1:200 dilution; Abcam), and Armenian hamster anti-mouse MCP-1 (1:400 dilution; BioLegend). The following secondary Abs were used: FITC-conjugated goat anti-Armenian hamster (1:500; Abcam) and Alexa Fluor 568–conjugated donkey anti-rabbit (1:1000; Invitrogen). Images were superimposed using ECLIPSE 80i/90i, as described elsewhere (16). The nuclei were counterstained with DAPI (blue).

**Flow cytometry**

Mouse hearts were digested with collagenase type IX (125 U/ml), collagenase type IS (450 U/ml), and hyaluronidase IS (60 U/ml) in 20 mM HEPES-PBS buffer. Cardiac cell suspensions were counted by 22 particles per cell counter (Beckman Coulter), and then stained with the indicated mAbs or isotype controls (BioLegend). Data were acquired by LSRFortessa (BD) and analyzed with FlowJo software (TreeStar). To profile CD8+ T cell activation status, CD45+CD3+CD8+ T cells were first gated, then followed by quantification of CD44 and CD62L expression (naïve, CD44+CD62L-; memory, CD44+CD62L+; active, CD44+CD62L+). Dead cells were excluded by propidium iodide staining (Invitrogen).

**Cell enrichment and adoptive transfer**

To isolate CD8+ T cells, single-cell suspensions of splenocytes and lymph nodes were subject to CD8+ selection according to the manufacturer’s manuals (EasySep; STEMCELL). The purity was >90%, as confirmed by flow cytometry. For macrophage isolation, mice were injected i.p. with 2 ml 4% thiglycollate broth to elicit peritoneal macrophages. Where needed, cells were cultured in RPMI 1640 (Thermo Scientific) supplemented with 3% FBS and 100 μl/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Life Technology). Adoptive transfer was performed as described previously (17). In brief, 1 d before and 3 d after Ang II infusion, various types of CD8+ T cells or peritoneal macrophages, as indicated, were resuspended in 150 μl PBS, passed through a 70-µm filter, and injected via tail vein into recipient mice. The full scale of immune cell reconstitution was verified by FACS analysis of PBMCs.

**Real-time PCR**

Total RNA was extracted from hearts using TRizol reagent (Invitrogen). Quantitative RT-PCR analyses were performed as previously described (18) in an ABI 7500 (ABI). Primers for MCP-1, INF-γ, TNF-α, and IL-6 were referred to in published works (19, 20). The housekeeping gene b-actin was used as an internal control.

**Coculture of CD8+ T cells and macrophages**

CD8+ T cells (2×106 per well) were mixed with macrophages (2×105 per well) in 96-well plates and cultured for 48 h in the presence of Abs to CD3 (10 ng/ml) and CD28 (5 μg/ml). For Transwell assays, CD8+ T cells (bottom well, 1×106) and macrophages (top insert, 1×105) were separated in a Boyden chamber device (24-well, 3-µm pore size; Corning Costar) and incubated at 37°C for 48 h, as previously described (17). Measurements of the cytokines/chemokines in the indicated media (10 μl) were analyzed by FLEXMAP 3D (Lumex, TX) using the MILLIPLEX Mouse Cytokine/Chemokine kit (Millipore). For macrophage migration assays, peritoneal macrophages (insert, 1×105) and CD3/CD28-activated CD8+ T cells (bottom well, 1×105), as indicated, were separated in a Boyden device (24-well, 5-µm pore size; Corning Costar) and incubated at 37°C for 48 h. Migratory macrophage cells moved through the pores toward the MCP-1 in the bottom wells, were rinsed with PBS, and were
fixed in 4% paraformaldehyde for DAPI staining. The number of cells on the membrane facing the bottom chamber was blind counted (20 view fields of two duplicate membranes) by two students and averaged from three independent experiments.

**Statistical analysis**

Data were expressed as the mean ± SEM. Differences between groups were assessed for statistical significance using the Student *t* test. A *p* value <0.05 denoted the statistically significant difference.

**Results**

**CD8⁺ T cells mediated the myocardial inflammatory response to hypertension**

Ang II infusion in mice raises blood pressure and initiates a spectrum of proinflammatory signaling in the heart (21). Application of a relatively higher dose of Ang II infusion (1500 ng/kg/min, 0.25 μl/h) effectively elevated blood pressure to 160 mm Hg (Fig. 1A) by the third day postinfusion (dpi) in C57BL/6 mice, accompanied by fibrogenesis in the left ventricle (Supplemental Fig. 1A). Cell counting of leukocytes in hearts showed cardiac infiltration of CD45⁺ leukocytes (see Supplemental Fig. 1B for Fig. 1A). Cell counting of leukocytes in hearts showed cardiac infiltration of CD45⁺ leukocytes (see Supplemental Fig. 1B for gating strategy) immediately after Ang II infusion, with the peak at 2 dpi and gradually receding by 7 dpi (Fig. 1B). Being dominated by macrophages (CD11b⁺F4/80⁺) and neutrophils (CD11b⁻F4/80⁻), however, infiltrated leukocytes consisted of <1000 per heart of CD4⁺ and CD8⁺ T cells that concomitantly migrated to myocardium (Fig. 1B, Supplemental Fig. 1C). The rest were CD45⁺CD3⁻CD11b⁻ leukocytes (Supplemental Fig. 1C). Because the role of CD4⁺ T cells in modulating hypertensive perivascular injury has been extensively studied by others (9–11), we decided to focus on the potential involvement of CD8⁺ T cells in the onset of proinflammatory response to the transient elevation of blood pressure. To avoid potential contamination from preparation of myocardial cells for cell counting, we further confirmed the influx of CD8⁺ T cells in the left ventricular area in response to Ang II (7 dpi) by immunohistochemistry (Fig. 1C) or by fluorescence microscopy (Fig. 1D), which depicted infiltration of adoptively transferred CD8⁺ T cells that had been isolated from GFP transgenic mice. More strikingly, phenotypic analysis showed that only cardiac infiltrated CD8⁺ T cells, but not peripheral or splenic CD8⁺ T cells, were preferentially activated (CD44⁺CD62L⁻) by Ang II (Fig. 1E), suggesting the involvement of the myocardial milieu in activation of CD8⁺ T cells (see also below). Therefore, these results suggested a potential role for activated CD8⁺ T cells in responding to and regulating transiently hypertensive inflammation that precedes cardiac injury and remodeling.

To verify this idea, we infused CD8 KO mice with Ang II (1500 ng/kg/min, 0.5 μl/h) for 7 d. Cardiac hypertrophic response to elevated blood pressure, systolic blood pressure (Supplemental Fig. 2A), hypertrophic growth of cardiomyocytes (Supplemental Fig. 2B), or left ventricular hypertrophy (Supplemental Fig. 2C) was indistinguishable in WT and CD8 KO mice. CD8⁺ cells are therefore not involved in modulation of cardiac hypertrophy. Strikingly, CD8 KO mice showed a much reduced fibrotic area, compared with WT, in the left ventricle (Fig. 2A, 7 dpi), which was associated with decreased proinflammatory and profibrotic cytokines in the lesion (Fig. 2B). To confirm the specific involvement of CD8⁺ T cells, WT mice were depleted of CD8⁺ T cells by Ab (see Supplemental Fig. 3A for depletion efficiency), which showed a reduction of cardiac profibrogenic inflammation similar to that in CD8 KO mice (Figs. 2C, 2D). In contrast, adoptive transfer of naive CD8⁺ T cells to CD8 KO mice (see Supplemental Fig. 4B for reconstitution efficiency) restored the profibrotic response to Ang II in interstitial myocardium (Fig. 2A, Supplemental Fig. 3B). Therefore, CD8⁺ T cells function as a profibrotic mediator that determines the cardiac inflammatory response to transient elevation of blood pressure. As expected,
mice lacking these proinflammatory genes, such as IL-6 KO (22), MCP-1 KO (Fig. 3C), or IFN-γ KO (see below), showed a resistance to Ang II–induced inflammatory response similar to that in CD8 KO mice. Of note, only adaptive T cells (αβ TCR+) were specifically involved in modulating the inflammatory response to Ang II, because mice depleted of innate T cells (γδ TCR+) by Ab failed to show alleviation of the inflammatory response to Ang II (Supplemental Fig. 3C). In all, these results demonstrated that conventional CD8+ T cells specifically mediated myocardial inflammatory response to acute elevation of high blood pressure.

**Fibrogenic macrophage infiltration and activation relied on CD8+ T cells**

Macrophage accumulation and activation are associated with hypertensive cardiac fibrosis (8) and perivascular hypertrophy (7). This finding was also true during the acute elevation of blood pressure after Ang II infusion (1500 ng/kg/min, 0.5 ml/h, 7 d), in which depletion of macrophages by clodronate liposomes (see also Supplemental Fig. 3D for depletion efficacy in heart) significantly reduced the fibrotic area in the left ventricle (Fig. 3A) and levels of inflammatory cytokines (Fig. 3B). Moreover, MCP-1 (20, 23), presumably produced by activated macrophages (24), is required for macrophage infiltration in fibrotic cardiomyopathy in Ang II–induced hypertension models. Indeed, gene targeting of MCP-1 reduced the myocardial fibrogenic response to Ang II (Fig. 3C), which was associated with reduced macrophage infiltration (Fig. 3D). Macrophages, albeit not the only cell source in the heart, might be the major MCP-1–producing cells at the onset of the cardiac inflammatory response to Ang II. This idea was indicated by drastic reduction of MCP-1 after mice were depleted of macrophages (Fig. 3B). It was further confirmed by adoptive transfer of macrophages to MCP-1 KO mice, which restored the fibrogenic effect of Ang II (Fig. 3C), associated with MCP-1 reinstatement in the left ventricle (Fig. 3D). Therefore, macrophage infiltration and activation did not require MCP-1, but were required for cardiac inflammation (Fig. 3A) and MCP-1 production (Fig. 3B, 3D), even though endothelial cells may also produce MCP-1 in myocardium (20).

The independence of MCP-1 for macrophage infiltration and activation in response to Ang II (Fig. 3D) prompted us to investigate whether CD8+ T cells are involved. Immunohistochemical analysis showed that macrophage infiltration (Mac-2 staining) accompanied activation (MCP-1 staining) in the lesion, which peaked around 4 dpi (Fig. 3A). Strikingly, cardiac enrichment (Fig. 4B) and activation (Fig. 4C) of macrophages upon Ang II infusion were nearly annihilated in the CD8 KO heart (residual fibrogenic response).

**FIGURE 3.** Deleterious roles of macrophage-derived MCP-1 in hypertensive cardiac fibrosis. WT, clodronate liposome–treated (∆Mφ) mice (A, B), or MCP-1 KO, reconstituted MCP-1 KO (MCP-1 KO + MφWT) mice (C, D) (n = 6 for each group) were infused with Ang II for 7 d. (A) Cardiac fibrosis was assessed as in Fig. 2A. Scale bars, 500 μm (top) and 100 μm (bottom). (B) Fold changes of mRNA expression of MCP-1, IL-6, TNF-α, IFN-γ and TGF-β, as indicated, were analyzed by real-time PCR. (C) Cardiac fibrosis level of WT, MCP-1 KO, or reconstituted MCP-1 KO mice was assessed as in Fig. 2A. Scale bars, 500 μm (top) and 100 μm (bottom). (D) Immunohistochemical staining of Mac-2 and MCP-1 expression in the ventricular sections. Scale bars, 50 μm. Error bars represent mean ± SEM. * p < 0.05, ** p < 0.01; a t test was used.
macrophage infiltration/activation in hearts may contribute to the systematic activation effect of Ang II on macrophages and were fully restored if CD8 KO mice had undergone adoptive transfer with CD8+ T cells (Fig. 4B, 4C). Therefore, CD8+ T cells were both necessary and sufficient for macrophage recruitment and activation in the cardiac inflammatory response to Ang II. Effector T cells can activate mononuclear phagocytes via cell–cell contact or long-distance action through cytokines (26). Macrophages not only comigrated with CD8+ T cells to the heart (Fig. 1B) in a CD8+ T cell–dependent manner (Fig. 4B) but were also in physical proximity to CD8+ T cells in the Ang II–infused heart, as revealed by immunohistochemical staining (Fig. 4D). CD8+ T cells activated macrophages to produce MCP-1 (Fig. 4E) when cocultured in vitro. Moreover, CD8+ T cells might activate macrophages via direct contact, because proinflammatory cytokine and chemokine production by macrophages was significantly reduced if CD8+ T cells were separated from macrophages in Boyden chambers (Fig. 4F). The dependence of macrophage MCP-1 on CD8+ T cells was further confirmed by immunofluorescence colocalization analysis in vivo (Supplemental Fig. 3E). Finally, macrophages were chemotactically attracted with a similar efficiency by recombinant MCP-1 and by MCP-1 produced by autologous macrophages in the presence of CD8+ T cells (Fig. 4G). Macrophage migration was, however, nondirectional because it occurred irrespective of whether CD8+ T cells (the source of MCP-1, for example) were present in the upper or bottom chamber. Therefore, macrophage recruitment and activation in myocardium relied on cell–cell contact with activated CD8+ T cells upon Ang II infusion.

CD8+ T cells activated macrophages in a TCR-independent manner

We and others have previously observed that T cells inhibit innate inflammatory responses by direct contact with macrophages in a TCR-independent manner (20, 25). It is therefore of interest to assess whether TCR would involve CD8+ T cell activation of macrophages at the onset of hypertensive cardiac inflammation. CD8+ T cells of OT-I mice express a surrogate TCR transgene specific for OVA (TCROVA). Intriguingly, OT-I mice showed a degree of cardiac fibrosis similar to that in WT mice in response to Ang II (Fig. 5A), suggesting that Ag-specific TCR was dispensable for profibrotic CD8+ T cells in modulating the cardiac inflammatory response. CD8+ T cells in either OT-I or WT mice were indeed activated similarly (CD44+CD62Llow) by Ang II infusion.
fusion (Fig. 5B). Activated CD8+ T cells all carried OVA-specific TCR, as indicated by uniform staining with transgene-specific Vα2 Ab before and after Ang II infusion (Supplemental Fig. 4A). This feature would exclude the possibility that the residual WT TCR and/or its editing (26), if any, would contribute to the proinflammatory response of CD8+ T cells to Ang II in OT-I mice. Measurement of the cytokine profile in the coculture experiments (Fig. 5C) further demonstrated that CD8+ T cells isolated from OT-I mice (CD8OT-I) activated macrophages in a manner indistinguishable from that of WT CD8+ T cells (CD8WT). These results suggested that CD8+ T cells activated macrophages via a cell contact–dependent, but TCR-independent, mode in mediating the sterile immune response to hypertensive cardiac inflammation. Of note, TCR-independent activation of macrophages by CD8+ T cells in hypertensive myocardium was in sharp contrast to that in MCP-1–mediated lung injury, in which Ag-specific cytolytic CD8+ T cells are required to activate macrophages (27, 28).

Considering the importance and hierarchy of CD8+ T cells in the activation of macrophages, it is therefore critical to determine how CD8+ T cells were recruited to and activated in the heart. Intriguingly, IFN-γ production upon Ang II infusion required CD8+ T cells (Fig. 2B), and IFN-γ KO mice showed a much reduced cardiac inflammatory response to Ang II (Fig. 6A, 6B), similarly to CD8 KO mice (Fig. 2A). This finding would suggest a functional link between IFN-γ and CD8+ T cell activation in the Ang II–infused heart. Strikingly, both number of infiltrated CD8+ T cells and percentage of the activated CD8+ T cells therein were significantly reduced in the IFN-γ KO or IFN-γR KO heart after Ang II infusion (Fig. 6C). Therefore, the IFN-γ/IFN-γR axis played a critical role in cardiac recruitment and activation of CD8+ T cells. Because CD8+ T cells can produce IFN-γ per se, whether CD8+ T cells were activated by autologous or heterologous IFN-γ should be determined. To address this issue, we reconstituted CD8 KO mice with CD8+ T cells (see Supplemental Fig. 4C for efficiency) isolated from WT (CD8WT), IFN-γ KO (CD8IFN-γ), or IFN-γR KO mice (CD8IFN-γR). Flow cytometric analysis showed that, compared with adoptive transfer of CD8WT, there were many fewer CD8+ T cells and a lower frequency of activated CD8+ T cells in the CD8IFN-γR, but not CD8IFN-γ, reconstituted heart upon Ang II infusion (Fig. 6D). Consequently, such a block of CD8+ T cell infiltration and activation in the CD8IFN-γR heart showed significant reduction in proinflammatory cytokines and chemokines, as measured by quantitative RT-PCR (Fig. 6E). Therefore, IFN-γ produced by myocardium cells, probably vascular endothelial or fibroblast cells, would signal IFN-γR–bearing CD8+ T cells to infiltrate and be activated (Supplemental Fig. 4D). Finally, activation of macrophages did not require IFN-γ by CD8+ T cells or IFN-γR in CD8+ T cells, because in vitro coculture experiments showed that both CD8IFN-γR and CD8IFN-γ+ T cells activated macrophages with similar efficiency (Fig. 6F). These results suggested that Ang II inflamed myocardial cells to secrete IFN-γ, which then recruited and activated IFN-γR+ CD8+ T cells in the establishment of the cardiac inflammatory cascade. In conclusion, CD8+ T and innate cells in the left ventricle appeared to form a sterile immune circuit that played a critical role in

**FIGURE 5.** TCR was not required for a CD8+ T cell–mediated inflammatory response to Ang II. (A) The WT or OT-I mice (*n* = 6 per group) were infused with Ang II, and cardiac fibrosis was assessed as in Fig. 2. Scale bars, 500 μm (top) and 100 μm (bottom). (B) CD8+ T cells (CD45+CD3+CD8+) isolated from Ang II–infused WT or OT-I hearts (*n* = 6 per group) were assessed by FACS for different phenotypes: naive, CD44+CD62L−; memory, CD44+CD62L−; activated, CD44+CD62L−. (C) Peritoneal macrophages (2 × 10^5^) were cocultured with splenic CD8+ T cells (2 × 10^5^) isolated from WT or OT-I mice (CD8WT or CD8OVA, respectively) for 48 h. Proinflammatory lymphokines MCP-1, IFN-γ, IL-6, TNF-α in the medium were measured by Luminex assays. Data represented the average of three independent experiments. Error bars represent mean ± SEM. *p < 0.05, **p < 0.001; a t test was used.
processing high blood pressure–induced cardiac inflammation and immunopathological alteration leading to ventricular remodeling.

**Discussion**

Ang II triggers a broad spectrum of proinflammatory signaling in hypertension (21), with both innate and adaptive immunity essentially involved (29–32). Most work on this topic reveals a preferential involvement of various CD4+ Th cells and/or CD8+ CD8+ cells (most likely NK, NKT, or γδT cells) in the regulation of vascular dysfunction, which is associated with Th1 cytokine polarization in circulation (33, 34), as well as an imbalance of deteriorative Th17 cells (10) over protective regulatory T cells (11), in the chronic progression of hypertension.Little has been addressed on how CD8+ T cells, in particular, modulate the immunopathological response to acute blood pressure overload in myocardium. The work that has been done has indicated that CD8+ T cell infiltration of the heart is both necessary and sufficient to initiate a proinflammatory response to acute elevation of blood pressure. Ang II–induced acute elevation of blood pressure and resultant cardiac inflammation may be relevant to hypertensive emergencies, in which uncontrolled blood pressures lead to progressive or impending end-organ dysfunction, such as acute left ventricular dysfunction, acute pulmonary edema, myocardial ischemia/infarction, and aortic dissection (35).
The Ang II receptor, AT,R, presents on most lymphocytes and monocytes (36), suggesting that Ang II, besides elevation of blood pressure, may directly activate and mobilize phagocytes and leukocytes for potential cardiac inflammation. Intriguingly, Ang II seems to specifically activate myocardium-infiltrated CD8+ T cells, but not CD8- T cells in the peripheral blood or spleen (Fig. 1E). This finding suggests a tissue-specific role for the CD8+ T cell/macrophage axis in the inflammatory response to Ang II. Furthermore, our previous results indicate that lowering of blood pressure by administration of hydralazine in mice can inhibit cardiac inflammation and fibrosis even in the presence of Ang II (37). This observation further suggests that hypertensive stress, rather than direct activation of leukocytes by Ang II, could contribute to cardiac inflammation. We therefore postulated that the proximal signaling circuit of IFN-γ from inflamed cardiomyocytes/endothelial cells onto IFN-γR-deficient CD8+ T cells may account for the onset of the cardiac inflammatory response to Ang II. IFN-γR-deficient mice show reduced cardiac damage by Ang II infusion, compared with that in WT mice (38).

Our previous work suggests that INF-γ production by T cells activates macrophages, which accounts for the cardiac fibrotic response to Ang II (39). In contrast, this current work shows that IFN-γ made by vascular endothelial cells and fibroblasts (Supplemental Fig. 4D) may be responsible for recruitment and activation of CD8+ T cells. More convincingly, IFN-γ production by CD8+ T cells is not involved in macrophage activation, because adoptive transfer of CD8+ T cells lacking IFN-γ can still render CD8 KO mice sensitive to Ang II–induced cardiac inflammation (Fig. 6D) and CD8+ T cells lacking IFN-γ can still activate macrophages in vitro (Fig. 6F). Therefore, albeit CD8+ T cells can produce IFN-γ, it is dispensable for macrophage recruitment and activation. Rather, direct contact between CD8+ T cells and macrophages is critical for macrophage activation and cardiac inflammation. Our work, therefore, clarifies the ambiguity of our previous report and identifies a new sterile immune signaling circuit critically involved in cardiac inflammation. The importance of IFN-γ signaling for migration, proliferation, and activation of recipient CD8+ T cells has been well documented in acute viral infection (40) and development of gastrointestinal graft-versus-host disease (41, 42). Further studies, of course, are needed to pinpoint the mechanism of how blood pressure overload triggers IFN-γ secretion in the heart.

We have previously shown that T cells are both necessary and sufficient to temper the TLR-mediated inflammatory response to pathogens, in a TCR-independent manner (17). TCR independence thus highlights that T cells possess evolutionarily conserved characteristics of innate immune cells, if the time frame of response and Ag nonspecificity are considered (43, 44). The finding in this study of TCR-independent activation of macrophages by CD8+ T cells therefore reveals yet another novel innate function of T cells: in addition to suppressing the innate inflammatory response to infections, CD8+ T cells are required to initiate and augment the innate inflammatory response to danger signals. It has become clearer that the conventional boundary between innate and adaptive immunity becomes blurred (26, 44, 45). Of note, such TCR-independent activation of macrophages by CD8+ T cells in hypertensive cardiac inflammation is different from the inflammatory cascade in the fat tissue of obesity. CD8+ T cell infiltration and concomitant decrease of CD4+ T cells in epidermides adipose tissue are prerequisites for recruitment and activation of macrophages early on in the development of obesity (46). Furthermore, in those cases, either macrophage infiltration does not require cell–cell contact with CD8+ T cells (46) or Ag-reactive TCR seems to be required for CD4+ T cells to control obesity-associated glucose homeostasis (47). In other words, sterile innate immunity in both myocardial and adipose tissues requires CD8+ T cells, but the requirement for TCR differs; thus, involvement of the innate facet of CD8+ T cells diverges.

In conclusion, our results have elucidated a novel mechanism whereby innate cell-like CD8+ T cells play a pivotal role in profibrotic cardiac inflammation. Infiltrated CD8+ T cells in response to stromal IFN-γ signal initiate and propagate the recruitment and activation of macrophages in an IFN-γR-dependent, but TCR-independent, manner. The orchestrated infiltration and activation of CD8+ T cells and macrophages in vascular fibrotic lesions might reveal a new immunopathological circuit that contributes to hypertensive cardiac fibrogenesis.

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Disclosures

The authors have no financial conflicts of interest.

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Fig. S1 FACS analysis of cardiac leukocytes.
(a) Mice were treated as in fig. 1a. Fibrosis of the left ventricular sections were analyzed at days 4 and 14 post Ang II infusion by Masson’s trichrome staining as in Fig. 2a. Scale bars: 500 μm (top) and 100 μm (bottom) (b) Diagram of gating strategy. (c) Direct comparison of cardiac leukocyte infiltration in Wt mice at day 0, 2, 4, 7 post Ang II infusion.
Fig. S2 Model of Ang II induced cardiac inflammation and injury.
(a) Wt and CD8 KO mice (n=6 for each group) were treated as in fig. 2a, systolic blood pressure (SBP) were measured as in fig. 1a. (b) WGA staining was analyzed for cardiac hypertrophy at day 7 after angiotensin II infusion. scale bar:50μm. Data represent the mean ± SEM. Student t test. ***P<0.001 vs. wt saline group; ###P<0.01 vs. cd8 ko saline group, (c) Echocardiography was performed at day 7 after angiotensin II infusion. LV mass was evaluated after angiotensin II infusion at day 7. Data represent the mean ± SEM. Student t test. **P<0.01 vs. wt saline group; ##P<0.01 vs. cd8 ko saline group.
**Fig. S3** CD8+ T cells were required for cardiac inflammatory response to hypertension.  
**(a)** Mice were treated as in fig. 2c, and then FACS stain of periphery blood lymphocytes was performed to verify depletion efficiency.  
**(b)** Mice were treated as in fig. 2a, Expression of pro-fibrotic factors were depicted by immunohistochemical staining for collagen-I, TGF-β, α-SMA and positive areas were plotted from 20 independent sections for each group. Scale bars: 50 μm.  
**(c)** γδT cells were depleted by i.p injection of 150 ug UC7 antibody or control IgG 1 day prior and 3 days after infusion. Mice were then infused with Ang II for 7 days and left ventricular sections were stained with Masson’s trichrome. Scale bars: 500 μm (top) and 100 μm (bottom).  
**(d)** Immunofluorescent stain of Mac-2 (red) in the ventricular sections of mice as treated in fig. 3a. The nuclei were counterstained with DAPI (blue). Scale bars: 50 μm.  
**(e)** MCP-1 induction depends on CD8 T cells. Immunofluorescent stain of MCP-1 (green) and Mac-2 (red) in the ventricular sections of mice as indicated (n=3 per group). The nuclei were counterstained with DAPI (blue). Scale bars: 50 μm.
Fig. S4 Reconstitution of CD8 KO mice by adoptive transfer of purified CD8+ T cells. (a) OT-I mice were treated as in fig. 5b, and then Vα-2 expression on CD8+ T was analyzed by FACS stain. (b) Mice were treated as in fig. 2a, and FACS stain of periphery blood lymphocytes was performed to verify transfer efficiency. (c) Mice were treated as in fig. 6d, and FACS stain of periphery blood lymphocytes was performed to verify transfer efficiency. (d) Production of IFN-γ in endothelial and fibroblast after Ang II infusion. Immunofluorescent co-localization of endothelial (anti-CD31, red) or fibroblast (anti-α-SMA, red) with IFN-γ (green) in the ventricular sections (n=6 per group) during Ang II infusion. The nuclei were counterstained with DAPI (blue). Arrows indicate co-localization. Scale bars: 50 μm. Error bars represented mean±SEM. NS, no statistical significance. t test.