JCI The Journal of Clinical Investigation

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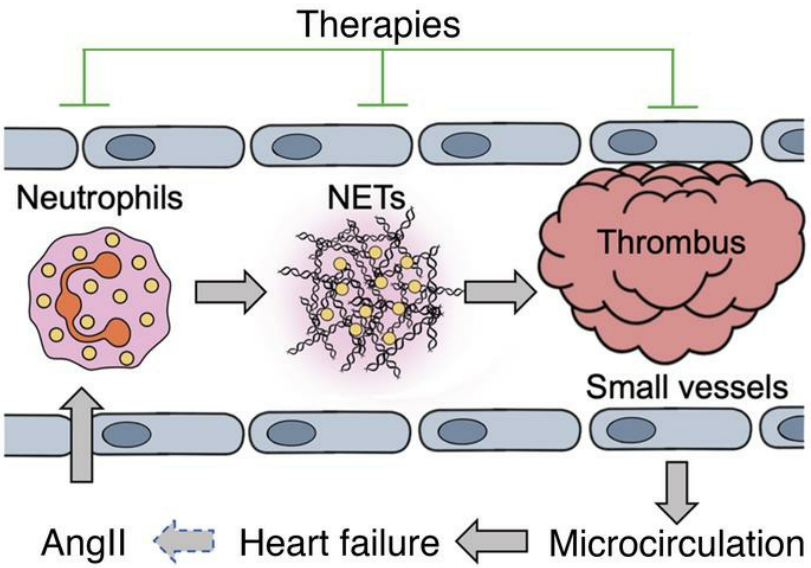
J Clin Invest. 2021. https://doi.org/10.1172/JCI147191.

Research In-Press Preview Cardiology Inflammation

Graphical abstract



KLF2 regulates neutrophil activation and thrombosis in cardiac hypertrophy and heart failure progression

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Abstract

It is widely recognized that inflammation plays a critical role in cardiac hypertrophy and heart failure. However, clinical trials targeting cytokines have shown equivocal effects indicating the need for a deeper understanding of the precise role of inflammation and inflammatory cells in heart failure.

Leukocytes from human subjects and a rodent model of heart failure were characterized by a marked reduction in expression of KLF2 mRNA. Using a mouse model of Angiotensin II-induced non-ischemic cardiac dysfunction, we showed that neutrophils played an essential role in the pathogenesis and progression of heart failure. Mechanistically, chronic Angiotensin II infusion activated a neutrophil KLF2-NETosis pathway that triggered sporadic thrombosis in small myocardial vessels leading to myocardial hypoxia, cell death, and hypertrophy. Conversely, targeting neutrophils, NETs or thrombosis ameliorated these pathological changes and preserved cardiac dysfunction. KLF2 regulated neutrophil activation in response to Angiotensin II at the molecular level, partly through the crosstalk with HIF1 signaling.

Taken together, our data implicate neutrophil-mediated immunothrombotic dysregulation as a critical pathogenic mechanism leading to cardiac hypertrophy and heart failure. This neutrophil KLF2-NETosis-thrombosis mechanism underlying chronic heart failure can be exploited for therapeutic gain by therapies targeting neutrophils, NETosis, or thrombosis.

Keywords:

Neutrophils, NETosis, Immunothrombosis, Myocardial microcirculation, Cardiac hypertrophy

Introduction

Although the development and progression of cardiac hypertrophy and failure have traditionally been viewed as hemodynamic and neurohormonal disorders, there is increasing awareness that inflammation also plays a critical role (1, 2). More than three centuries ago, physicians observed myocardial inflammation in the diseased heart (1). In the 1950s, C-reactive protein (CRP) was first identified as an inflammatory biomarker for acute myocardial infarction (AMI), and decades later, it was also shown to be associated with cardiac hypertrophy and failure (3-7). More recently, studies have found that pro-inflammatory cytokines, such as TNFα, and members of the interleukin 1 (*IL1*) and 6 (*IL6*) families are elevated in heart failure patients. Further, experimental overexpression of these cytokines to clinically relevant levels drives cardiac hypertrophy and failure in animal models (8, 9). Hence, it was hypothesized that elevated pro-inflammatory cytokines are significant contributors to cardiac hypertrophy and failure (10, 11). However, despite robust preclinical data, placebo-controlled double-blinded clinical trials targeting cytokines in heart failure patients yielded neutral or negative effects on outcomes (12). A plausible explanation for such failures is the lack of temporal and spatial precision to modulate inflammation properly. Such considerations speak to the need for a deeper understanding of how inflammation affects myocardial function.

Neutrophils are the most abundant leukocytes in human blood and are the first responders to infection and injury. During an infection or tissue damage, pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) are sensed by pattern recognition receptors (PPRs) on tissue-resident cells, which produce chemokines such as CXCL1 and CXCL2 to recruit neutrophils (13). In addition, PAMPs and DAMPs may also directly activate neutrophils to induce recruitment and pro-inflammatory activation (14). During sterile tissue injuries, neutrophils participate in the clearance of cellular debris to restore tissue to homeostasis. Neutrophils display a wide range of effector mechanisms such as phagocytosis, cytokine release, matrix protease secretion, and reactive oxygen species (ROS) generation, and the formation of neutrophil extracellular traps (NETs) through a process termed NETosis (15). These neutrophil functions must be tightly regulated as a dysregulated neutrophil activity contributes to persistent inflammation leading to tissue damage. Regarding cardiac diseases, it has been reported that neutrophils participate in AMI by contributing to thrombosis, removing debris, and eventually orchestrating wound healing (16). However, the role of neutrophils in non-ischemic heart diseases and chronic heart failure (HF) remains very poorly understood.

Krüppel-like factors (KLFs) are a family of zinc-finger transcription factors conserved from nematodes to humans. They are essential for a broad spectrum of biological processes, including cell stemness, differentiation, proliferation, malignancy, metabolism, and immunity (17-20). Over the past two decades, studies from our group and others have identified KLF2 as a potent repressor of myeloid pro-inflammatory activation (21, 22). In homeostatic conditions, KLF2 inhibits NFκB and HIF1 signaling in myeloid cells, thereby maintaining cellular quiescence. Upon pro-inflammatory stimulation, myeloid KLF2 expression is rapidly diminished to release repression and facilitate myeloid activation. Macrophages and neutrophils that are deficient in KLF2 exhibit more robust pro-inflammatory activation, rendering hosts resistant to infection but sensitive to systemic inflammation (21). In the current study, we provide multiple lines of evidence to demonstrate that neutrophils are requisite for cardiac pathogenesis in response to chronic non-ischemic stress. Further, we use chronic Angiotensin II (AngII) infusion as a classic non-ischemic cardiac disease model (23) to show a critical role of neutrophil KLF2 in regulating the development and progression of AngII-induced cardiac hypertrophy.

Results

Heart failure is associated with reduced KLF2 expression in circulating neutrophils.

Inflammation is thought to play a critical role in cardiac hypertrophy and failure, but the underlying mechanisms remain elusive (12). Here we aimed to investigate how immune cells shape cardiac adaptations to pathological stress. Given that KLF2 is a tonic repressor of inflammation, we assessed *Klf2* mRNA levels by quantitative RT-PCR (qPCR) in peripheral blood leukocytes harvested from a cohort of HF patients and non-HF controls (Supplemental Table 1). In addition, because the majority (50-70%) of human circulating leukocytes are neutrophils, we further determined *Klf2* mRNA expression in neutrophils. As shown in Figure 1A, HF is associated with a significant reduction in *Klf2* mRNA levels in both leukocytes and neutrophils. Similar results were observed in mice with 4-week AngII infusion, a classic neurohormonal non-ischemic HF model (Figure 1B). However, due to the lack of a high-quality KLF2 antibody, the assessment of KLF2 protein has been a challenge. Therefore, we generated a KLF2-tag knock-in mouse by inserting a 3xFLAG tag immediately after the ATG codon at the *Klf2* locus using the CRISPR-Cas9 gene-editing technology (Figure 1C). This KLF2-tag mouse line allows robust detection of KLF2 protein by commercial high-affinity antibodies to FLAG epitope, and we confirmed AngII-mediated reduction in KLF2 protein in blood neutrophils in vivo (Figure 1C). These results suggest an implication of neutrophil KLF2 in the pathogenesis of cardiac hypertrophy and failure.

Next, we purified bone marrow-derived neutrophils from C57BL6 WT mice and treated them with AngII in vitro. AngII treatment not only rapidly (within 30 min) reduced *Klf2* expression (Figure 1D) but also induced the expression of pro-inflammatory genes such as *Tnfa*, *Il6*, *Il1b,* and *Ccl2*, indicating AngII-induced pro-inflammatory activation of neutrophils (Figure 1E, Supplemental Figure 1A). Similar reductions in *Klf2* expression were observed in macrophages, another significant myeloid cell type, but with a much slower (after 24 hours) induction of pro-inflammatory genes (Supplemental Figure 1B). These cell-based studies suggest that AngII regulates KLF2 expression through cell-autonomous signaling pathways and that AngII can induce pro-inflammatory activation of myeloid cells. The AngII-mediated direct and rapid signaling in neutrophils further suggests a potential pathogenic role of myeloid KLF2 in cardiac hypertrophy and failure.

Myeloid KLF2 deficiency enhances AngII-induced cardiac hypertrophy.

To address whether KLF2 can affect HF by mediating myeloid cell quiescence, we employed a mouse model with myeloid-specific deletion of *Klf2* via Lyz2-Cre-mediated Cre-lox gene recombination. Previous studies from our group and others have shown the Lyz2-KLF2-KO mice (designated K2KO) exhibit enhanced pro-inflammatory activation of macrophages and neutrophils compared to the Lyz2-Cre controls (designated Cre) (21, 22).

To assess the effects on cardiac hypertrophy and failure, we used the well-established AngII infusion model. In this model, animals develop cardiac hypertrophy and fibrosis at a regular AngII dose of 1.0 μg/kg/min. A higher amount of AngII infusion (2.0 μg/kg/kg/min) can result in cardiac hypertrophy, failure, and death (24, 25). As the first step, we assessed myeloid cell numbers in the heart before and after AngII infusion. Hearts from vehicle-infused (Sham: PBS as the vehicle) or AngII-infused (AngII: 1.0 μg/kg/min in PBS) animals were analyzed by flow cytometry. AngII induced significant accumulation of Ly6G+ neutrophils in K2KO myocardium, which increased significantly by 1-week post-AngII infusion and returned to baseline after four weeks (Figure 2A). A similar accumulation of cardiac macrophages (CD45+CD11b+F4/80+Ly6G-) was observed in both Cre and K2KO groups, without significant difference between the two genotypes. This AngII-induced myeloid response in the heart was more robust at a higher dose of AngII (2.0 μg/kg/min), evidenced by significant accumulation of macrophages and neutrophils in Cre and K2KO hearts with higher neutrophil numbers in the K2KO group (Supplemental Figure 2, A&B). However, compared to the <10% post-surgery mortality with 1.0 μg/kg/min AngII infusion, AngII at 2.0 μg/kg/min resulted in ~50% mortality rate in Cre mice and ~80% mortality rate in

K2KO mice within two weeks (Supplemental Figure 2C). Therefore, we chose the regular dose of 1.0 μg/kg/min for most *in vivo* studies.

When subjected to the AngII infusion model for four weeks, the K2KO mice developed significantly worse cardiac dysfunction than Cre controls. As revealed by echocardiographic assessments (Figure 2B), Cre and K2KO demonstrated normal cardiac function at baseline. However, after AngII infusion, the K2KO mice exhibited significantly reduced left ventricular ejection fraction (LVEF), increased LV end-diastolic volume (LVEDV), and reduced systolic contraction (increased LV end-systolic volume, LVESV). Of note, this was not due to differences in blood pressure response following AngII infusion (Figure 2C). In addition, plasma levels of cardiac troponin (cTnT) were significantly increased in K2KO mice 1-week post-AngII, indicating myocardial injury at the disease onset (Figure 2D). Consistently, K2KO hearts showed increased transcription of cardiac hypertrophy genes (e.g., *NPPA*) and inflammation genes (e.g., *IL6*) (Figure 2E). At the histological level, we observed significant cardiac hypertrophy (enlarged cardiomyocyte cross-sectional area) and fibrosis (collagen-positive area) in the K2KO myocardium (Figure 2, F&G). These AngII-induced pathological changes in the Cre group were less severe than that of the K2KO. Cre mice after 4-week AngII infusion exhibited preserved LVEF indicating functional cardiac compensation but increased LVEDV, cardiac hypertrophy, and inflammation compared to the Sham group (Figure 2, B-G).

Collectively, these data demonstrated that myeloid KLF2 is critical in protecting against AngII-induced cardiac hypertrophy. In response to chronic AngII infusion, KLF2 deficiency likely enhances the pro-inflammatory activation of myeloid cells, leading to more severe myocardial injury and enhanced cardiac susceptibility to hypertrophy.

KLF2-deficient neutrophils are critical for AngII-induced cardiac hypertrophy.

Since Lyz2-Cre is operative in both macrophages and neutrophils, we asked which myeloid cell type plays a dominant role in regulating AngII-induced cardiac responses. To address the role of macrophages, we used a Cx3cr1-Cre line to silence *Klf2* exclusively in monocytes and macrophages, thereby maintaining neutrophil KLF2 expression (26). In sharp contrast to the Lyz2-K2KO mice with KLF2 deficiency in both macrophages and neutrophils, the Cx3cr1-Cre-driven KLF2-KO mice (designated Cx3cr1-K2KO) did not manifest severe cardiac hypertrophy in response to 4-week AngII infusion (Supplemental Figure 3). Furthermore, cardiac function was preserved in both Cx3cr1-Cre and Cx3cr1-K2KO groups, while the cardiac hypertrophy was even less in the Cx3cr1-K2KO group, suggesting the KLF2-deficient macrophages are not the primary

driver of AngII-induced cardiac dysfunction. Conversely, these data suggest a dominant role of KLF2-deficient neutrophils in HF pathogenesis.

To explore our hypothesis that neutrophils are the major contributors to cardiac phenotype, we treated K2KO mice with anti-Ly6G antibody (clone 1A8) to deplete neutrophils and subsequently subjected them to AngII infusion. Mice receiving normal rat IgG were used as the non-depletion control. Strikingly, neutrophil depletion protected K2KO mice from AngII-induced LV dysfunction (Figure 3A). Furthermore, consistent with preserved cardiac function, neutrophil depletion also reduced myocardial pathologies, including cardiac hypertrophy, fibrosis, and inflammation (Figure 3, B-D). As expected, anti-Ly6G administration essentially eliminated neutrophil infiltration in the myocardium but did not affect cardiac macrophages (Figure 3E), which corroborates the results from the Cx3cr1-K2KO study (Supplemental Figure 3). Collectively, these data demonstrate a critical role of KLF2-deficient neutrophils, rather than macrophages, in the regulation of AngII-induced cardiac hypertrophy.

Neutrophil extracellular traps (NETs) are a key mediator of cardiac responses to AngII.

Given the requisite role of neutrophils in AngII-induced cardiac hypertrophy, we sought to understand the underlying pathogenic mechanisms. One well-described mechanism of neutrophil activity is the formation of neutrophil extracellular traps (NETs), sticky web-like structures made of decondensed genomic DNA and decorated with histones and granular proteins such as neutrophil elastase (NE) and myeloperoxidase (MPO). Activated neutrophils release NETs to trap and kill pathogens but can also exacerbate sterile inflammation (15).

As we have shown in Figure 1D, AngII can promote the pro-inflammatory activation of neutrophils. To investigate the NETs formation specifically, we performed fluorescent immunostaining for citrullinated histone H3 (H3Cit), a NET-specific biomarker (27). We observed that 1-week AngII infusion resulted in significantly more H3Cit-positive NETs in the K2KO myocardium than the Cre, with little NETs in untreated hearts from either genotype (Figure 4A, Supplemental Figure 4A). We further confirmed that AngII induced NETs formation in vitro with isolated bone marrow neutrophils (Supplemental Figure 4B). Consistent with their pro-inflammatory nature, K2KO neutrophils formed more NETs than Cre cells. The data from these *in vitro* and *in vivo* studies demonstrate that AngII induces NETs formation and KLF2 deficiency facilitates this process. Moreover, the intracardiac NETs were very few after a 4-week AngII infusion (data not shown), indicating a potential pathogenic role of NETs at the disease onset.

To validate the pathogenic role of NETs *in vivo*, we treated K2KO mice with DNase I (4 mg/kg) during AngII infusion to clear NETs in vivo (28-31). Strikingly, DNase I administration significantly protected K2KO mice from cardiac dysfunction, hypertrophy, and fibrosis (Figure 4, B & C). Furthermore, at the disease onset, DNase I administration cleared intracardiac NETs and attenuated cardiac cell death as revealed by TUNEL staining (Figure 4D). Subsequently, the myocardial infiltration of neutrophils, but not cardiac macrophages, was also reduced (Supplemental Figure 4C).

In mouse neutrophils, peptidyl arginine deiminase 4 (PAD4) catalyzes the conversion of histone arginine to citrulline in a process termed histone citrullination. This conversion reduces the positive charge on histones, resulting in weakened histone-DNA binding, unwrapping of nucleosomes, and the release of decondensed DNA (32). This process is requisite for NETs formation *in vivo* as PAD4-KO mice cannot form NETs in response to physiological activators (33). Since DNase I may also degrade extracellular DNA other than NETs, we sought to target the NETosis pathway specifically by GSK-484, a small-molecule PAD4 inhibitor. Our in vivo studies demonstrated that GSK-484 had significant cardioprotective effects similar to DNase I (Figure 4, E-G). By four weeks, K2KO mice with GSK-484 treatment were significantly protected from AngII-induced cardiac dysfunction, hypertrophy, and fibrosis (Figure 4, E & F). Furthermore, at the disease onset, GSK-484 treatment eliminated NETs formation and cell death in the K2KO myocardium (Figure 4G). Collectively, these data demonstrate that NETs play a critical role in the pathogenesis of AngII-induced cardiac hypertrophy.

AngII-induced intravascular NET formation causes thrombosis in small vessels and impairs myocardial perfusion.

We next sought to understand how NETs regulate cardiac hypertrophy. We first tested if NETs or other neutrophil-derived factors could exert direct intercellular crosstalk to cardiomyocytes. Neonatal rat ventricular cardiomyocytes (NRVM) were isolated and treated with AngII to induce hypertrophy in vitro. Then, to recapitulate the neutrophil-cardiomyocyte crosstalk, we treated NRVM with neutrophil-conditioned medium (cell-free fraction). As expected, AngII-treatment caused hypertrophy of cardiomyocytes as indicated by the induction of hypertrophic marker gene *Nppb* (BNP) in NRVM. Still, no additional effect was observed from adding neutrophil conditioned medium (Supplemental Figure 5, A&B). Further, similar *in vitro* studies with cardiac fibroblasts did not reveal any detectable effects from neutrophil-conditioned medium to enhance the expression of fibrosis genes (Supplemental Figure 5C). These data suggest that the pro-hypertrophic, pro-

fibrotic results of neutrophils observed in vivo are more complicated than direct paracrine crosstalk and may involve other cells or systems in the myocardium.

The location of a cell in tissues can provide clues to function. Therefore, we carefully mapped the localization of neutrophils and NETs in the myocardium. When we overlaid the immunofluorescence of neutrophil marker Ly6G and endothelial marker CD31, we found that most neutrophils in the myocardium appeared to localize inside small vessels or on large vessel walls, with few in the intramuscular space (Supplemental Figure 6A). A similar localization pattern was observed with H3Cit and CD31, indicating that most NETs were formed inside small vessels (Supplemental Figure 6B). This observation raised the possibility that neutrophils and NETs contribute to thrombosis and resultant tissue ischemia in a process termed immunothrombosis to cause myocardial injury (27, 34). Indeed, NETs promote thrombosis in both acute infection (i.e., sepsis, COVID-19) and chronic inflammation (i.e., deep vein thrombosis) (27, 31, 35). And NETs can regulate coagulation at multiple levels: activation of intrinsic coagulation factors (i.e., factor XII, XI, X), enrichment in extrinsic coagulation initiator tissue factor, proteolytic degradation of tissue factor pathway inhibitor (TFPI), facilitation of platelet adhesion, and interaction with fibronectin and von Willebrand factor (vWF) for thrombus propagation (34, 36, 37). As such, we hypothesized that NETs in small vessels might cause sporadic thrombosis and subsequently impair blood perfusion of the myocardium.

In the AngII-treated myocardium, we found platelet-rich microthrombi (stained by P-selectin) localized inside small vessels (stained by vWF), a localization pattern similar to the NETs (stained by H3Cit) (Figure 5A, Supplemental Figure 6C). Significant microthrombi were observed in the K2KO myocardium, but they were scarce in the Cre group (Figure 5B, Supplemental Figure 6D). As a consequence of thrombosis, such affected regions in the K2KO myocardium would likely have suffered from ischemic stress. Indeed, TUNEL staining revealed significant cell death, mainly involving cardiomyocytes and a small number of vascular cells (Figure 5C). While the death of cardiomyocytes could account for the increased plasma levels of cTnT (Figure 2B), the death of vascular cells suggests NETs may trigger vascular injury secondary to thrombosis or through a direct mechanism such as ROS. In supporting the thrombotic hypothesis, myocardial ischemia was supported by the increased nuclear accumulation of hypoxia-inducible factor 1α (Hif1α) protein (Figure 5D) and induction of *Vegfa* expression (Figure 5E). Surprisingly, despite higher expression of *Vegfa*, the myocardial capillary density was significantly reduced in the AngII-infused K2KO heart compared to Cre (Figure 5F), which could impair blood perfusion and further enhance ischemia. Thus, the reduced capillary density in AngII-infused K2KO hearts could be

due to attenuated angiogenesis and/or enhanced capillary rarefaction secondary to microvascular thrombosis. Our data on myocardial *Vegfa* mRNA levels do not support an angiogenesis defect (Figure 5E). Still, the data on TUNEL-positive vascular cells support capillary rarefaction (Figure 5C, lower panels).

Nevertheless, these data support our hypothesis that AngII induces intravascular NET formation leading to sporadic thrombosis and hypoxia in the myocardium. Consistent with the cardiac phenotypes, AngII-induced pathological changes in thrombosis, ischemia, cell death, and capillary density were most significant in the K2KO group and undetectable in sham groups. In contrast, K2KO animals depleted of neutrophils (by anti-Ly6G) or NETs (by DNase I and GSK-484) were dramatically protected not only from AngII-induced cardiac dysfunction (Figures 3&4) but also from proximal events such as intracardiac NETs, thrombosis, cell death and capillary rarefaction (Supplemental Figure 7).

To determine if myocardial perfusion is indeed affected by the AngII-NETs-immunothrombosis mechanism hypothesized above, we assessed myocardial microcirculation using quantitative contrast echocardiography (Contrast-ECHO, as illustrated in Figure 6A). Briefly, mice were intravenously infused with a microbubble solution (contrast agent) at a constant flow rate to establish a stable concentration of microbubbles in the myocardium. Then a transient high-energy ultrasonic beam was applied at the precise time to destruct the microbubbles in the myocardium (Burst). Subsequently, intratissue microbubbles were replenished at a rate proportional to the blood flow rate of the myocardial microcirculation. Under continuous echocardiographic monitoring, the stable level of microbubbles in the myocardium (baseline, stable phase before the Burst), the “zero” level after destruction (clearance of microbubbles by the Burst), and the recovery to stable level (replenishment after the Burst) can be recorded in real-time by echocardiography (Figure 6B). Time-stamped contrast-ECHO images then can be calculated and fit into a one-phase decay exponential curve to estimate the myocardial blood flow rate (Figure 6C). The time constant (Tau) from the fitting curve was used as an indicator of flow rate. A more significant Tau value indicates a longer recovery time; that is, microbubbles from blood need a longer time period to replenish the myocardium, indicating a slower blood flow rate. As shown in Figure 6D, there was little difference between the Cre and K2KO mice at baseline. However, AngII infusion induced a profound change in myocardial perfusion in the K2KO mice with a Tau value about 2-fold larger than that of the Cre (2.51±0.36 vs. 1.13±0.10), indicating impaired microcirculation in the K2KO myocardium in response to AngII infusion. In addition, such

impairment in myocardial microcirculation was largely restored by DNase I administration (Figure 6E), supporting a causative role of NETs in this process.

The anticoagulant heparin ameliorates Ang-II induced cardiac dysfunction

Next, we investigated if anticoagulant therapies can be cardioprotective in this model. For this purpose, we used heparin, one of the most commonly used anticoagulants in the clinical setting (38). K2KO mice were subjected to AngII infusion (1.0 μg/kg/min) with or without the concurrent infusion of heparin (1.0 U/kg/min), and cardiac function and pathologies were subsequently assessed (Figure 7). By the end of 4-week AngII infusion, heparin treatment preserved cardiac function, reduced cardiac hypertrophy, attenuated myocardial fibrosis, and improved angiogenesis in the myocardium (Figure 7, A & B). Mechanistically, heparin infusion significantly attenuated intracardiac thrombosis, NETs formation, and cell death at the disease onset (Figure 7C).

Collectively, these data demonstrate that in the absence of KLF2 in neutrophils, AngII-induced intravascular NETs formation triggers thrombosis, particularly in small vessels with the narrow lumen (prone to blockade) and low flow rate (prone to clot), leading to reduced or absent perfusion in affected myocardial regions. Although sporadic, NETs-triggered thrombosis may result in localized myocardial ischemia, cell death, and capillary rarefaction, which are known pathogenic factors for cardiac hypertrophy and heart failure. As such, therapies that target neutrophils (Figure 3), NETs (Figure 4), or thrombosis (Figure 7) can be beneficial to the heart.

The AngII-NET axis is operative in wild-type mice.

Using the myeloid KLF2 deficient (K2KO) mice as a pro-inflammatory genetic model, we demonstrated that the neutrophil-KLF2-NETs axis is critical for regulating cardiac hypertrophy. Furthermore, given that the reduction of KLF2 expression in neutrophils is observed from both clinical HF patients and experimental HF mice (Figure 1) and that KLF2 reduction is known to enhance pro-inflammatory myeloid activation(39), we sought to validate if this pathogenic pathway is operative in WT animals, where the KLF2 gene is not deleted, but rather its expression is reduced in the setting of disease.

We first used an anti-Ly6G antibody to deplete neutrophils in WT mice to see if it protects the heart from hypertrophy. It is well known that C57BL6 mice do not develop severe cardiac hypertrophy in response to a regular dose of AngII (1.0 μg/kg/min). Therefore, we used a higher dose of AngII (2.0 μg/kg/min) to probe the protective roles of neutrophil depletion. At this high dose, AngII also induced a high mortality rate (Supplemental Figure 2C), likely due to aortic

dissection (data not shown). At four weeks post-AngII infusion, the surviving mice showed significant improvement in cardiac functions and hypertrophy in the Ly6G antibody-treated (neutropenia) group compared to the normal IgG antibody-treated (control) groups (Supplemental Figure 8), indicating a pathogenic role of WT neutrophils that is similar to the KLF2-null condition.

Next, we assessed whether neutrophilia established by adoptive transfusion could augment cardiac dysfunction in WT mice. During the 4-week AngII infusion, 5 million neutrophils isolated from WT donors' bone marrow were transfused every week via intravenous injection (i.v.). It has been reported that transfusion of 5 million neutrophils is well tolerated by normal mice without severe adverse effects (30), resulting in acute neutrophilia within two hours and slightly increased leukocyte counts after 24h but no change in platelet counts (Supplemental Figure 9). In mice with AngII infusion, neutrophil transfusion significantly accelerated cardiac hypertrophy resulting in reduced LV contractility and enlarged hearts (Figure 8A). Plasma cTnT levels were also elevated in the transfusion group, indicating more severe myocardial injury (Figure 8B). Pathological analyses revealed more severe cardiomyocyte hypertrophy, myocardial fibrosis, cell death, and NET formation in neutrophil-transfused hearts (Figure 8, C&D). Consistent with the pathogenic roles of NETs, simultaneous administration of DNase I with neutrophil transfusion almost completely blocked the adverse effects (Figure 8, A-D). These data demonstrated enhanced neutrophil function accelerates AngII-induced cardiac hypertrophy, likely through the NETs-thrombosis axis.

To determine the clinical relevance of the neutrophil-KLF2-NETs axis, we analyzed plasma samples from HF patients and non-HF control subjects for NETs-related biomarkers, including histone-associated DNA fragment and cell-free DNA (cfDNA). Compared with the non-HF group, we detected significantly higher levels of histone-associated DNA fragments and cfDNA in the HF group (Figure 8E), suggesting increased NETs formation in HF patients. Given that HF patients often have heightened activity of the renin-angiotensin system (RAS) and reduced neutrophil KLF2 levels, these results suggest that the AngII-neutrophil-KLF2-NETs axis may contribute to human HF.

KLF2 is critical for the transcriptional regulation of neutrophils in cardiac hypertrophy.

To understand mechanistically how changes in the neutrophil transcriptome governed by neutrophil KLF2 regulates cardiac hypertrophy, we sorted the Ly6G+ neutrophils from the myocardium and performed RNA-seq studies. We picked the 1-week post-AngII infusion time point for neutrophil isolation because it is both the peak of neutrophil infiltration and the onset of heart disease. Since there were very few neutrophils in the myocardium at baseline (Figure 2A),

we only focused on the AngII-infused groups. In total, about 5000 neutrophils were included in each sample (Cre vs. K2KO, n=4) for RNA extraction. RNA-seq study was performed using the low-RNA-input protocol. We Identified 1740 differentially expressed genes (DEG) with significant p-value < 0.05 and 2-fold change in expression levels (Supplemental Figure 10A).

The gene ontology (GO) and pathway enrichment analyses with DEGs (Figure 9A) in GO and KEGG pathways were enriched in protein translation (ribosome function), cytokine/chemokine signaling, inflammation, leukocyte migration, and adhesion, which strongly support the pro-inflammatory phenotypes we observed with K2KO neutrophils *in vivo* and *in vitro*. Furthermore, the GO terms on apoptosis and programmed cell death may be associated with NETosis, a unique form of programmed cell death. Hallmark pathway analysis revealed 30 Hallmark pathways with ($p<0.00001$), including inflammation-related pathways (TNFα, P53, ROS, TGFβ, apoptosis), metabolic pathways (glycolysis, hypoxia), and coagulation pathway (Figure 9A, Supplemental Figure 10B). Together, findings from these transcriptomic analyses support a crucial role of KLF2 in neutrophil biology in response to AngII.

We next investigated the transcriptional network that KLF2 may control in neutrophils in response to AngII. Among the 1740 DEGs, we identified 39 transcription factors that were changed over twofold ($|FC|>2$), including 26 down-regulated and 13 upregulated transcription factors (Figure 9B). As expected, *Klf2* was found to be the most significantly ($p<0.00005$) down-regulated transcription factor in K2KO neutrophils. Conversely, the top 3 most significantly upregulated transcription factors were *Nfe2*, *Bcl6*, and *Hif1a*. NFE2 (nuclear factor erythroid 2) is associated with myeloproliferative neoplasms and polycythemic disorders (40). BCL6 (B-cell lymphoma 6) has been reported to regulate apoptosis in neutrophils, which may be involved in NETosis (41). Notably, HIF1α is an oxygen-sensing subunit of the heterodimeric transcription factor hypoxia-inducible factor 1 alpha (HIF1α), a master regulator of myeloid cells (42). HIF1α transcriptionally regulates glycolysis, the dominant metabolic pathway that supports ATP synthesis in pro-inflammatory myeloid cells and many cytokine genes such as IL1β (43). HIF1α also induces the expression of NFE2 (44). Therefore, we focused on investing the potential cross-talks between KLF2 and HIF1α signaling pathways.

KLF2 has been reported to negatively regulate HIF1α signaling in myeloid cells and other cell types (21, 45). As revealed by RNA-seq, KLF2 deficiency augmented HIF1α expression in neutrophils in response to AngII (Figure 9B). This response was recapitulated *in vitro* using isolated mouse bone marrow neutrophils. The results showed that KLF2-deficient neutrophils expressed higher levels of HIF1α mRNA both at baseline and after AngII treatment (Figure 9C).

To confirm a KLF2-HIF1α -axis in vivo, we generated Lyz2-Cre-driven, myeloid-specific, KLF2-HIF1α double knockout mice (designated DKO) and subjected them to the AngII infusion model. At baseline, the DKO mice are grossly normal without any visible defects. However, after a 4-week AngII infusion, the cardiac function of DKO mice was well preserved to a level comparable to Cre mice (Figure 9D). Furthermore, AngII-induced myocardial neutrophil infiltration, NETs formation, and cell death were primarily abolished in the DKO myocardium (Figure 9, E&F). These data demonstrate that, in response to AngII, compound deficiency of both KLF2 and HIF1α restrains neutrophil pro-inflammatory activation and preserves cardiac function. This further suggests that hyperactivation of HIF1α signaling resulting from neutrophil KLF2 deficiency exacerbates inflammation and worsens cardiac hypertrophy. Collectively, these transcriptomic and genetic studies indicated that the KLF2- HIF1α axis is critical for AngII-induced neutrophil activation and cardiac hypertrophy, likely through the regulation of NETosis and resultant thrombosis in small vessels of the myocardium.

Neutrophils orchestrate the myocardial inflammatory responses.

Neutrophils are the first responders to injury or inflammatory stimuli, capable of forming complex crosstalk with other cells, including immune cells and mural cells. To understand how neutrophils orchestrate the myocardial responses to AngII, we performed single-cell RNA sequencing (scRNA-seq) with live non-cardiomyocytes sorted from the Cre and K2KO hearts.

We subjected Cre and K2KO mice to a 1-week AngII infusion to capture the pathogenic changes at the disease onset. After enzymatically dispersing cardiac tissue, cells were sorted using flow cytometry. Dead cells were excluded by live-dead dye, and live cells were sorted to enrich CD45+ cells and CD31+ cells. Single-cell capturing and library preparation was performed using a 10X Genomics Chromium Single Cell 3' GEM, Library and Gel Bead Kit v3, followed by next-generation sequencing using Illumina platform. After preprocessing raw sequencing data and the quality control of cell barcodes and UMIs (unique molecular identifiers), 8716 cells from the Cre group and 8540 cells from the KO group were subject to downstream bioinformatics analysis. Uniform Manifold Approximation and Projection for dimension reduction (UMAP) and unsupervised clustering analysis using Seurat pipeline identified seven distinct cell populations from the total 17,256 cells (Figure 10A). Gene expression patterns of established canonical markers of various immune cell lineages (C1qa, C1qb, S100a8, S100a9, Cd3g, Ly6d, Cd79a, etc.), endothelial cells (Cdh5, Cav1, Kdr, etc.), and fibroblasts (Dcn, Mgp, Col1a2, etc.) allowed the assignment of putative biological identities to each cluster, namely neutrophils (Neu), macrophages (Mac), B cells, T/NK cells, conventional endothelial cells (EC), mitotic endothelial

cells (mEC), and fibroblasts (Fib). Cell type-specific markers are shown as Heatmap of Top 50 marker genes for each cluster (Figure 10B), Feature-Plot depicting gene expression on UMAP (Figure 10C), and Dot-Plot for Top 4 marker genes for each cluster (Supplemental Figure 11A). Between the Cre and K2KO groups, it appeared that the neutrophils (Neu) cluster was increased (2.67% in Cre vs. 5.62% in K2KO). In comparison, the mitotic endothelial cell cluster (mEC) was reduced (4.04% in Cre vs. 1.59% in K2KO) in the K2KO group (Figure 10, D & E), faithfully recapitulating the excessive neutrophil infiltration (Figure 2A) and capillary rarefaction phenotypes (Figure 5F) at the mRNA level.

Next, we performed gene ontology (GO) analysis with differentially expressed genes (DEGs, K2KO vs. Cre, adjP<0.05), particularly in the significant four cell types: neutrophils, macrophages, endothelial cells, and cardiac fibroblasts (Supplemental Figure 11B). Upregulated DEGs in K2KO neutrophils were enriched in GO terms related to protein synthesis (ribosome assembly, cytoplasmic translation), energy metabolism (ATP metabolic process and electron transfer chain), purine ribonucleotides and purine-containing compounds metabolism, ribose phosphate metabolic process, and ribonucleoprotein complexes related processes, indicating a robust activation status of the K2KO neutrophils (Figure 11A). In particular, GO terms related to ribonucleoprotein complexes and energy metabolism likely supported the enhanced NETosis observed in K2KO neutrophils (46). Furthermore, purine ribonucleotides and purine-containing compounds are classic biomarkers of neutrophil activation and mediators of EC dysfunction (47, 48). In contrast, upregulated DEGs in Cre neutrophils were enriched in GO terms related to classic immune functions, including neutrophil activation, leukocyte activation, chemotaxis, leukocyte migration, cytokine-mediated signaling pathway, responses to infection (viral and other organisms), and wounding (Supplemental Figure 12).

K2KO macrophage showed enrichment in GO terms "myeloid cell differentiation," "positive regulation of cytokine production," and “response to molecule of bacteria origin,” indicating pro-inflammatory activation of cardiac macrophages during cardiac hypertrophy (Figure 11A). Cre macrophage showed enrichment in GO terms related to antigen presentation, mitochondrial function, RNA splicing, protein folding and catabolic processes, and ribose/nucleoside metabolism (Supplemental Figure 12). We have shown that AngII induced similar levels of macrophage accumulation in Cre and K2KO myocardium, but the GO analysis demonstrated vast differences in their function.

Consistent with the myocardial fibrosis phenotype, K2KO fibroblast GO terms were enriched in TGF-beta signaling, gene transcription, and translation-related related processes,

proteasomal protein degradation, and Golgi vesicle transport processes. At the same time, there was minimal GO enrichment in Cre fibroblasts (Figure 11A & Supplemental Figure 12). In K2KO endothelial cells, GO terms were enriched in angiogenesis, EC development and migration, and regulation of cellular protein localization. In contrast, Cre EC showed enrichment in "cytoplasmic translation," "establishment of endothelial barrier," ATP synthesis-related processes, protein folding, etc. (Figure 11A & Supplemental Figure 12). Thus, the EC GO analysis suggests heightened endothelial cell activation and impaired endothelial barrier function in the K2KO myocardium, underscoring myocardial perfusion and capillary density impairments.

Finally, to gain insight into the complex myocardial responses orchestrated by neutrophils, particularly the KLF2-deficient neutrophils, we performed cell-cell interactome analysis, comparing Cre vs. K2KO groups. The most significant changes (i.e., the biggest fold changes) of ligand-receptor communication between different cell types revealed a complex immune checkpoint network among all major non-cardiomyocyte cell types (Figure 11B). Interestingly, among genes related to an immune checkpoint, TNF superfamily member 9 (Tnfsf9, also known as 4-1BB ligand) is one of the most significantly upregulated ligands both in neutrophils and macrophages of the K2KO group and transacting signals with multiple cell types. The cluster-specific expression of Tnfsf9 was shown in Figure 11C, separately. It has been reported that TNF signaling accelerates thrombosis and fibrosis *in vivo* (49), and Tnfsf9 is implicated in lung inflammation and fibrosis (50). Our results thus suggest the potential role of Tnfsf9 in heart failure. Collectively, these scRNA-seq data demonstrate that neutrophils are the crucial regulator orchestrating myocardial inflammation because alterations in neutrophil function (due to KLF2 deficiency) significantly reshape the immune responses from all significant non-cardiomyocyte cell types in the myocardium.

Discussion

The pathogenesis and progression of HF are multifactorial, and accumulating evidence suggests that inflammatory cells and related pro-inflammatory cytokines play a pathogenic role. In the present study using chronic AngII infusion as a classic non-ischemic HF model, we show a requisite role of neutrophils in cardiac dysfunction (summarized in Figure 12). Our data demonstrate that AngII activates neutrophils to mediate cardiac hypertrophy through a KLF2-NETosis-thrombosis pathway. Activated neutrophils adhere to vascular walls and release NETs, leading to thrombotic occlusion of small vessels and impaired myocardial microcirculation. Chronic micro-thrombosis may cause capillary rarefaction to further worsen the hypoxic condition in the myocardium. In HF patients, the hyperphysiological levels of AngII due to heightened RAS

activity may propel this vicious cycle during HF pathogenesis and progression (Figure 12, dashed arrow). Our model underscores the clinical findings that small vessel dysfunction is a strong independent predictor of HF deterioration and death, regardless of ischemic or non-ischemic HF (51-54). Identifying a neutrophil-KLF2-NETosis-thrombosis pathway for chronic non-ischemic cardiac diseases provides novel pathogenic mechanisms and promising therapeutic targets for HF.

An essential aspect of our work relates to identifying neutrophils as critical regulators of chronic non-ischemic heart diseases. Surprisingly, despite being the predominant leukocytes in human blood, little is known about neutrophils in HF. It has been reported that, as the first responders to injury, neutrophils are recruited to the heart during AMI to mediate wound healing (16). Neutrophils are also found in AMI thrombi, suggesting they may participate in thrombosis that causes AMI (55). However, the role of neutrophils in chronic HF and other non-ischemic cardiac diseases is poorly understood. Indeed, neutrophils can regulate both acute and chronic inflammation. Several chronic inflammatory and autoimmune diseases are characterized by a sustained influx of neutrophils, such as cystic fibrosis, chronic obstructive pulmonary disease, systemic lupus erythematosus, and rheumatoid arthritis, to name a few (56).

Moreover, metabolic diseases, such as diabetes and obesity that posit risk for HF, are associated with persistent low-grade neutrophil activation and NETosis (55). COVID-19 is also associated with heart failure, and a high neutrophil-lymphocyte ratio significantly prognoses severe outcomes (57). This study found that neutrophils are activated by chronic AngII infusion, likely via neutrophil-autonomous KLF2-dependent signaling, as suggested by in vitro findings (Figure 1, D & E). Meanwhile, cardiac tissue injury triggered by AngII-dependent or AngII-independent mechanisms can also indirectly recruit and activate neutrophils in the heart. Future studies will be needed to delineate the detailed mechanisms by which neutrophils are activated in the course of HF.

Nonetheless, neutrophil activation leads to long-term effects on cardiac hypertrophy in our mouse model, likely through a KLF2-NETosis pathway that is also associated with HF patients. Our findings are aligned with the clinical observations that the neutrophil-lymphocyte ratio is a prognostic marker for acute and chronic HF hospitalization and mortality. Furthermore, the beneficial effects of ACE inhibitors in HF might be partly due to effects on neutrophils, including anti-inflammation and neutropenia(58). Given that neutrophils are the most abundant leukocytes in human blood, identifying neutrophils as crucial regulators of HF is of great importance for basic research and clinical applications.

Recently, studies from our group and others have revealed the distinct roles of monocytes and macrophages in the heart (59). Although we found that KLF2 deficiency in macrophages was not the critical determinant of AngII-induced cardiac hypertrophy in this study, one should not exclude the functions of macrophages in HF. On the one hand, numerous studies using the same AngII model and other cardiac models have shown that macrophages exhibit pro-fibrosis, pro-inflammatory roles in the heart (60). Our scRNA-seq data demonstrated the pro-inflammatory activation of cardiac macrophages, which was exacerbated in the K2KO group (Figure 11A & Supplemental Figure 12). On the other hand, we found that alterations in neutrophils (KLF2 deficiency, neutrophil depletion, DNase I treatment, etc.) did not affect cardiac macrophage numbers. This, however, does not mean that the functions of cardiac macrophages are not affected by neutrophils. It has been reported that neutrophils regulate macrophage functions in response to infection or tissue injury (61, 62). Finally, AngII (and AngII-induced cytokines) can also activate signal transduction in macrophages unrelated to neutrophils (60). For example, we showed that AngII did not induce neutrophil infiltration to KLF2-HIF1α-DKO hearts, but it significantly increased the cardiac macrophage numbers (Figure 9E).

Given that depletion of neutrophils or neutrophil-derived products (NETs, microthrombi) profoundly ameliorated HF, our findings suggest neutrophils may function upstream of the macrophages and monocytes in the development of HF. In this study, we also explored the crosstalk between neutrophils and other cells in the myocardium by the single-cell RNA-seq (scRNA-seq) approach. Major immune cell types identified from the myocardium are macrophages, neutrophils, and lymphocytes, with significant changes in neutrophil numbers between genotypes. Furthermore, the interactome analysis suggests that neutrophils are the key regulator of myocardial inflammation because KLF2-deficient neutrophils, which exhibit enhanced pro-inflammatory functions, significantly reshape the immune responses from all major cell types in the K2KO myocardium (Figure 11B). Future studies are warranted to study the crosstalk between neutrophils and other immune cells in the setting of HF.

Another important aspect of our work relates to neutrophil control of small vessel thrombosis and microcirculation in a chronic non-ischemic disease setting. Our studies found that the effects of neutrophils on cardiac hypertrophy were not mediated by paracrine cytokines but rather through the regulation of hypoxic stress in the myocardium. In AngII-infused mice, particularly those with KLF2-deficient pro-inflammatory neutrophils, we showed strong evidence of sporadic thrombosis in small vessels throughout the myocardium, associated with capillary rarefaction and cell death. Conversely, treatment with classic anticoagulant heparin prevented

thrombosis, cell death and subsequently improved cardiac function and myocardial pathologies. Further, these defects were also rescued by administering DNase-I or GSK-484, indicating a crucial role of NETs. Consistently, depletion of neutrophils also ameliorated these pathological events. Therefore, we proposed a neutrophil-NETs-thrombosis hypothesis to interpret these cardiac responses. Based on this hypothesis, AngII-activated neutrophils are recruited to the heart, forming intravascular NETs that trigger thrombosis. In particular, small vessels prone to occlusion are affected the most, leading to sporadic myocardial ischemia, death of cardiomyocytes and vascular endothelial cells, and subsequent capillary rarefaction. These pathological changes are factors known to cause cardiac hypertrophy and fibrosis (63, 64). Collectively, our findings suggest that non-ischemic cardiac diseases, such as cardiac overload secondary to hypertension or neurohormonal stress, may share pathogenic characteristics similar to ischemic cardiac disorders. This notion is consistent with the clinical observations of myocardial capillary rarefaction in HF patients (54), as well as experimental studies demonstrating that inadequate myocardial perfusion due to impaired angiogenesis is a critical determinant of transition from compensatory hypertrophy to heart failure (65-67).

The neutrophil-NET-thrombosis hypothesis is well-supported by our data from this study and literature showing neutrophils are critically involved in thrombotic processes (36). In mouse studies of arterial thrombosis, neutrophils are the first cells at the site of damage, arriving even earlier than platelets (68). Neutrophils are also found in the venous thrombi from a mouse model of inferior vena cava ligation (31). Mechanistically, the pro-thrombotic activity of neutrophils has been linked to NETs (35). Nucleosomes, significant components of NETs, can directly activate coagulation factors and platelets. The neutrophil-derived serine proteases, elastase, and cathepsin G deposited onto NETs promote proteolysis of the coagulation suppressor TFPI (tissue factor pathway inhibitor), releasing the brakes of coagulation (37, 69). Tissue factor, the initiator of the extrinsic coagulation cascade, is also found on NETs. Fibrin also connects with NETs, which may be critical for immunothrombosis.

Collectively, these data demonstrate that a KLF2-HIF1 axis is operative in neutrophils in response to chronic sterile inflammation. Furthermore, the fact that neutrophil-deficiency of KLF2 or HIF1 was able to skew cardiac responses so profoundly suggests that, for each HF patient, the pathogenesis and progression of HF can be dictated by the condition of their immune system. Heart failure affects more than 26 million people worldwide and has a devastating mortality rate comparable to many malignant cancers (70-72). However, current therapies to reduce neurohormonal stress and improve hemodynamics are only partially effective, underscoring the

need for orthogonal strategies based on newly identified molecular and cellular mechanisms. The studies here highlight the previously unidentified essential roles of neutrophils in an experimental model of heart failure. Further, they suggest that inhibiting neutrophil activation, blocking NETs formation or promoting NETs clearance, or targeting the downstream micro-thrombosis, can be exploited for therapeutic gain in the clinical management of HF.

Methods

Detailed materials and methods can be found in Supplemental Information online. RNA-seq data are deposited in the Gene Expression Omnibus (Bulk RNA-seq data accession no. GSE186468; scRNA-seq data accession no. GSE185756).

Study Approval

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Case Western Reserve University School of Medicine and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The human study was approved by the Institutional Review Board of Shanghai Minhang Hospital of Integrated Traditional Chinese and Western Medicine (2019005). Informed consent was obtained from all the participants included in the study.

Author contributions

XT, RZ, IW, EC, VV, LN, Stephanie L, Sarah L, AM, DRS, XL performed animal-based and cell-based experiments and analyzed the data. XT, PW, JL, JF, TZ did human studies and analyzed data. VV, HWJ, RHA performed bioinformatics. XL conceived the project. TZ, XL, MKJ supervised the project. XL, MKJ, TZ, XT wrote the paper with input from all authors.

Acknowledgments

This work was supported by the AHA Transformative Project Award 18TPA34230033 (to X. Liao), the National Natural Science Foundation of China grant 82074049 (to T Zhang), NIH grant 1R35HL135789, the Elisabeth Severance Prentiss Foundation grant, the American Heart Association – Paul G. Allen Frontiers Group grant 19PABHI34580006 (to MK Jain) and the Leducq Foundation grant 18CVD03 (to RH Adams, MK Jain). This research was supported by the Cytometry & Imaging Microscopy Shared Resource of the Case Comprehensive Cancer Center (P30CA043703), the CWRU Genomics Core and Applied Functional Genomics Core (AFGC) at Case Western Reserve University School of Medicine. The authors acknowledge the

Flow Cytometry Core and the Genomics Core at the Cleveland Clinic Lerner Research Institute for cell sorting and 10x single-cell omics services. Our special thanks go to Michael Sramkoski for flow cytometry, and Selena Liao for artistic illustrations.

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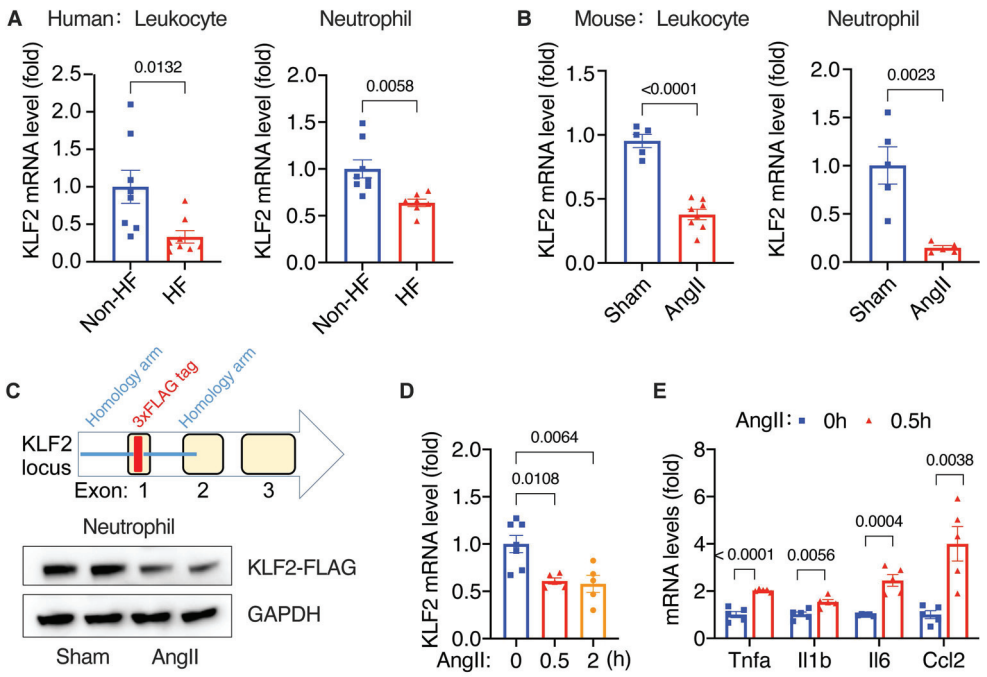

Figure 1. Heart failure is associated with reduced KLF2 expression in circulating leukocytes and neutrophils.
(**A**) KLF2 mRNA expression in human peripheral blood leukocytes (n=8) and neutrophils (n=7-8). HF: patients with heart failure (n=15). Non-HF: aged-matched patients without heart failure (n=16). (**B**) KLF2 mRNA expression in peripheral blood leukocytes and neutrophils from WT mice with AngII (1.0 μg/kg/min) or PBS (Sham) infusion (n=5-8). (**C**) KLF2-tag mice were generated using the CRISPR/Cas9 method. Protein levels of 3xFLAG-KLF2 in blood neutrophils were detected by M2 anti-FLAG antibody (Sigma, F3165). Each lane represented one animal. (**D** and **E**) Mouse bone marrow-derived neutrophils treated with AngII (100 nmol/L) in vitro (n=5-7). P values were from 2-tailed unpaired Student t test (A, B, E) and post-hoc test with Tukey correction of One-way ANOVA (D).

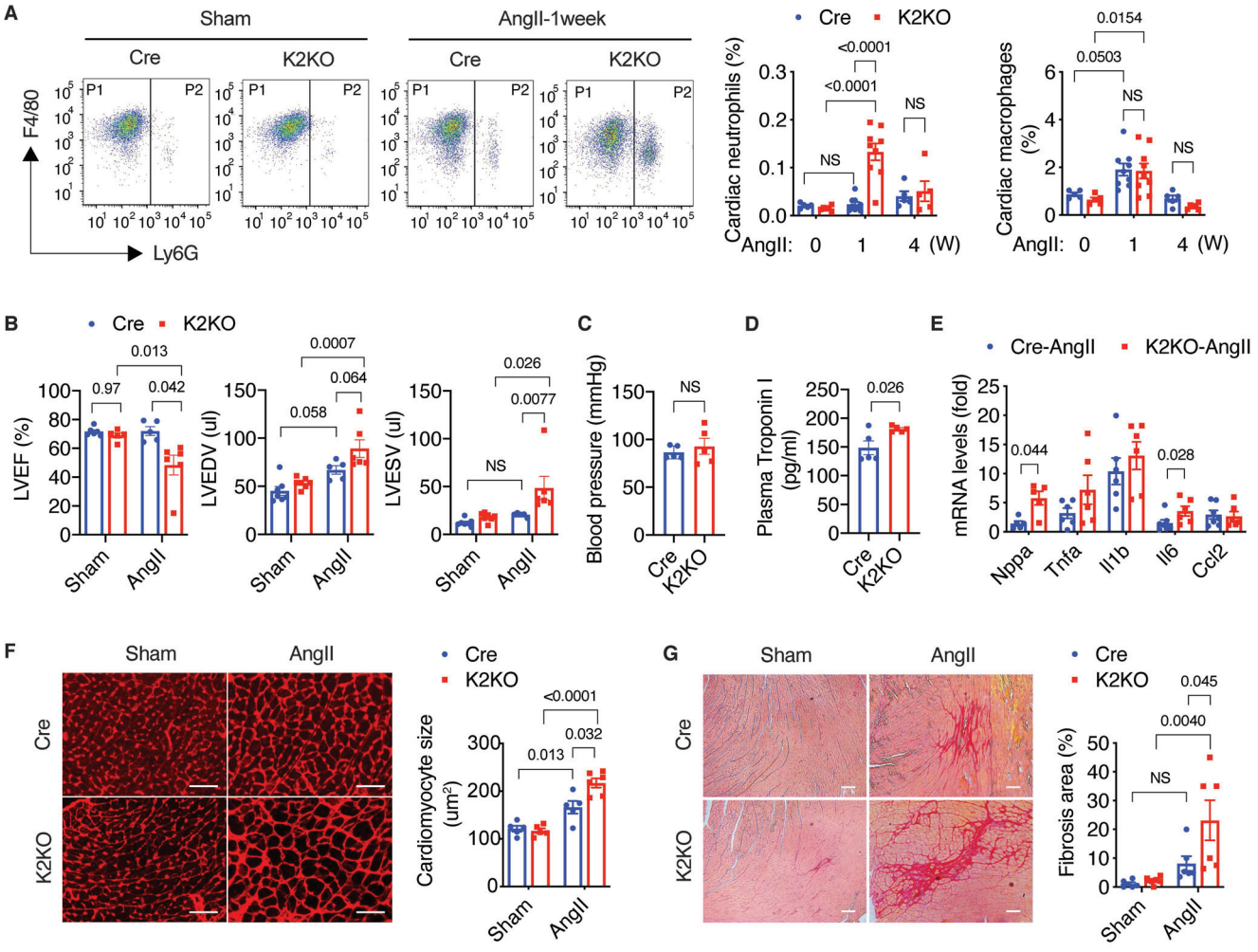

Figure 2. Myeloid KLF2 deficiency enhances AngII-induced cardiac hypertrophy.
(**A**) FACS analysis of myeloid cells in the myocardium (n=5-9). P1: CD45+CD11b+F4/80+Ly6G- macrophages; P2: CD45+CD11b+Ly6G+ neutrophils. (**B**) Echocardiography assessments of mouse left ventricular (LV) functions after 4-week infusion (n=5-6). LVEF: left ventricular ejection fraction; LVEDV: LV volume at end of diastole. LVESV: LV volume at end of systole. (**C**) Mean blood pressure measured from the right common carotid artery by invasive hemodynamics (n=5). (**D**) Plasma levels of cardiac troponin (cTnT) after 1-week of AngII infusion (n=5). (**E**) Expression of hypertrophy and inflammation genes in the heart (n=6-7). (**F**) Cardiomyocyte cross-sectional area analysis by Alexa Fluor 594 conjugated wheat germ agglutinin (WGA) staining (n=5-6). (**G**) Myocardial fibrosis analysis by Picro Sirius Red staining (n=6). P values were from 2-tailed unpaired Student t test and post-hoc test with Tukey correction when applicable. Two-way ANOVA was applied for (B, F, G). NS: not significant (P>0.5). Scale bar: 25 μm.

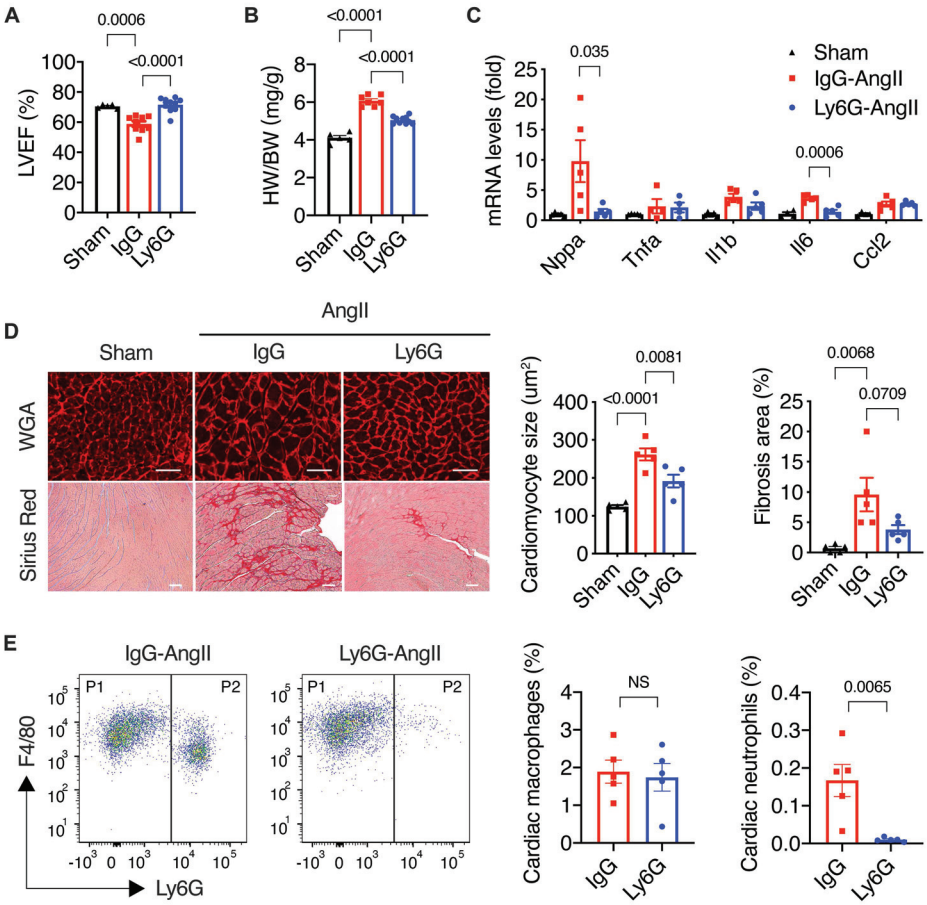

Figure 3. KLF2-deficient neutrophils are critical for AngII-induced cardiac hypertrophy. (**A**) Cardiac function (n=5-10). (**B**) Heart weight (HW) normalized to body weight (BW) (n=5-7). (**C**) Myocardial gene expression (n=4-5). (**D**) Cardiac hypertrophy (WGA staining) and fibrosis (Sirius Red staining) after 4-week AngII infusion in K2KO mice (n=5). AngII-treated mice subjected to anti-Ly6G antibody (Ly6G) or IgG control antibody (IgG) treatments. LVEF: left ventricular ejection fraction. Scale bar: 25 μm. (**E**) FACS analysis of macrophages and neutrophils in K2KO hearts after 1-week AngII infusion and antibody treatments with anti-IgG or anti-Ly6G (n=5). P values were from 2-tailed unpaired Student t test and post-hoc test with Tukey correction of One-way ANOVA. NS: not significant.

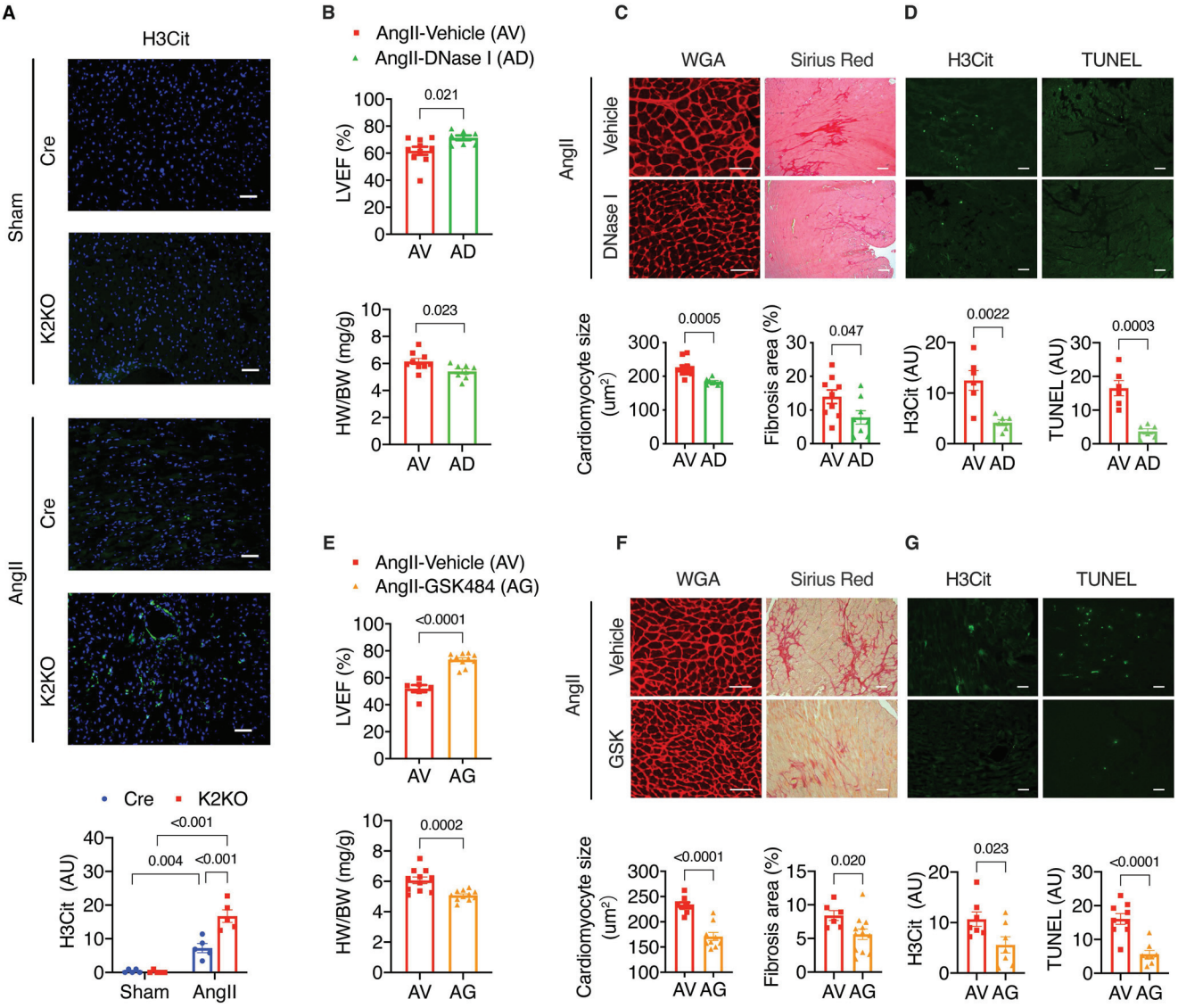

Figure 4. Neutrophil extracellular traps (NETs) as a critical mediator of cardiac responses to AngII. (**A**) Immunostaining of citrullinated histone H3 (H3Cit) from Cre and K2KO hearts (n=5). (**B-D**) DNase I administration (n=6-10) and (**E-G**) GSK-484 administration (n=6-11) in AngII-infused K2KO mice. (**B** and **E**) LV function and heart weight. (**C** and **F**) Cardiac hypertrophy (WGA staining) and fibrosis (Sirius Red staining). (**D** and **G**) Intracardiac NETs formation (H3cit immunofluorescence) and cell death (TUNEL immunofluorescence). AngII infusion: (A, D, G) 1 week; (B, C, E, F) 4 weeks. P values were from Tukey correction of Two-way ANOVA (A) and 2-tailed unpaired Student t test (B-F). Scale bar: 25 μm.

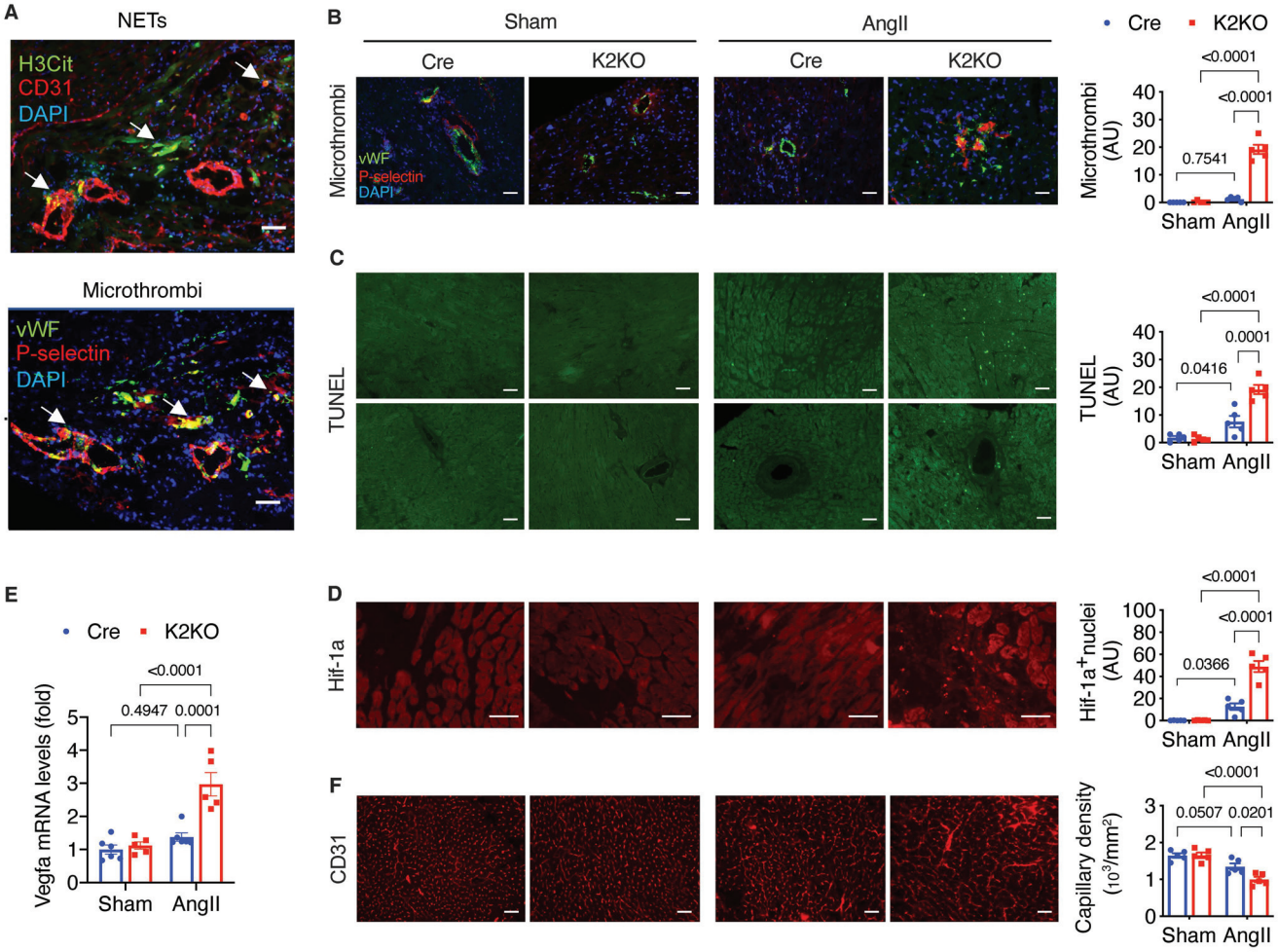

Figure 5. AngII-induced NETs formation triggers micro-thrombosis and myocardial injury.
(**A** and **B**) Immunostaining of H3Cit, vWF, P-selectin, and CD31. (**C**) TUNEL staining to assess cell death. Upper: intramuscular regions. Lower: perivascular regions. (**D**) Immunostaining of Hif1α protein. Hif1α positive nuclei were counted. (**E**) Myocardial expression of Vegfa mRNA. (**F**) Myocardial capillary density assessed by CD31 immunostaining. AngII infusion: (A-D) 1-week; (E and F) 4 weeks. P values were from post-hoc test of Two-way ANOVA with Tukey correction for (B-F). Representative images from 5-6 mice in each group. Scale bar: 25μm.

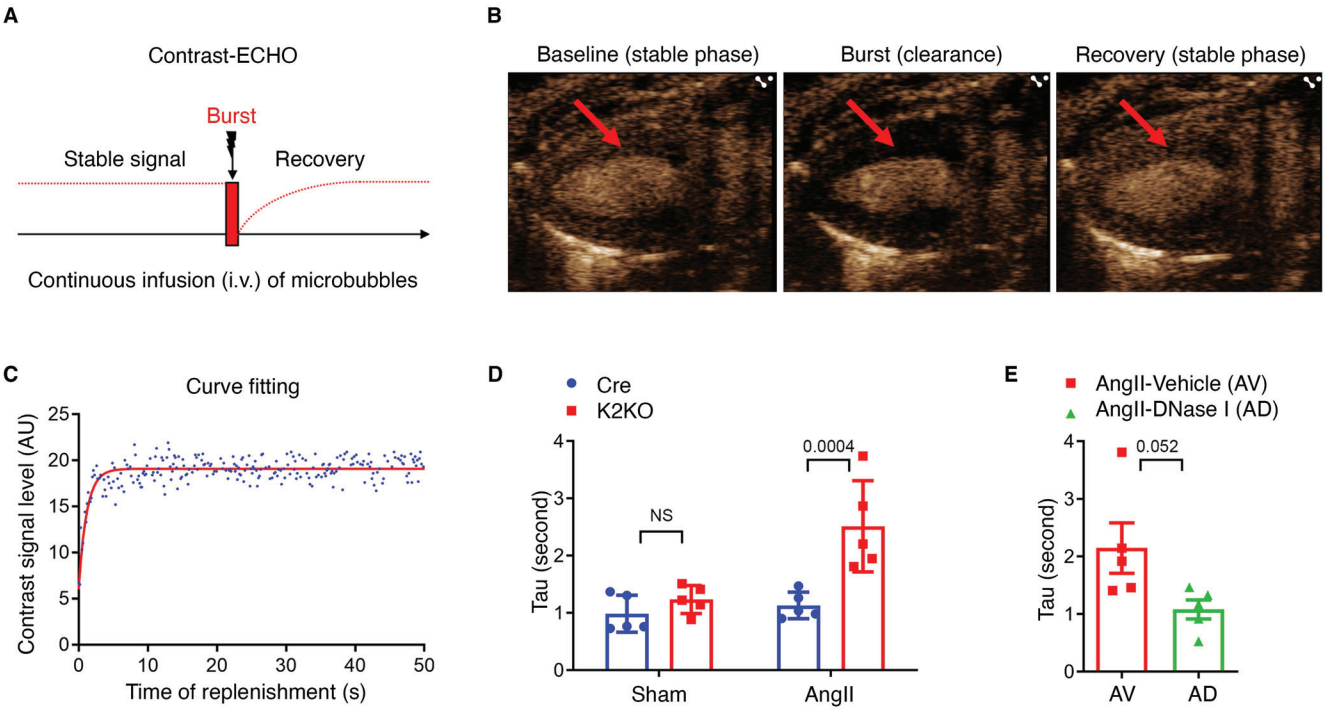

Figure 6. AngII infusion impairs microcirculation in the K2KO myocardium.
(**A**) The experimental design of contrast-ECHO showing three phases of the contrast signal: basal stable level; clearance by a burst of high-energy ultrasound beam; and recovery. The rate of contrast signal recovery is correlated with the microcirculatory blood flow rate. (**B**) Representative contrast-ECHO images showing "baseline," "burst," and complete "recovery" phases. Arrows indicate LV wall. (**C**) Representative data analysis showing cure fitting of a one-phase exponential decay curve. The recovery rate (blood flow rate) can be estimated by Time Constant (Tau) of the curve. A more considerable Tau value indicates slower blood flow. (**D**) Contrast-ECHO data from Cre and K2KO mice before and after 4-week AngI infusion (n=5). P(interaction)=0.0143 by Two-way ANOVA. P values shown as post-hoc test. NS: not significant. (**E**) The effect of DNase I administration on myocardial microcirculation assessed by contrast-ECHO (n=5). P value from 2-tailed unpaired Student t test.

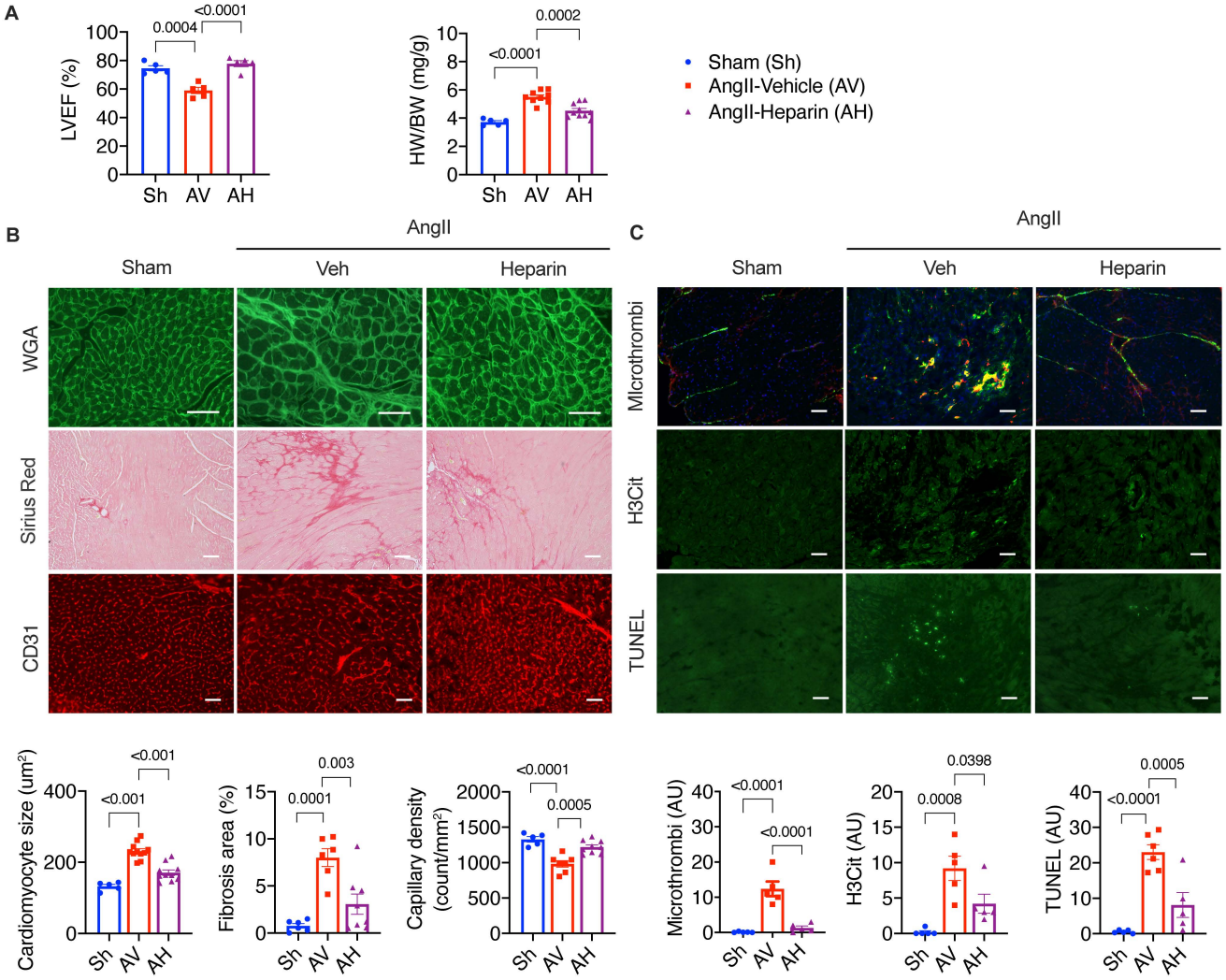

Figure 7. Heparin administration ameliorates AngII-induced cardiac dysfunction in K2KO hearts. (**A**) Cardiac function and hypertrophy (n=5-10). (**B**) Myocardial hypertrophy (WGA-Alexa Fluor 488 staining), fibrosis (Sirius Red staining), and capillary density (CD31 immunofluorescence). n=5-11. (**C**) Intracardiac microthrombosis (vWF/P-selectin immunofluorescence), NETs formation (H3cit immunofluorescence), and cell death (TUNEL immunofluorescence). Infusion: (A and B) 4 weeks; (C) 1 week. P values were from post-hoc test of One-way ANOVA with Tukey correction. Representative images from individual animal (n=5-11 in each group). Scale bar: 25μm.

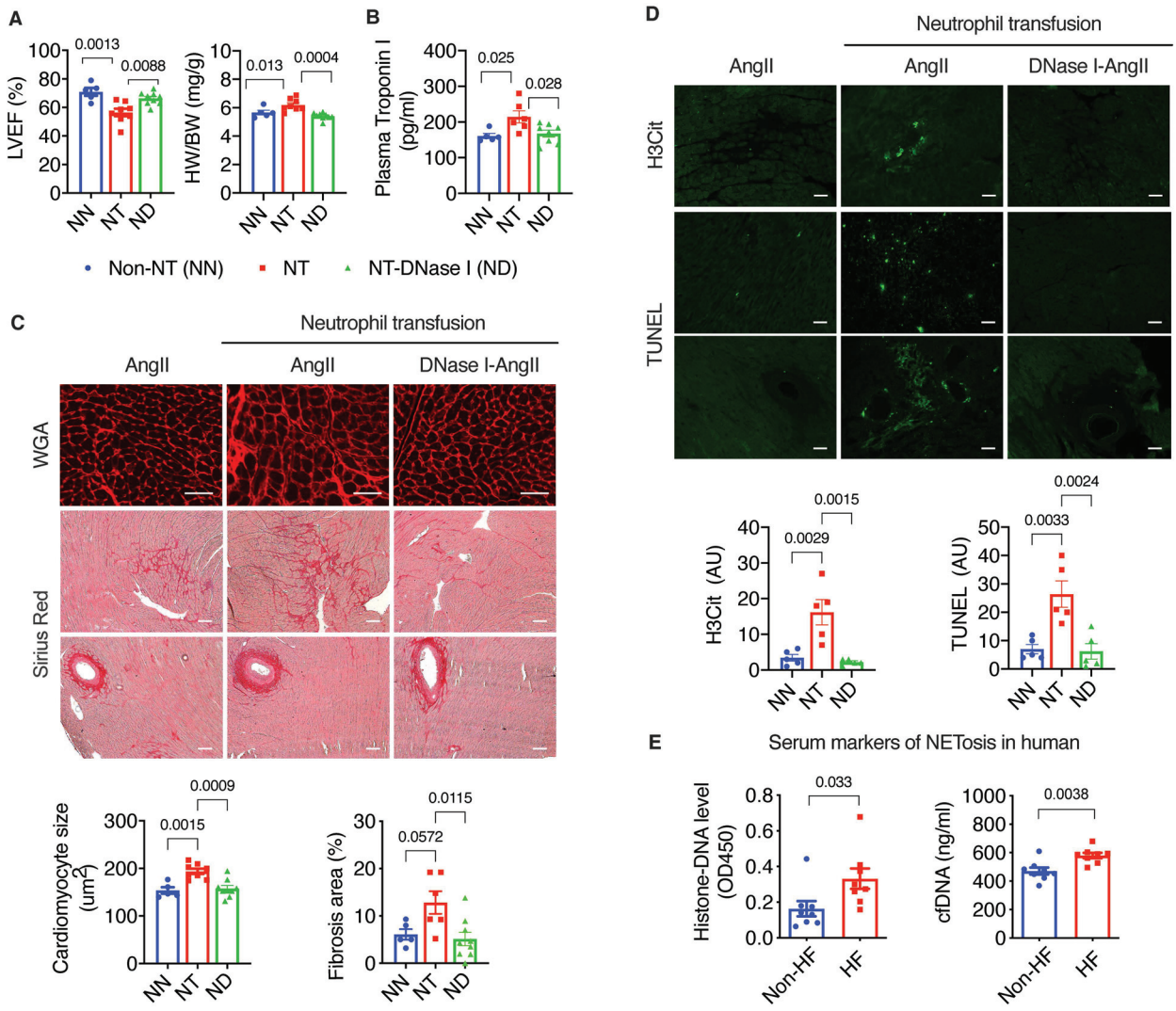

Figure 8. Neutrophilia by adoptive neutrophil transfusion accelerates AngII-induced cardiac hypertrophy.

(**A**) LV function and hypertrophy. Non-NT: no neutrophil transfusion, NT: neutrophil transfusion, NT-DN: Neutrophil transfusion plus DNase I treatment. All groups received a 4-week AngII infusion (n=5-9). (**B**) Plasma cardiac troponin I (cTnT) levels after 1-week AngII infusion (n=5-8). (**C**) Myocardial hypertrophy (WGA staining) and fibrosis (Sirius Red staining). (**D**) Intracardiac NETs (H3Cit) and cell death (TUNEL). (**E**) Histone-associated DNA fragments and cell-free DNA (cfDNA) in the plasma of HF patients and non-HF controls (n=8). (A-D) P values were from post-hoc test of One-way ANOVA with Tukey correction. (E) P value from 2-tailed unpaired Student t test. Representative images from individual animal (n=5-9 in each group). Scale bar: 25 μm.

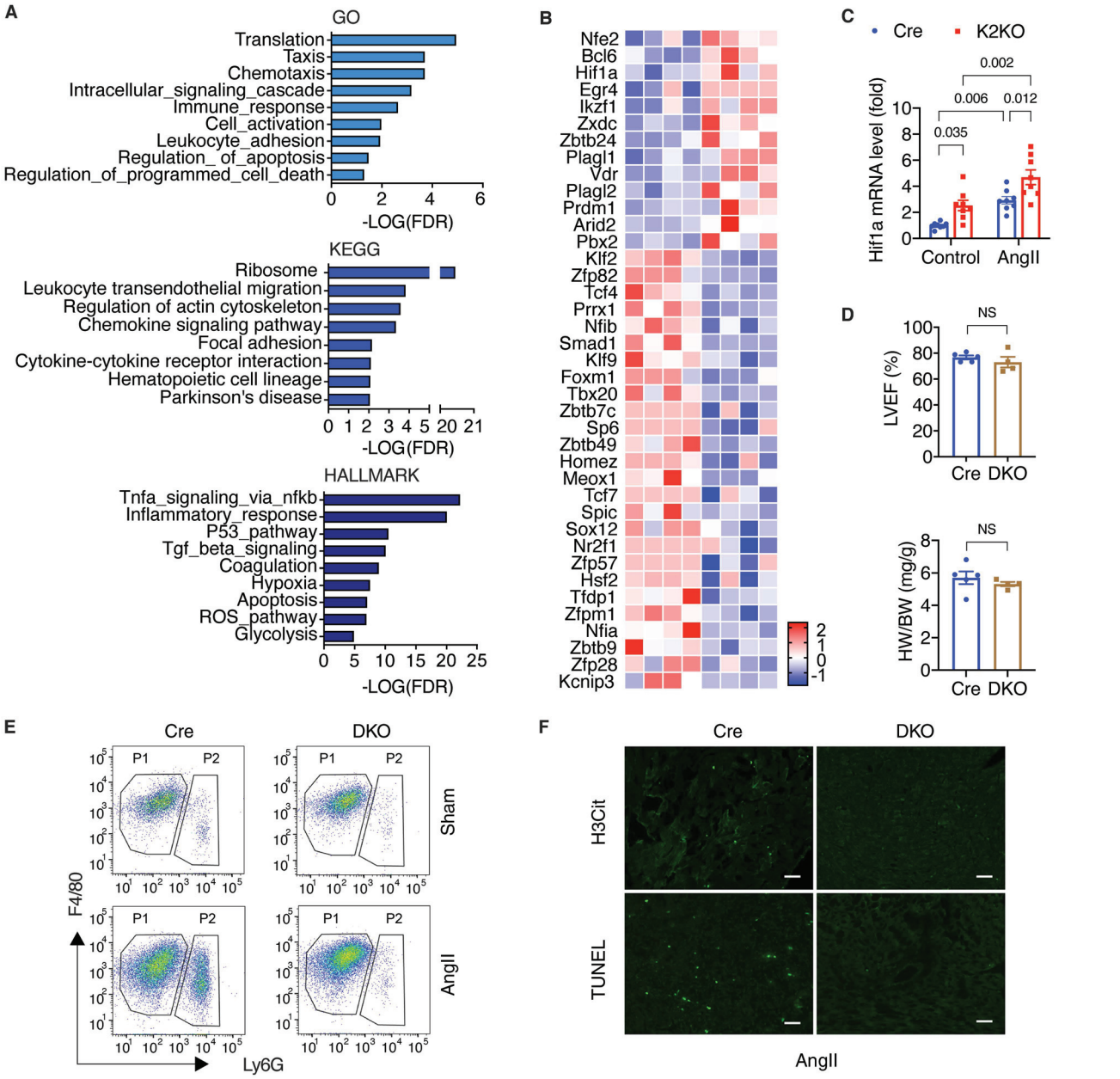

Figure 9. Transcriptomic studies identify KLF2 as a nodal regulator in neutrophils.
(**A** and **B**) Pathway enrichment analysis and Heatmap of all transcription factors in neutrophil DEGs (Cre-AngII vs. K2KO-AngII). RNA-seq studies included 4 animals in each group. (**C**) HIF1α expression in neutrophils (n=6). Treatment: AngII 100 nmol/L for 0.5h in vitro. (**D-F**) Lyz2-Cre (Cre) vs. Lyz2-Cre-KLF2-HIF1a double knockout (DKO) mice (n=4-5). (D) Cardiac function and hypertrophy. (**E**) FACS analysis of cardiac myeloid cells. (**F**) Intracardiac NETs formation (H3cit) and cell death (TUNEL). Scale bar: 25 μm. Representative FACS and immunofluorescence images from individual animal (n=5). AngII infusion: (D) 4 weeks. (E and F) 1 week. (C) P values were from post-hoc test of Two-way ANOVA with Tukey correction. (D) NS indicates not significant by 2-tailed unpaired Student t test.

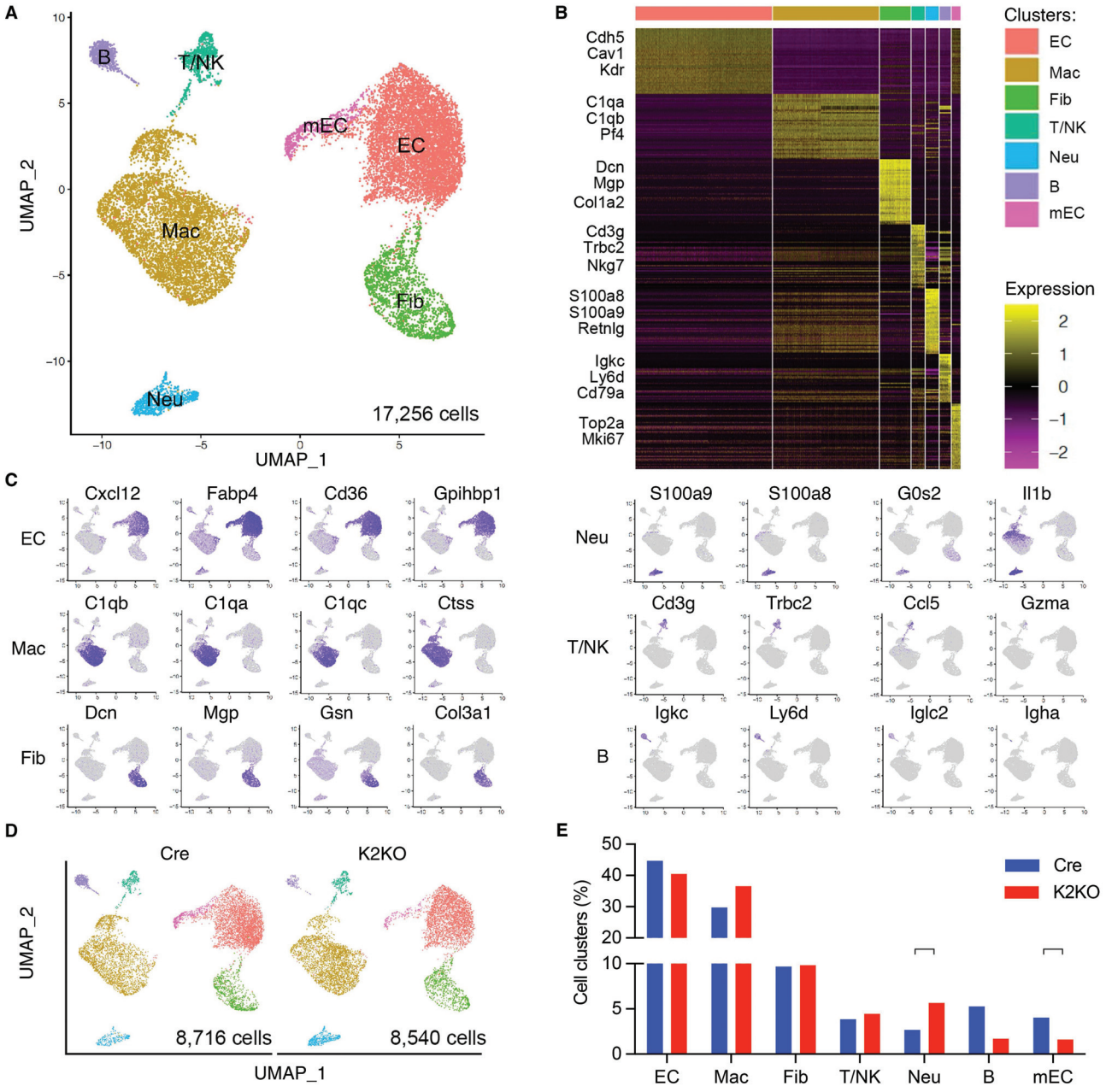

Figure 10. Single-cell RNA-seq study identifies the major non-cardiomyocyte cell types that regulate cardiac dysfunction.

(**A**) Uniform Manifold Approximation and Projection for dimension reduction (UMAP) and unsupervised clustering analysis using Seurat pipeline identified seven distinct cell populations from the total 17,256 cells. EC: endothelial cell; Mac: macrophage; Fib: fibroblast; T/NK: T cell and NK cell; Neu: neutrophil; B: B cell; mEC: mitotic endothelial cell. (**B**) Heatmap of Top 50 marker genes for each cluster. Selected cell type-specific markers labeled. (**C**) FeaturePlot depicting gene expression on UMAP. (**D**) UMAP of 8,716 Cre cells and 8,540 K2KO cells showing seven cell populations. (**E**) Percentage of each cell cluster in Cre and K2KO groups. Differences in neutrophils (Neu) and mitotic endothelial cells (mEC) were noted. Cells isolated from 3 mice in each group were pooled before FACS sorting. Two pooled samples (Cre vs. K2KO) were single-cell captured and sequenced.

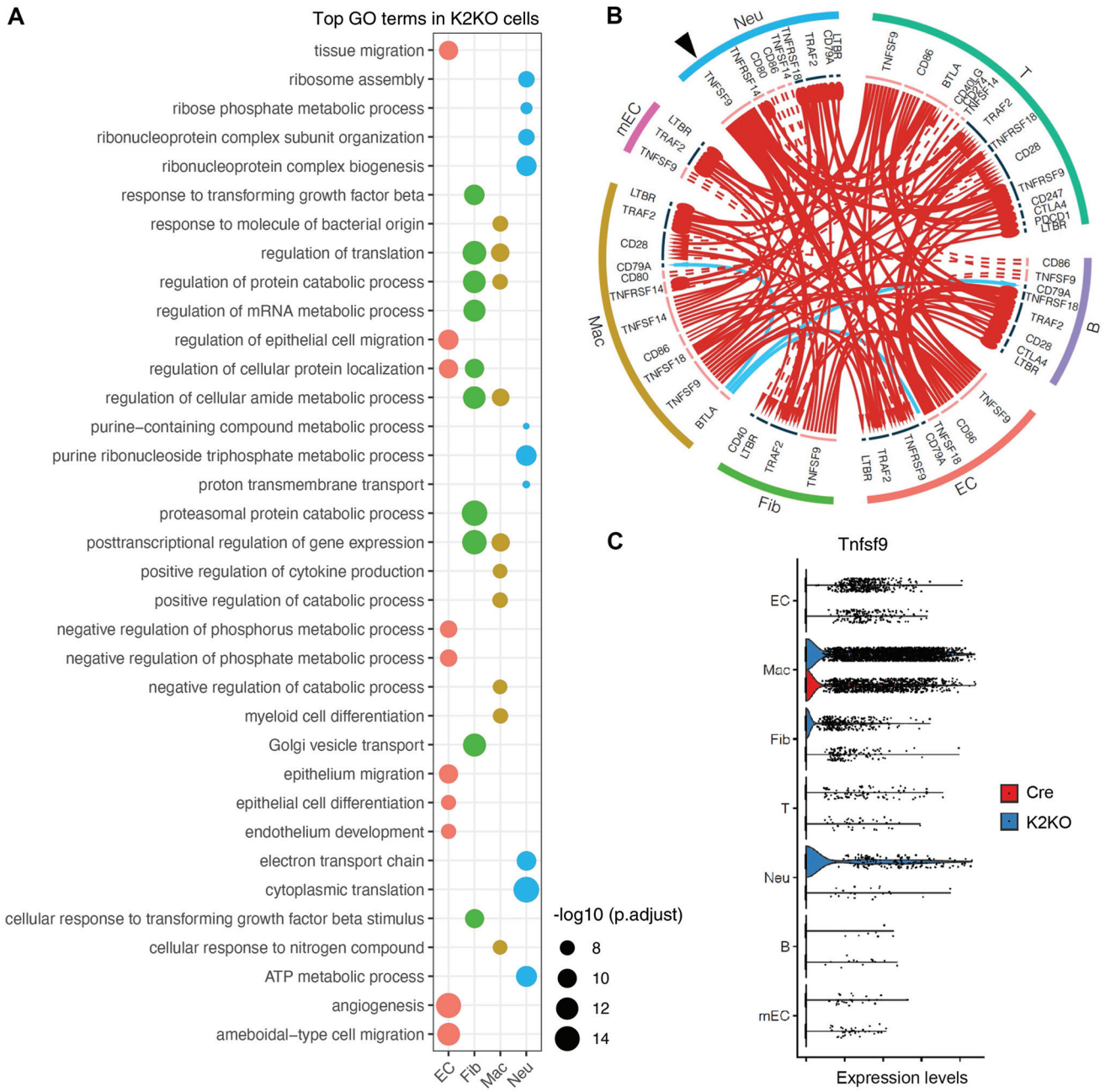

Figure 11. Neutrophils orchestrate myocardial inflammation and adaptation to AngII stress.
(**A**) Gene ontology (GO) analyses with K2KO DEGs from 4 major cell types: neutrophils, macrophages, endothelial cells, and fibroblasts, showing Top 10 Biological Process (BP) GO terms according to adjusted P values (p.adjust). (**B**) Cell-cell interactome analysis of all significant seven cell types based on the ligand-receptor communication. Arrow: red = up-regulated, blue = down-regulated, arrowhead = receptor level changed, circle head = receptor level NOT changed, solid line = ligand level changed, dotted line = ligand level NOT changed, line thickness and head size represent relative fold change values. (**C**) Tnfsf9 mRNA expression levels in all significant seven cell types are shown as Violin plot.

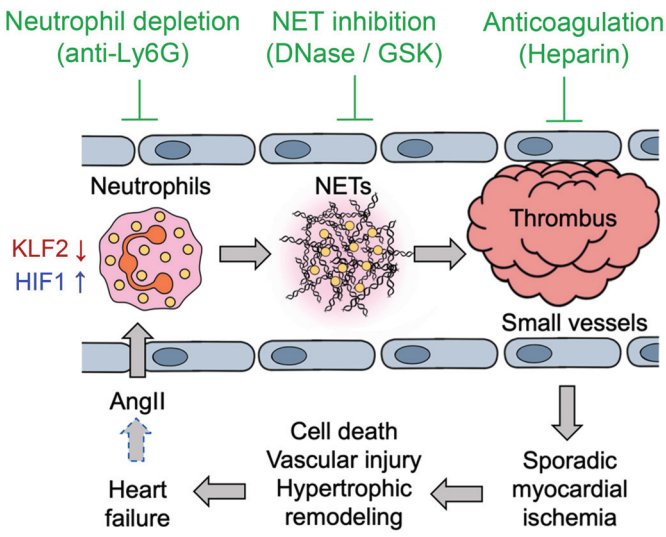

Figure 12. Working model.
AngII-induced NETosis results in microthrombosis and sporadic ischemia in the myocardium, promoting cardiac hypertrophy. In HF patients, hyperphysiological AngII levels due to heightened RAS may propel this vicious cycle (dashed arrow). This model suggests novel therapeutic approaches for HF by targeting neutrophils, NETs, or thrombosis.