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PRECLINICAL RESEARCH

Clec4e-Receptor Signaling in Myocardial Repair After Ischemia-Reperfusion Injury

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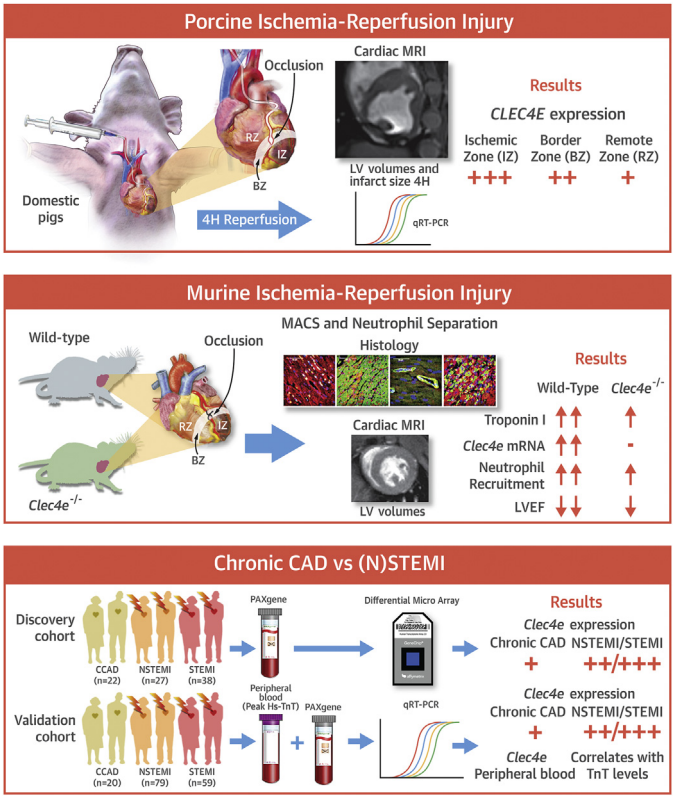
VISUAL ABSTRACT



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HIGHLIGHTS

- **The role of the CLEC4E during myocardial healing after ischemia-reperfusion injury is unknown.**
- **CLEC4E deletion is associated with reduced cardiac injury, inflammation, and left ventricular structural and functional remodeling.**
- **CLEC4E is a promising target to modulate myocardial inflammation and enhance repair after ischemia-reperfusion injury.**

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The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

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ABBREVIATIONS AND ACRONYMS

ACS = acute coronary syndrome

AMI = acute myocardial infarction

ANOVA = analysis of variance

CAD = coronary artery disease

Car3 = carbonic anhydrase 3

CLEC4E = C-type lectin domain family 4 member E

CMC = cardiac myocyte

Cxcl2 = CXC chemokine ligand 2

Cxcr2 = CXC chemokine receptor 2

DAMP = damage-associated molecular pattern

ECM = extracellular matrix

Efna2 = ephrin A2

ESV = end-systolic volume

Grk2 = G protein–coupled receptor kinase 2

hs-TnI = high-sensitivity troponin I

I/R = ischemia-reperfusion

LAD = left anterior descending coronary artery

LV = left ventricular

MPO = myeloperoxidase

MRI = magnetic resonance imaging

NS = not significant

PRR = pattern recognition receptor

qRT-PCR = quantitative reverse transcription polymerase chain reaction

RNA = ribonucleic acid

SMC = smooth muscle cell

STEMI = ST-segment elevation myocardial infarction

TnT = troponin T

WT = wild-type

SUMMARY

The bacterial C-type lectin domain family 4 member E (CLEC4E) has an important role in sterile inflammation, but its role in myocardial repair is unknown. Using complementary approaches in porcine, murine, and human samples, we show that *CLEC4E* expression levels in the myocardium and in blood correlate with the extent of myocardial injury and left ventricular (LV) functional impairment. *CLEC4E* expression is markedly increased in the vasculature, cardiac myocytes, and infiltrating leukocytes in the ischemic heart. Loss of *Clec4e* signaling is associated with reduced acute cardiac injury, neutrophil infiltration, and infarct size. Reduced myocardial injury in *Clec4e*$^{-/-}$ translates into significantly improved LV structural and functional remodeling at 4 weeks' follow-up. The early transcriptome of LV tissue from *Clec4e*$^{-/-}$ mice versus wild-type mice reveals significant upregulation of transcripts involved in myocardial metabolism, radical scavenging, angiogenesis, and extracellular matrix organization. Therefore, targeting *CLEC4E* in the early phase of ischemia-reperfusion injury is a promising therapeutic strategy to modulate myocardial inflammation and enhance repair after ischemia-reperfusion injury. (J Am Coll Cardiol Basic Trans Science 2021;6:631–646)

Cardiac repair after myocardial infarction is a highly orchestrated and complex cascade of events characterized by an initial inflammatory phase with immune cell infiltration and release of danger-associated molecular patterns (DAMPs) that subsequently transits into a reparative and proliferative phase (1-3). The early inflammatory response with binding of DAMPS to cognate pattern recognition receptors (PRRs) needs to be properly and timely coordinated to allow subsequent tissue repair and prevent maladaptive left ventricular (LV) remodeling, defective scar formation, and impaired patient outcome (4,5). Excessive or prolonged inflammation would result in ventricular dilatation and systolic dysfunction, putting patients at increased risk for developing heart failure (6), whereas deficient early inflammation would fail to clear necrotic cardiac cells and matrix components and hamper subsequent tissue repair. This complex and tightly regulated innate immune activation response emphasizes the critical need for a better understanding of the cellular and molecular pathways governing this biphasic repair process after ischemia-reperfusion (I/R) injury.

Previously, we investigated the temporal changes in transcriptional profile of patients with an acute myocardial infarction (AMI) in circulating blood cells (7). We confirmed a robust activation of pro-inflammatory pattern recognition receptors, including the C-type lectin domain family 4 member E receptor (CLEC4E) that is usually expressed on leukocytes and activated in response to bacteria (8,9). This innate immune receptor senses necrotic material induced by ischemic injury in vitro (10) and adversely modulates the inflammatory response in experimental murine brain injury (11,12). However, the role of CLEC4E in myocardial I/R injury remains unknown.

The current study investigated the role of CLEC4e signaling on the early inflammatory response and the subsequent healing phase after acute myocardial ischemic injury, and explored its potential as a new target for intervention in myocardial I/R injury.

METHODS

ETHICS. All animal procedures were performed according to the Belgian law on care and use of experimental animals and were approved by the Ethics Committee for Animal Experimentation at KU Leuven (P244/2014 and P064/2017). The patient study protocol complied with the Declaration of Helsinki, was approved by the regional ethical committee, and all patients signed informed consent (Ethical Committee ML8525, Belgian trial no. B322201214942, S54129) (7).

PORCINE MODEL OF I/R INJURY AND CARDIAC MAGNETIC RESONANCE IMAGING. Ten domestic pigs (body weight: 20-30 kg) were sedated, anesthetized, and underwent I/R injury by transient balloon occlusion of the left anterior descending coronary artery (LAD) distal to the first diagonal branch for 50 minutes, followed by 4 hours' reperfusion, as previously described (7). After 4 hours' reperfusion, a subgroup of 6 pigs underwent 3-T cardiac magnetic resonance imaging (MRI) (Prisma-Tim, Siemens) imaging to evaluate infarct size (MI/left ventricle), end-systolic volume (ESV), end-

diastolic volume, and ejection fraction, followed by euthanasia (13). Biopsy specimens from ischemic, border, and remote zones of the left ventricle were collected for differential gene expression analysis and histopathology. Details are provided in the Supplemental Materials and Methods, and Supplemental Table 1.

MOUSE MODEL OF I/R INJURY AND CARDIAC MRI. Twelve- to 14-week-old male C57Bl6/J wild-type (WT) mice were cross-bred with *Clec4e*$^{-/-}$ mice (031936-UCD), obtained at the Mutant Mouse Resource and Research Center. Mice underwent I/R injury by 60 minutes' LAD ligation followed by reperfusion, as described previously (14). They were randomly allocated to evaluate arms with 24 hours' (WT, n = 12; Clec4e$^{-/-}$, n = 14), 72 hours' (WT, n = 5; Clec4e$^{-/-}$, n = 5), or 4 weeks' (WT, n = 17; Clec4e$^{-/-}$, n = 16) reperfusion. Cardiac MRI data were acquired at 4 weeks' reperfusion on a Bruker BioSpec 70/30 7T MRI system (Bruker BioSpin) by investigators blinded to the genotype of the mice. After euthanasia, blood was collected from the inferior caval vein to measure high-sensitivity troponin I (hs-TnI) as a surrogate marker of cardiac injury and to isolate neutrophils to study their phenotype and migration capacity; LV tissue was collected for histological analysis and transcriptome studies at 24 hours after I/R injury. Organs were perfused with saline for 10 minutes before harvesting for histological and differential gene expression analysis using ribonucleic acid (RNA)-sequencing and quantitative real-time polymerase chain reaction (qRT-PCR). Additional details are provided in the Supplemental Materials and Methods.

IN VITRO ANALYSIS OF CLEC4E GENE DELETION ON CHEMOKINE SIGNALING. Peripheral blood neutrophils were isolated from WT and *Clec4e*$^{-/-}$ mice by using the Neutrophil Isolation Kit for mice (130-097-658, Miltenyi), according to the manufacturer's instructions. To study the effect of Clec4e on neutrophil migration, a transwell migration assay toward a Cxcl2 gradient (10 ng/mL) was performed by using WT and *Clec4e*$^{-/-}$ neutrophils. To investigate whether Clec4e influences Cxcr2 protein expression, bone marrow cells were collected from the femurs of WT and *Clec4e*$^{-/-}$ mice as previously described (15). Proteins were then isolated by using radioimmunoprecipitation assay buffer supplemented with protease inhibitors, and Cxcr2 expression was analyzed according to immunoblot analysis. Additional details are provided in the Supplemental Materials and Methods.

ANALYSIS OF MICROARRAY CLEC4E EXPRESSION AND VALIDATION WITH qRT-PCR IN PATIENTS WITH AMI. We identified *CLEC4E* as one of the top upregulated transcripts in a profiling study in 65 patients with acute coronary syndrome (ACS) (GEO accession number GSE123342) (7). We validated *CLEC4E* expression at the time of admission in an extended cohort of 138 patients with AMI via qRT-PCR and in 20 patients with stable coronary artery disease (CAD). CLEC4E whole blood transcript levels were then correlated with peak high-sensitivity troponin T (TnT) levels and LV ejection fraction before discharge.

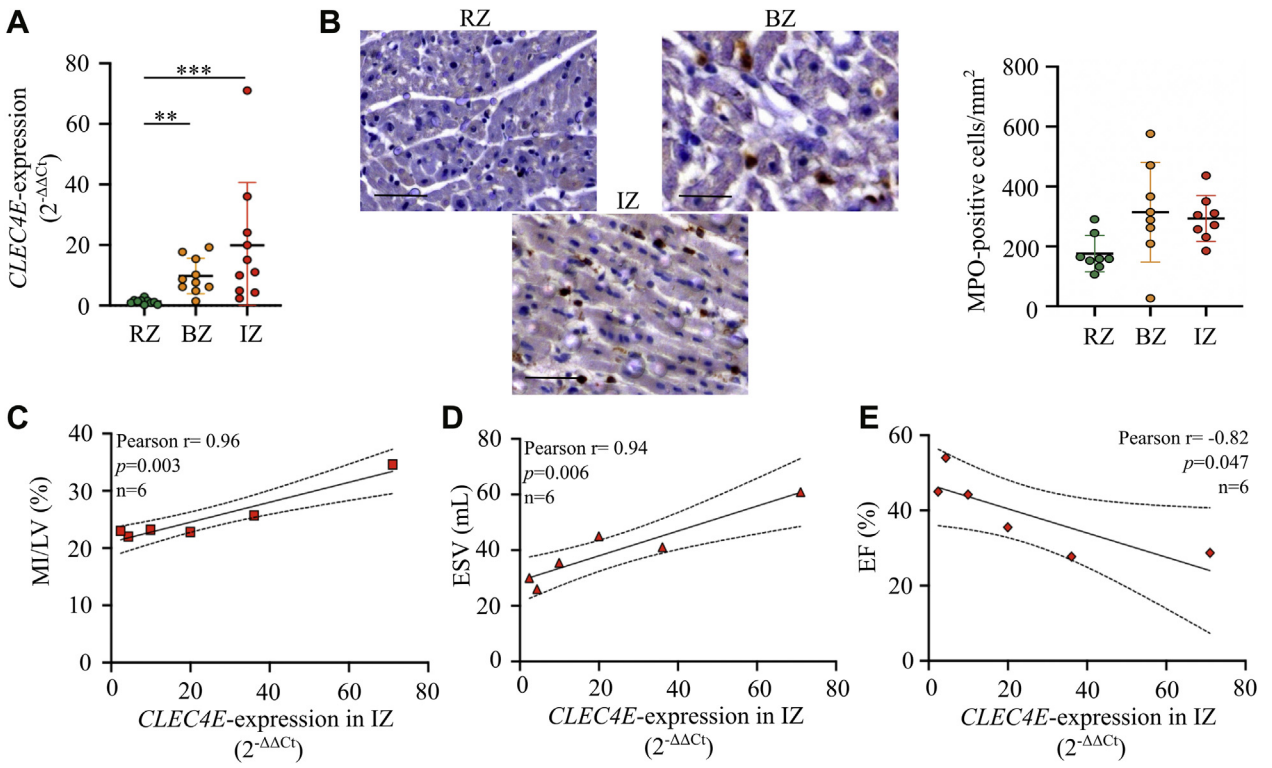
STATISTICAL ANALYSIS. Data are shown as mean ± SD or median (interquartile range) for the number of animals studied. Data analysis was performed blinded, and normal distributions were tested by using Shapiro-Wilk and Kolmogorov-Smirnov tests. *CLEC4E* expression in porcine tissue was analyzed by using one-way analysis of variance (ANOVA) with Tukey's correction for multiple pairwise testing. *CLEC4E* expression in human peripheral blood samples was analyzed by using a Kruskal-Wallis test with Dunn's correction for multiple comparisons. Intergroup differences were analyzed by using a 2-tailed Student's *t*-test for normally distributed data or nonparametric Mann-Whitney *U* test for non-normally distributed data. For experiments performed at different time points (24 and 72 hours), 2-way ANOVA with Šidák's correction for multiple testing was performed. Pearson correlations were used for normally distributed data and Spearman correlations for non-normally distributed data to analyze linear or general associations between variables, respectively. Categorical variable differences were determined with the chi-square test or Fisher exact test and are presented with the odds ratio and 95% confidence intervals. P values < 0.05 were considered statistically significant. The statistical analyses were performed by using Prism version 8.0 software (GraphPad Software).

Details regarding the extended methods are provided in the Supplemental Materials and Methods section, and in Supplemental Figures 1, 2, 3, and 4.

RESULTS

CLEC4E EXPRESSION LEVELS IN THE ISCHEMIC PORCINE MYOCARDIUM AND CORRELATION WITH LV FUNCTION. To examine whether *CLEC4E* expression is upregulated in the heart after I/R injury, we collected 3 different regions of porcine heart tissue 4 hours after I/R injury (remote zone, border zone, and ischemic core, n = 10) and measured *CLEC4E* expression levels. *CLEC4E* expression was significantly upregulated in both the ischemic core and border zone, with the highest levels in the ischemic

FIGURE 1 ***CLEC4E*** **Is Significantly Upregulated in the Ischemic Myocardium and Correlates With Infarct Size and Left Ventricular Function in the Acute Inflammatory Response After I/R Injury in Pigs**



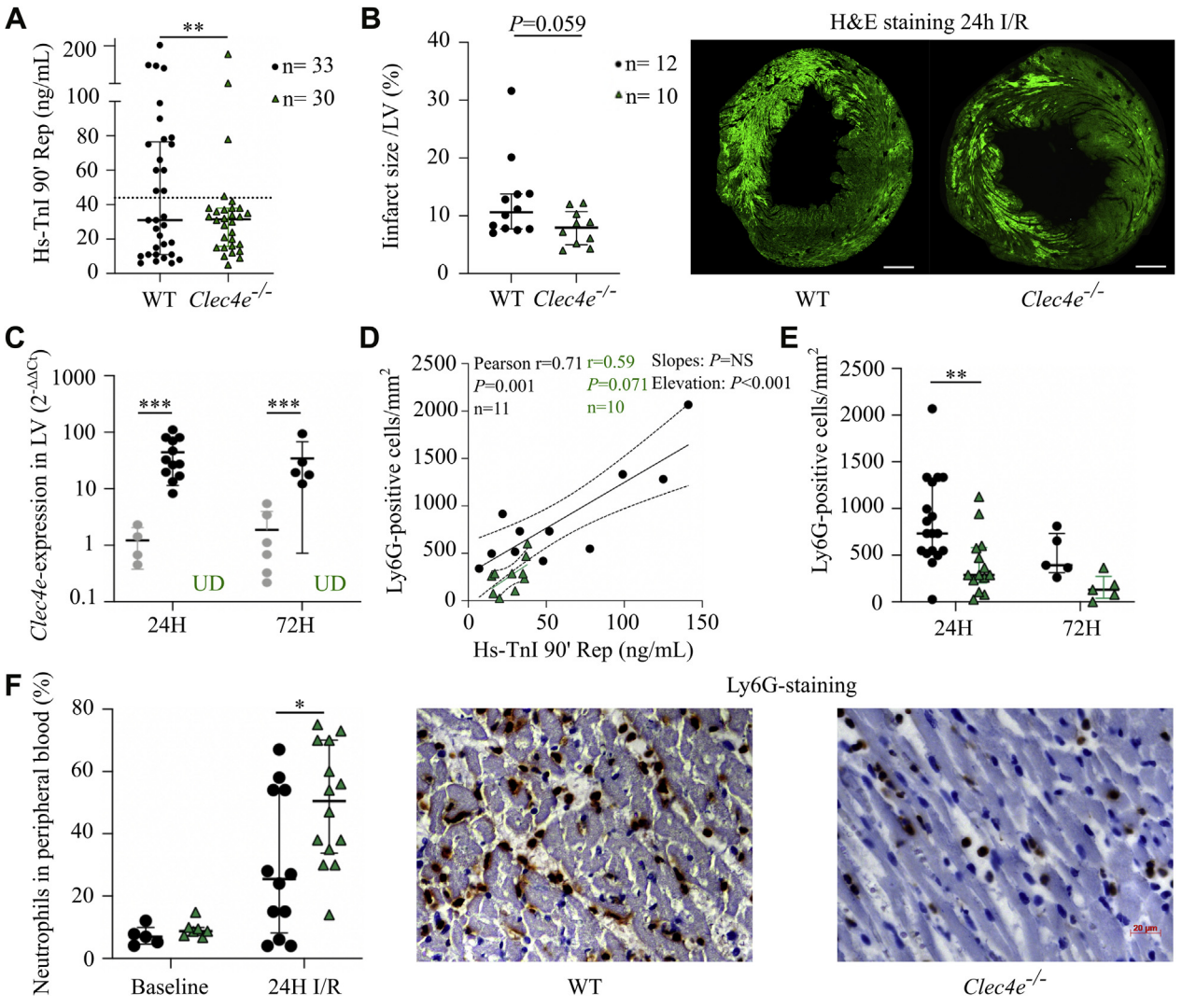
(A) Quantitative reverse transcription polymerase chain reaction of C-type lectin domain family 4 member E (CLEC4E) expression (mean with SD, $2^{-\Delta\Delta Ct}$) in porcine remote zone (RZ), border zone (BZ), and ischemic core zone (IZ) heart tissue, normalized to peptidylprolyl isomerase A (n = 10). **(B)** Quantification of myeloperoxidase (MPO)-positive cells/mm^2 (mean ± SD) in RZ, BZ, and IZ heart tissue. **(C to E)** Pearson correlations of CLEC4E expression in IZ versus infarct size as percentage of left ventricular mass (myocardial infarction [MI]/left ventricle [LV], %), left ventricular end-systolic volume (ESV), and ejection fraction (EF) (n = 6). One-way analysis of variance test, with Tukey's correction for multiple testing. **Dotted lines** indicate 90% CI. $^{**}P < 0.01$, $^{***}P < 0.001$. Scale bar = 20 μm. I/R = ischemia-reperfusion.

core (P = 0.004 and P < 0.001 vs remote zone, respectively) (Figure 1A).

To investigate whether increased *CLEC4E* expression in the border zone and ischemic core can be accounted for by infiltrating inflammatory cells, which in the acute phase are predominantly neutrophils, we performed immunohistochemical stains for neutrophil-specific myeloperoxidase (MPO) and measured the number of MPO-positive cells (Figure 1B). However, neutrophil infiltration did not correlate with LV *CLEC4E* expression (Pearson r = 0.12; P = not significant [NS]; n = 8), suggesting that *CLEC4E* was also upregulated in resident or parenchymal cells within the ischemic myocardium. The latter is consistent with our findings that *CLEC4E* expression in the ischemic core positively correlates with MRI-based measurements of infarct size (MI/left ventricle) (r = 0.96; P = 0.003) (Figure 1C) and LV ESV (r = 0.94; P = 0.006) (Figure 1D), and inversely relates to LV ejection fraction (r = -0.82; P = 0.047) (Figure 1E).

LEUKOCYTE RECRUITMENT AND *CLEC4E*-EXPRESSION IN THE INFARCTED MURINE HEART DURING THE ACUTE INFLAMMATORY PHASE OF I/R INJURY. To investigate how *Clec4e* gene function modulates the myocardial response to acute ischemic injury, transient ligation of the LAD was performed in *Clec4e*$^{-/-}$ mice and WT control mice. We first examined whether *Clec4e* gene function affects the initial ischemic injury. Significantly greater ischemic injury 90 minutes after reperfusion was measured in WT mice (n = 33) versus *Clec4e*$^{-/-}$ mice (n = 30), as evidenced by the greater proportion of mice with plasma hs-TnI levels exceeding the average value of 44 ng/mL (P = 0.006) (Figure 2A). The data suggest that the odds of having

FIGURE 2 ***Clec4e*** **Expression Is Upregulated in the Murine Heart, and** ***Clec4e*** **Signaling Influences the Recruitment of Leukocytes After I/R Injury**

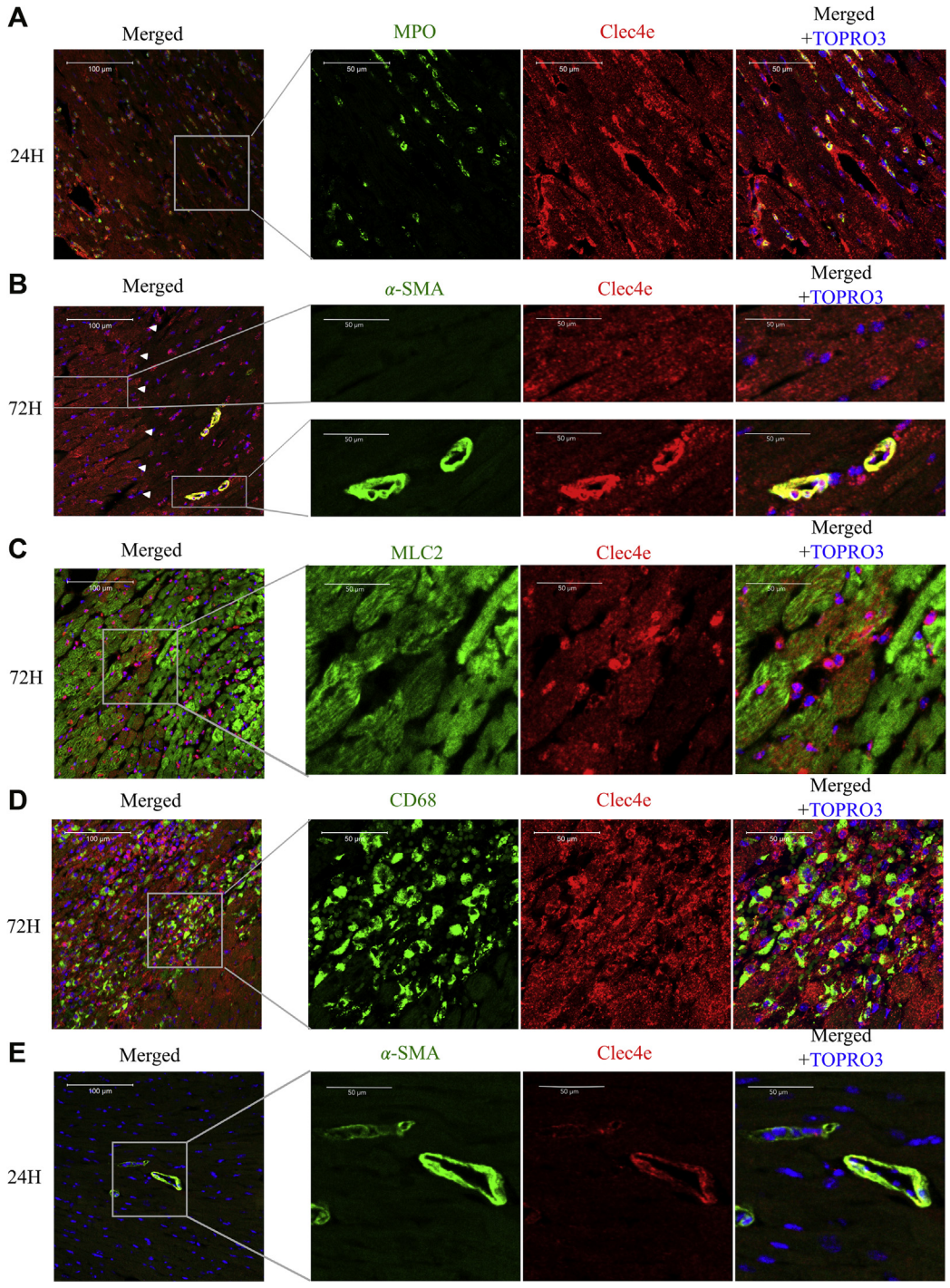


(A) High-sensitivity troponin I (hs-TnI) plasma levels at 90 minutes' reperfusion (Rep) after 60 minutes' ischemia (wild-type [WT], n = 33; *Clec4e*$^{-/-}$, n = 30); Mann-Whitney *U* test. **(B)** Infarct size in the left ventricle (LV) based on hematoxylin and eosin (H&E) staining under 488 nm wavelength light to visualize autofluorescence of necrotic cells, with representative sections per genotype. Scale bar = 500 μm. **(C)** Quantitative reverse transcription polymerase chain reaction of *Clec4e* expression (log_2-fold) in left ventricular tissue in sham-operated mice **(gray)**, WT control mice **(black)**, and *Clec4e*$^{-/-}$ mice **(green)** 24 hours (n = 4, n = 12, and n = 10, respectively) and 72 hours (n = 6, n = 5, and n = 5) after I/R injury. Two-way analysis of variance (ANOVA) with Šidák's correction. **(D)** Pearson correlation between TnI at 90 minutes' reperfusion versus lymphocyte antigen 6 complex locus G6D (Ly6G)-positive cells at 24 hours' reperfusion in WT (n = 11) and *Clec4e*$^{-/-}$ (n = 10) mice. **(E)** Number of Ly6G-positive cells in the LV/mm^2 in WT control mice (and *Clec4e*$^{-/-}$ mice (n = 14) at 24 hours (n = 18 and n = 14, respectively) and 72 hours (n = 5 and n = 5). Two-way ANOVA with Šidák's correction. **(F) Left:** Percent neutrophils in peripheral blood at baseline and 24 hours (n = 12 and n = 14). Two-way ANOVA with Šidák's correction. **Right:** Ly6G-staining of left ventricular tissue in WT and *Clec4e*$^{-/-}$ mice at 24 hours. Scale bar = 20 μm. Results are shown as mean with SD or as median with interquartile range. **Dotted lines** indicate 95% confidence interval. *P < 0.05, **P < 0.01, ***P < 0.001. NS = not significant; UD = undetectable; other abbreviations as in Figure 1.

TnI above the average of 44 ng/mL is 5.4 times higher in WT mice compared with *Clec4e*$^{-/-}$ mice (odds ratio: 5.4; 95% CI: 1.6-16.5), despite having comparable risk areas after occlusion of the LAD (Supplemental Figure 1A). The difference observed in cardiac necrosis markers at 90 minutes is consistent with a trend toward reduced infarct size (P = 0.059) in *Clec4e*$^{-/-}$ mice 24 hours after I/R injury (Figure 2B).

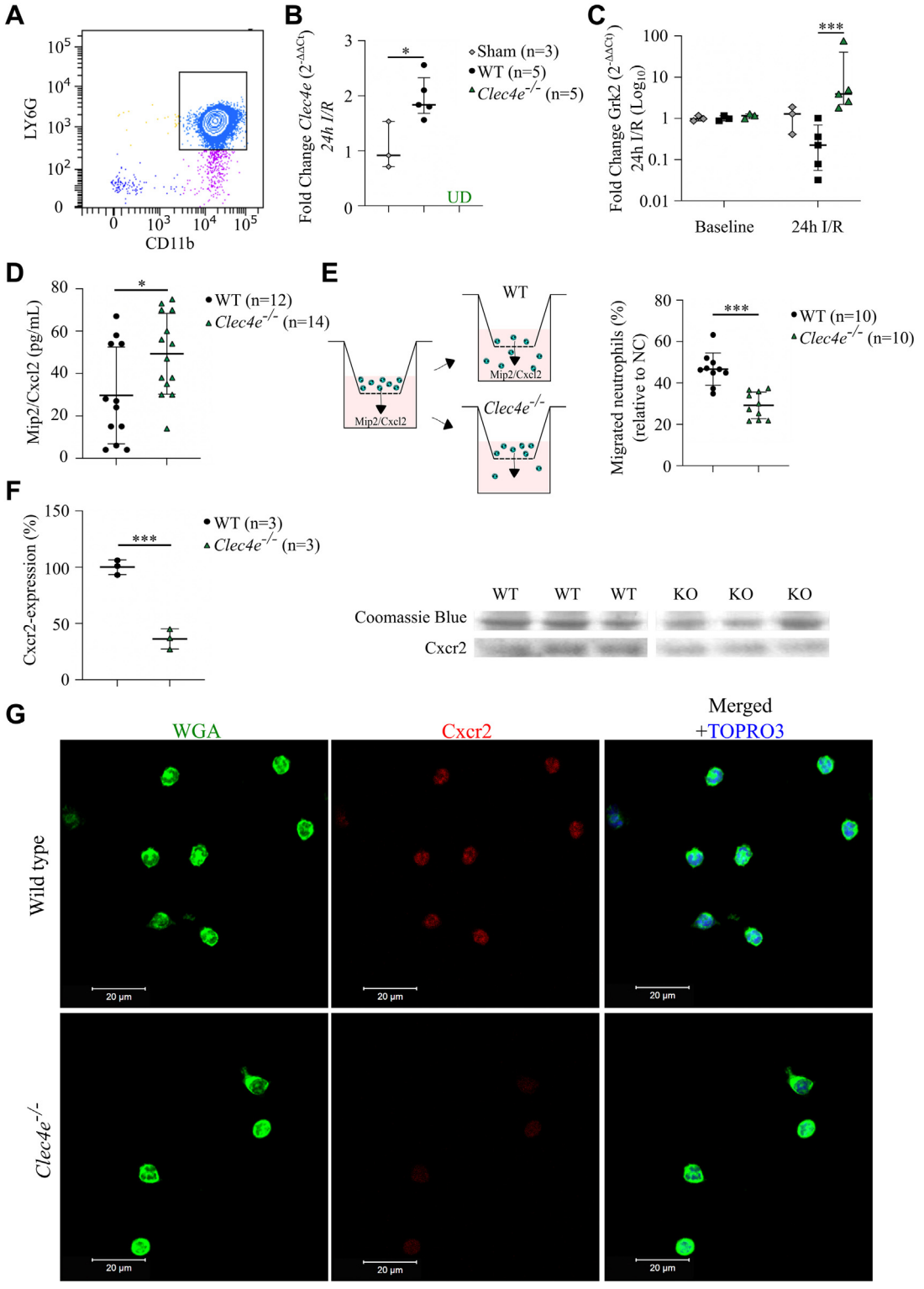
We next measured *Clec4e* expression in the murine heart at 24 and 72 hours after I/R injury using qRT-

FIGURE 3 Temporal and Spatial Clec4e Expression in the Ischemic Heart 24 and 72 Hours After I/R Injury



Heart tissue was stained for Clec4e **(red)** in combination with antibodies against MPO to identify neutrophils **(green)** at 24 h' post-I/R injury **(A)** and *α-smooth muscle actin* (α-*SMA*) to highlight smooth muscle cells **(green)** 72 h' post-I/R injury **(B)**. **Upper panels** show viable BZ tissue, and **lower panels** show IZ tissue. MLC2 to visualize cardiomyocytes **(green) (C)** and CD68 identified infiltrating monocytes/macrophages **(D)** at 72 h' post-I/R injury. **(E)** Sections from the RZ were stained with α-SMA antibody at 24 h' post-I/R injury. Nuclei are counterstained with TO-PRO-3 (**blue;** Life Technologies). Scale bar is 100 μm in **left panel** (merged) and 50 μm in 3 **right panels**. **Left panel in A**, 20× magnification; **3 right panels,** 40× magnifications. Abbreviations as in Figure 1.

FIGURE 4 **Potential Mechanism for Reduced Neutrophil Infiltration in Response to I/R Injury**



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PCR and found significant upregulation of *Clec4e* transcript levels in WT mice compared with sham mice at 24 and 72 hours (both, $P < 0.001$) (Figure 2C). To investigate whether increased *Clec4e* signaling is associated with recruitment of different leukocyte subpopulations in the infarcted myocardium during the early post-I/R inflammatory phase, we measured neutrophil infiltration in the left ventricle at 24 hours' reperfusion and monocyte/macrophage infiltration at 72 hours' reperfusion. Early TnI plasma levels significantly correlated with the number of infiltrating neutrophils at 24 hours in a similar way in both genotypes (P = NS) (Figure 2D), but the slope in *Clec4e*$^{-/-}$ was significantly lower ($P < 0.001$). The number of infiltrating lymphocyte antigen 6 complex locus G6D-positive neutrophils in *Clec4e*$^{-/-}$ mice at 24 hours was significantly decreased compared with WT mice ($P = 0.003$) (Figure 2E), while neutrophil counts in peripheral blood were significantly higher ($P = 0.025$) (Figure 2F). At 72 hours, monocytes/macrophages (lysosome-associated membrane protein 2 [Mac3]) infiltration was significantly and similarly increased in the left ventricle of WT and *Clec4e*$^{-/-}$ mice compared with findings at the 24-hour time point (both, $P < 0.001$). Consistent with our earlier observations in pigs, no correlation between *Clec4e* expression in the ischemic murine myocardium and the number of infiltrating neutrophils was observed (Pearson r = -0.38; P = NS; n = 12).

To determine whether Clec4e expression is increased in parenchymal cardiac cells upon I/R injury, we performed immunohistochemical analysis on midventricular heart sections of WT C57Bl6/J mice at 24 and 72 hours after I/R injury. Clec4e protein expression co-localizes with MPO-positive cells, with cardiac myocytes (CMCs), and in muscularized vasculature 24 hours after I/R injury (Figure 3A). At 72 hours after I/R injury, Clec4e expression is markedly increased in smooth muscle cells (SMCs), infiltrating macrophages, and CMCs (Figures 3B to 3D), more specifically in viable CMCs at the border zone (Figure 3B, left inset). Interestingly, Clec4e expression also co-localizes with CD68, a marker for monocytes and macrophages in the ischemic myocardium. In remote, nonischemic myocardium, Clec4e immunoreactivity was only detectable in SMCs, although much less than in ischemic myocardium (Figure 3E). Together, these results indicate that Clec4e is not only expressed by infiltrating inflammatory cells but also by resident myocardial parenchymal and vascular cells.

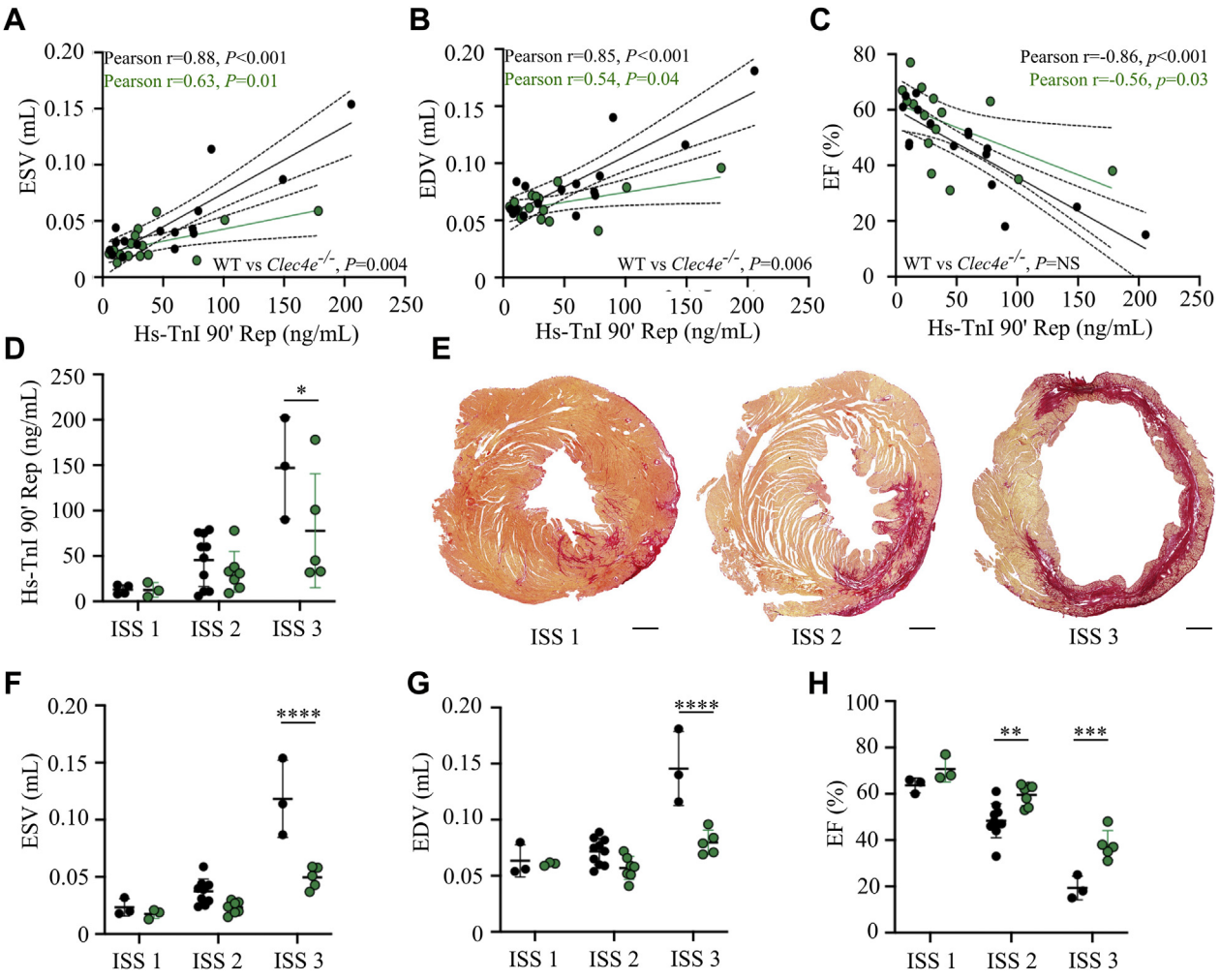
Effects of *Clec4e* deletion on chemokine signaling. To explore the potential mechanism of reduced neutrophil infiltration in *Clec4e*$^{-/-}$ mice, we investigated the effect of *Clec4e*$^{-/-}$ on chemokine signaling. One of the most important chemokine receptors responsible for neutrophil recruitment is CXC chemokine receptor 2 (CXCR2), post-translationally regulated by the G protein–coupled receptor kinase 2 (Grk2) (16). Grk2 is responsible for the phosphorylation of chemokine receptors, leading to internalization, receptor desensitization, and reduced responsiveness of neutrophils to migrate toward chemokine gradients produced during inflammation (17,18). To investigate whether Clec4e signaling alters *Grk2* expression in the setting of sterile inflammation induced by I/R injury, we isolated peripheral blood neutrophils from WT and *Clec4e*$^{-/-}$ mice 24 hours after I/R injury (Figure 4A) and performed qRT-PCR for *Clec4e* and *Grk2*. We measured significant upregulation of *Clec4e* in WT mice compared with sham mice ($P = 0.016$) (Figure 4B). There was no difference in *Grk2* expression levels in neutrophils from WT (n = 3) and *Clec4e*$^{-/-}$ (n = 3) mice at baseline (Figure 4C). Interestingly, at 24 hours' post-I/R injury, *Grk2* expression levels were downregulated in WT mice but significantly increased in *Clec4e*$^{-/-}$ mice ($P < 0.001$). In addition, increased Cxcl2 levels were recorded in plasma at 24 hours' post-I/R injury ($P = 0.025$) (Figure 4D).

To determine whether *Clec4e*$^{-/-}$ neutrophils showed reduced migration toward the Cxcr2 receptor CXC chemokine ligand 2 (Cxcl2), we performed a Cxcl-induced migration assay, using freshly isolated

FIGURE 4 Continued

(A) Flow cytometry confirms isolation of neutrophils, positive for both LY6G and cluster of differentiation 11 b (CD11b) antigens. **(B)** Quantitative reverse transcription polymerase chain reaction of isolated neutrophils from sham (n = 3), WT (n = 5), and *Clec4e*$^{-/-}$ mice 24 h' post-I/R injury. *Clec4e* expression. **(C)** Baseline (n = 3 vs 3) and 24-h G protein–coupled receptor kinase 2 (*Grk2*) expression in WT and *Clec4e*$^{-/-}$ mice (n = 5 vs 5). Two-way ANOVA with Šidák's correction. **(D)** Cxcl2 plasma levels in WT (n = 12) and *Clec4e*$^{-/-}$ (n = 14) mice 24 h' post-I/R injury; Student's *t*-test. **(E)** Neutrophil migration assay toward Mip2/Cxcl2-chemokine gradient in WT (n = 10) and *Clec4e*$^{-/-}$ (n = 10) mice, normalized to negative control (NC) (medium alone); Student's *t*-test. **(F) Right**: Cxcr2 expression levels in isolated bone marrow–derived cells, normalized to average Cxcr2 expression in WT value in n = 3 for both genotypes. **Left:** Image of Coomassie blue stain (loading control) and Cxcr2 Western blot. Results are shown as mean ± SD or median with interquartile range. **(G)** Neutrophils isolated from health WT and Clec4e-/- mice were stained for the chemokine receptor Cxcr2 **(Red)** in combination with wheat-germ agglutinin (WGA; **Green**) to delineate cell membranes. Nuclei are counterstained with TO-PRO-3 (**Blue**; Life Technologies). Scale bar is 20 μm. *$P < 0.05$, ***$P < 0.001$. Scale bar = 20 μm. KO = knockout; *WGA = wheat germ agglutinin*; other abbreviations as in Figures 1 and 2.

FIGURE 5 **Cardiac MRI in WT Control Mice and Clec4e$^{-/-}$ Mice 4 Weeks After I/R Injury**



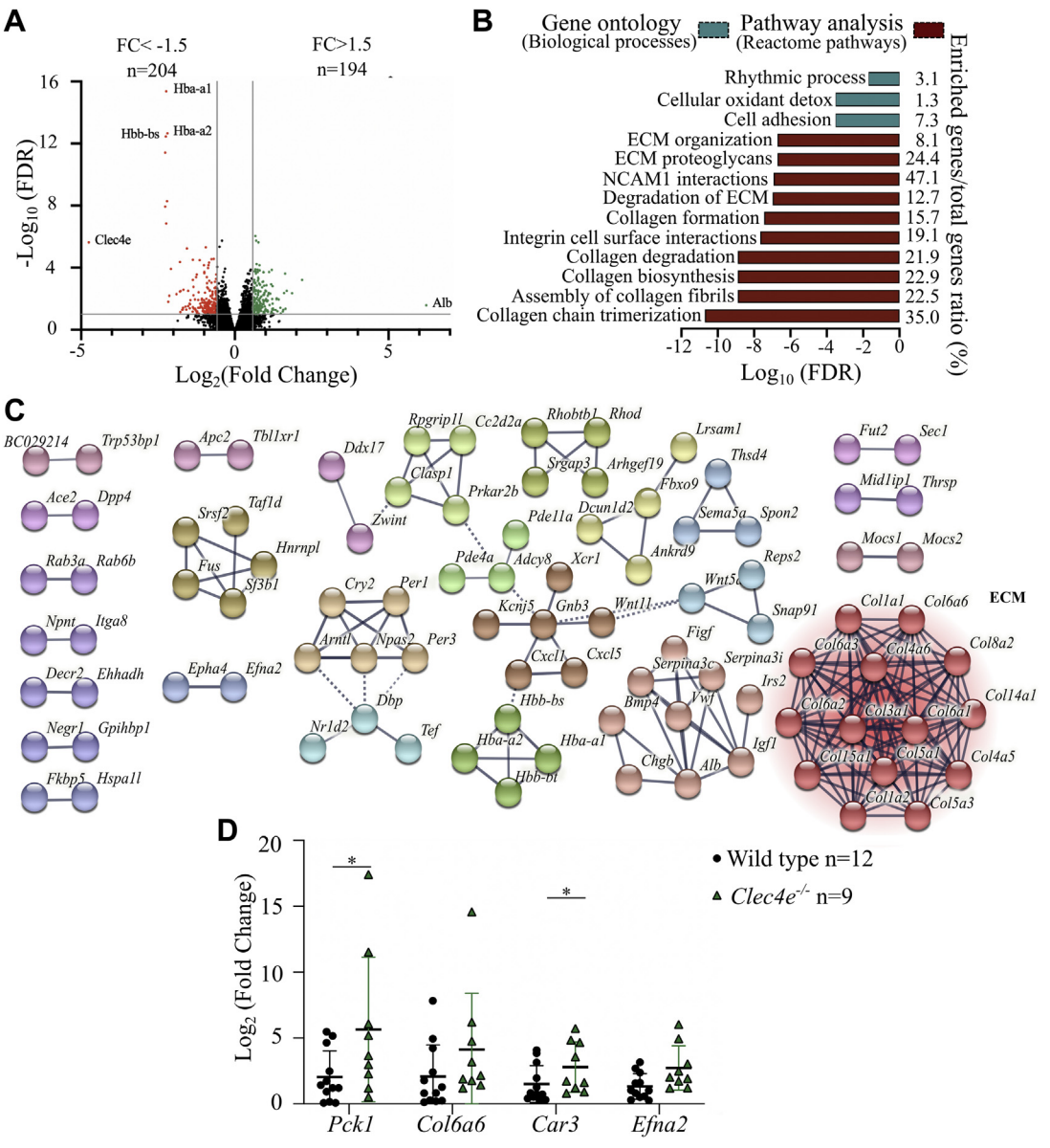
Pearson correlation between high-sensitivity troponin I (hs-TnI) plasma levels measured at 90 min' reperfusion (Rep) and ESV **(A)**, end-diastolic volume (EDV) **(B)**, and EF **(C)** in WT control mice (n = 16) and *Clec4e*$^{-/-}$ mice (n = 15). **(D)** hs-TnI at 90 min' reperfusion for WT and *Clec4e*$^{-/-}$ mice (mean ± SD). **(E)** Representative mosaic images of fibrosis staining for each infarct severity score (ISS). **(F)** ESV, **(G)** EDV, and **(H)** EF. To compare the 2 genotypes and the 3 levels of infarct severity, a 2-way ANOVA with Šidák's correction was performed. Scale bar = 500 μm. **Dotted lines** indicate 95% CI. *P < 0.05, **P < 0.01, ***P < 0.001. Abbreviations as in **Figures 1 and 2**.

neutrophils from WT and *Clec4e*$^{-/-}$ mice (**Figure 4E**). Of note, *Clec4e*$^{-/-}$ neutrophils exhibited significantly reduced migration compared with WT neutrophils ($P < 0.001$).

We next evaluated whether reduced neutrophil migration could be caused by lower Cxcr2 receptor expression in *Clec4e*$^{-/-}$ cells and performed immunoblot analysis on isolated bone marrow-derived cells from both genotypes. The results revealed significantly lower expression of Xxcr2 in *Clec4e*$^{-/-}$ cells compared with WT cells ($P < 0.001$) (**Figure 4F**). To validate these results in isolated peripheral blood neutrophils from WT and *Clec4e*$^{-/-}$ mice, we performed confocal microscopy using Cxcr2-specific antibody and confirmed markedly reduced total Cxcr2 expression in isolated neutrophils from *Clec4e*$^{-/-}$ mice compared with WT mice (**Figure 4G**).

LV remodeling and function of the murine heart 4 weeks after I/R injury. To investigate whether *Clec4e* signaling and the altered neutrophil infiltration in early post-I/R injury influences LV functional and structural remodeling, we performed cardiac MRI

FIGURE 6 RNA Sequencing of WT Versus *Clec4e*$^{-/-}$ LV Tissue 24 Hours After I/R Injury Shows Differential Expression of Genes Involved in ECM Regulation



(A) Volcano plot of differentially expressed genes. **Red dots** reflect 204 significantly downregulated transcripts, and **green dots** reflect 194 significantly upregulated transcripts (adjusted $P < 0.10$). **(B)** Gene ontology and pathway analysis shows significant differential regulation of biological processes and pathways involved in extracellular matrix (ECM) regulation. **(C)** STRING network confirms clustering of genes involved in ECM regulation. **(D)** Polymerase chain reaction analysis of selected transcripts. Two-way ANOVA. Mean values ± SD in WT mice (n = 12) and *Clec4e*$^{-/-}$ mice (n = 10). *$P < 0.05$. *Car3* = carbonic anhydrase 3; *Col6a6* = collagen type IV alpha 6 chain; *Efna2* = ephrin A2; FC = fold change; FDR = false discovery rate; *NCAM* = neural cell adhesion molecule; *Pck1* = phosphoenolpyruvate carboxykinase 1; other abbreviations as in Figures 1 and 2.

analysis and fibrosis staining of LV tissue at the 1-month follow-up in 17 WT mice and 16 *Clec4e*$^{-/-}$ mice. Cardiac MRI analysis revealed a significantly higher number of *Clec4e*$^{-/-}$ mice with ejection fraction >52% (odds ratio: 6.0; 95% CI: 1.1-24.6; $P = 0.032$) and thus better preserved LV function after I/R injury, consistent with the smaller initial ischemic injury. Linear regression analysis to quantify the relationship between cardiac MRI-based LV end-systolic and end-diastolic dimensions at 1-month follow-up with the

initial TnI plasma levels exhibited significant genotype-dependent differences in slopes for the regression lines ($P = 0.004$ and $P = 0.006$, respectively) (**Figures 5A and 5B**). This was not observed for LV ejection fraction (**Figure 5C**). These data suggest significantly better LV functional and structural LV remodeling after I/R injury in *Clec4e*$^{-/-}$ mice.

The degree of replacement fibrosis at 4 weeks as a percentage of the LV surface area paralleled the severity of the initial ischemic injury, reflected by hs-TnI levels at 90 minutes in both genotypes (WT, Pearson $r = 0.92$, $P < 0.001$; *Clec4e*$^{-/-}$, Pearson $r = 0.39$, P = NS). In an additional exploratory analysis, 3 independent and blinded researchers scored and stratified mice according to tertiles of replacement fibrosis in the left ventricle (post-mortem histopathological analysis) (**Figure 5E**). WT and *Clec4e*$^{-/-}$ mice with an infarct severity score of 1 (lowest tertile or replacement fibrosis <8% of LV surface area; WT, n = 4; *Clec4e*$^{-/-}$, n = 3) had similar hs-TnI plasma levels at 90 minutes' reperfusion (**Figure 5D**), MRI-based LV dimensions (**Figures 5F and 5G**), and LV global function parameters (**Figure 5H**). In contrast, *Clec4e*$^{-/-}$ mice with a severity score of 2 (middle tertile with fibrotic area between 8% and 25% of the LV surface area; WT, n = 10; *Clec4e*$^{-/-}$, n = 8) and with a severity score of 3 (upper tertile with replacement fibrosis >25% of the LV surface area; WT, n = 3; *Clec4e*$^{-/-}$, n = 5) exhibited better preservation of LV function and a lesser degree of adverse remodeling; these findings were evidenced by significantly smaller ESV and end-diastolic volume (both, $P < 0.001$) and significantly higher LV ejection fraction in *Clec4e*$^{-/-}$ mice with an infarct severity score of 2 to 3 ($P = 0.003$ and $P = 0.001$, respectively). Of note, hs-TnI plasma levels at 90 minutes' reperfusion were again significantly lower in *Clec4e*$^{-/-}$ mice in those with an infarct severity score of 3 ($P = 0.039$).

Transcriptome of the left ventricle during the early inflammatory response after I/R injury. To explore potential pathogenic mechanisms associated with increased *Clec4e* expression, we performed RNA-sequencing of LV tissue (apex to mid-left ventricle) extracts from 5 WT mice and 5 *Clec4e*$^{-/-}$ mice at 24 and 72 hours after I/R injury. We detected 398 differentially expressed genes, of which 204 were downregulated and 194 were upregulated ($P < 0.100$) (**Figure 6A**). The top 20 significantly differentially expressed genes (fold-change) included genes involved in cardiac remodeling (Mir22 host gene [*Mir22hg*]), gluconeogenesis (phosphoenolpyruvate carboxykinase 1 [*Pck1*]), angiogenesis, endothelial barrier function (UBX domain protein 10 [*Ubxn10*], ephrin A2 [*Efna2*]), secondary messenger signaling (adenylate cyclase 8 [*Adcy8*], phosphodiesterase 11A [*Pde11a*]), scavenging of reactive oxygen species (carbonic anhydrase 3 [*Car3*]), and extracellular matrix (ECM) (collagen type VI alpha 6 chain [*Col6a6*]) (Supplemental Table 2).

Gene ontology analysis of these 398 transcripts with the DAVID (Database for Annotation, Visualization and Integrated Discovery) functional gene annotation tool revealed their involvement in cell adhesion and cellular oxidant detoxification (**Figure 6B**, blue). In addition, network analysis with the online STRING database confirmed clustering of these genes related to ECM organization (**Figure 6C**). Moreover, pathway analysis with the Reactome Pathway Database showed enrichment of genes involved in collagen chain trimerization pathways, assembly of collagen fibrils, and collagen biosynthesis (**Figure 6B**, red). We validated the profiling data by RT-qPCR for *Pck1*, *Col6a6*, *Car3*, and *Efna2* in WT (n = 12) and *Clec4e*$^{-/-}$ (n = 10) mice and confirmed significant upregulation of *Pck1* ($P = 0.047$) and *Car3* ($P = 0.047$) and a trend for *Efna2* ($P = 0.056$) (**Figure 6D**).

At 72 hours after I/R injury, only 8 genes were significantly differentially regulated (Supplemental Figure 5), suggesting that CLEC4E signaling plays a more prominent role in the early inflammatory response during the first 24 hours after I/R injury.

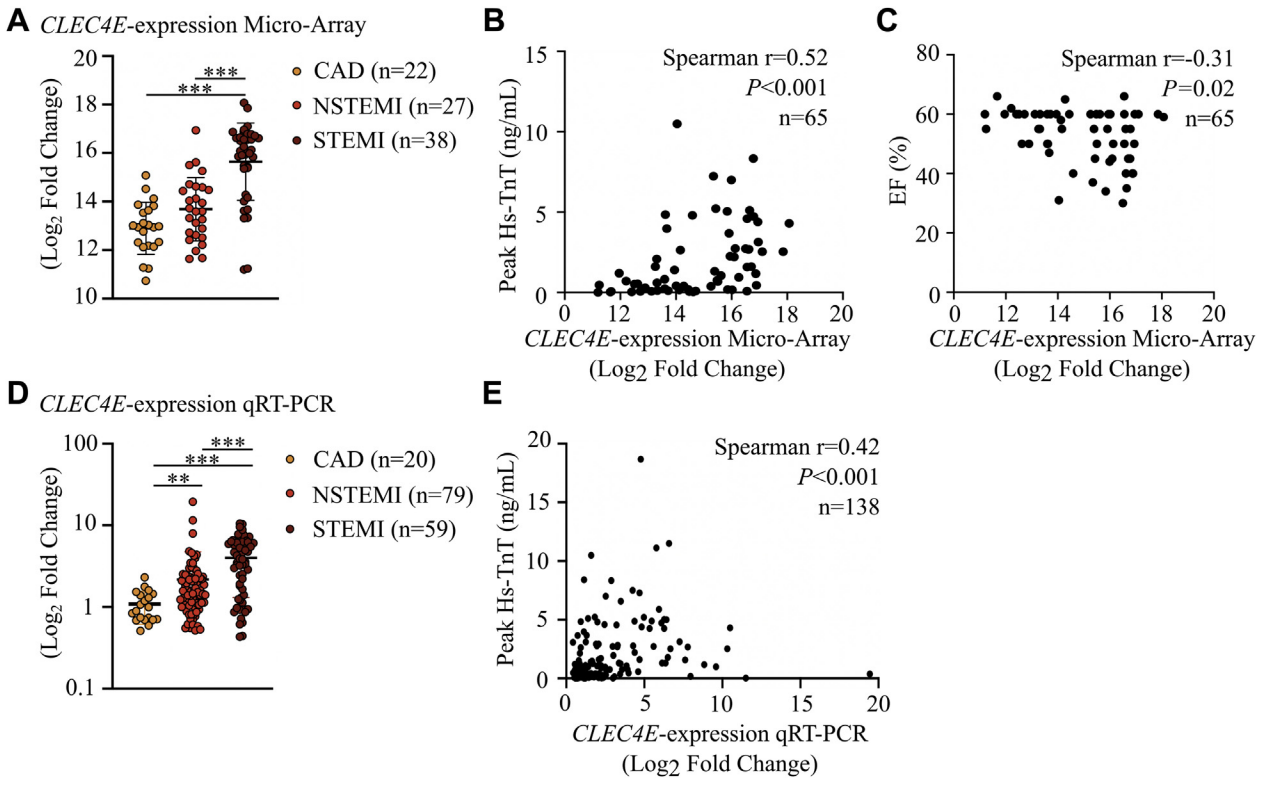
CLEC4E expression in whole blood of patients with AMI in the acute phase of AMI. We detected significant upregulation of *CLEC4E* in patients with ST-segment elevation myocardial infarction (STEMI) compared with patients with chronic CAD ($P < 0.001$) (**Figure 7A**). *CLEC4E* expression significantly correlated with peak TnT levels ($r = 0.52$; $P < 0.001$) (**Figure 7B**) and ejection fraction ($r = -0.31$; $P = 0.018$) (**Figure 7C**) in these patients with ACS (n = 38 with STEMI and n = 27 with non-STEMI).

We then validated *CLEC4E* expression in an extended cohort of 138 patients with AMI using qRT-PCR. We confirmed the expression data from the microarray with a significant increase of *CLEC4E* in patients with non-STEMI ($P = 0.004$) and STEMI ($P < 0.001$) versus patients with chronic CAD (**Figure 7D**) and a significant correlation with peak TnT levels ($r = 0.42$; $P < 0.001$) (**Figure 7E**).

DISCUSSION

In the current study, we report for the first time that the CLEC4E receptor of the innate immune system is upregulated during the acute inflammatory phase in porcine and murine I/R injury. Confocal analysis showed that the early induction at 24 hours after I/R

FIGURE 7 CLEC4E Is Significantly Upregulated in the Acute Phase of AMI and Correlates With Cardiac Damage on Admission

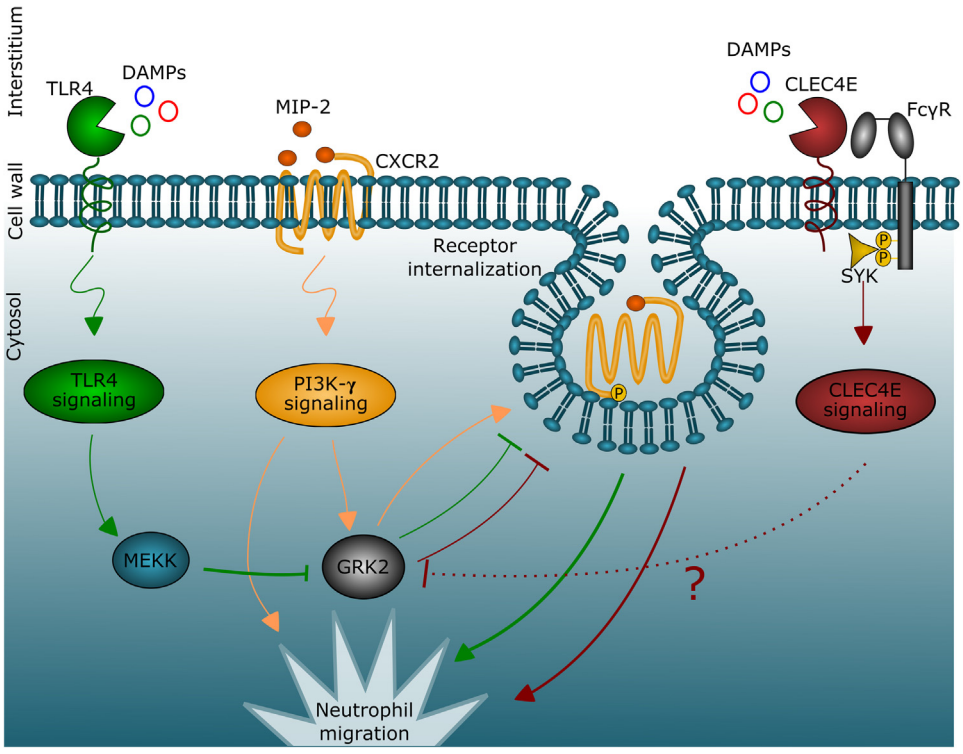


(A) Microarray analysis of CLEC4E expression in 65 patients with acute coronary syndrome (ACS) on admission and 22 control subjects with chronic coronary artery disease (CAD). Spearmann correlation between CLEC4E expression determined by microarray and peak high-sensitivity troponin T (hs-TnT) levels **(B)** and EF **(C)** on admission. n = 65. **(D)** Quantitative reverse transcription polymerase chain reaction (qRT-PCR) validation of CLEC4E expression in a cohort of 158 patients with ACS. **(E)** Spearmann correlation between CLEC4E expression determined by PCR and peak TnT levels in 138 patients. Results are shown as mean ± SD. To test the differences in CLEC4E expression, a Kruskal-Wallis test with Dunn's correction for multiple comparisons was performed. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. NSTEMI = non-ST-segment elevation myocardial infarction; STEMI = ST-segment elevation myocardial infarction; other abbreviations as in Figure 1.

injury is accounted for in part by infiltrating neutrophils and in part attributable to increased expression in resident CMCs and vascular SMCs, with a further increase at 72 hours in infiltrating monocytes/macrophages. *Clec4e* deletion in mice was associated with significantly less acute ischemic cardiac injury, reduced early neutrophil infiltration, and a strong trend toward smaller infarct size at 24 hours' post-I/R injury. Isolated neutrophils from *Clec4e*$^{-/-}$ mice 24 hours' post-I/R injury showed increased expression of *Grk2* kinase, known to desensitize CXCR2-receptor signaling, and *Clec4e*$^{-/-}$ neutrophils exhibited reduced migration toward CXCL2 in vitro. Furthermore, isolated bone marrow cells from *Clec4e*$^{-/-}$ mice displayed a significant reduction in baseline Cxcr2 protein expression. Reduced early ischemic injury translated into favorable functional and structural LV remodeling 4 weeks after I/R injury. Differential gene expression and STRING network analysis of LV tissue samples identified a transcriptome in *Clec4e*$^{-/-}$ mice that was predominantly enriched in biological processes and pathways involved in ECM regulation. Finally, exploratory analysis of patients with AMI confirmed a highly significant correlation between *CLEC4E* expression levels in whole blood on admission, peak high-sensitivity TnT levels, and LV ejection fraction. Taken together, our data highlight for the first time an important role for CLEC4E signaling during acute myocardial ischemic injury, not only as a marker of infarct severity and adverse LV remodeling but also as a novel target for pharmacologic intervention.

The first key observation of our study relates to the temporal and spatial dynamics of myocardial *CLEC4E* expression in the setting of acute ischemic damage. We measured a gradient of increased *Clec4e*

FIGURE 8 **Schematic Overview of the Interplay Between Chemokine-Signaling and PRR-Signaling Pathways**



The release of the chemokine MIP-2 during I/R injury triggers the activation of the CXC chemokine receptor 2 (CXCR2) receptor on neutrophils, leading to downstream phosphatidylinositol 3-kinase-γ (PI3K-γ) signaling and subsequent neutrophil chemotaxis. The CXCR2 receptor also directly regulates its own expression via upregulation of GRK2, which leads to CXCR2 phosphorylation, receptor internalization, and recycling. During sterile inflammation, neutrophil recruitment is required, and the negative feedback induced by CXCR2 needs to be reduced. When *Toll-like receptor 4* (TLR4) signaling is activated by damage-associated molecular patterns (DAMPs), it downregulates GRK2 in an MEKK-dependent manner, and promotes neutrophil chemotaxis. In addition to TLR4, the CLEC4E receptor also promotes neutrophil migration by downregulating GRK2. Together, the triggers for neutrophil chemotaxis outweigh the negative feedback of chemokine signaling, but when CLEC4E gene function is deleted, the balance will shift toward increased GRK2 expression, CXCR2 receptor desensitization, and reduced chemotaxis. MEKK = MAP/ERK Kinase Kinase; PRR = pattern recognition receptor; other abbreviations as in Figures 1, 2, and 4.

expression toward the most vulnerable ischemic area that was not only attributable to early leukocyte recruitment in the porcine or murine ischemic myocardium. Confocal analysis confirmed that Clec4e immunoreactivity is not restricted to infiltrating immune cells but is also widely present in CMCs and in vascular SMCs. Although endothelial cells express different types of PRRs that are partially responsible for endothelial cell activation (19) and subsequent inflammatory cell extravasation, we did not detect *Clec4e* expression in endothelial cells. These pathological observations are in line with bone marrow chimera experiments by Arumugam et al (12) in a murine brain injury model, which also revealed marked *Clec4e* expression in resident brain cells.

The second key observation is that *Clec4e* seems to play a major role in the induction and propagation of ischemic inflammation in response to the release of DAMPs after I/R injury. Our data support the widely held model of *Clec4e* as a necrotic cell receptor directing inflammatory responses to areas of necrotic cell damage. It was long thought that PRRs were predominantly expressed on leukocytes, but recent studies also showed an important role of PRRs on CMCs and SMCs. CMCs have been shown to express functional PRRs and increase inflammatory signaling in response to myocardial injury (20,21). Increased inflammatory signaling and downstream chemokine production reportedly exert cardioprotective effects in vitro. Tarzami et al (22) showed that 2 important early-phase chemokines (Mip2/Cxcl2 and Mcp1/Cxcl1)

reduce hypoxia-induced cell death in cultured CMCs. In line with the protective effects of *Clec4e*$^{-/-}$ we showed on acute injury and leukocyte infiltration after I/R injury, our results suggest that *Clec4e* deletion increases viability of CMCs in response to hypoxic stress, which might be due to a change in chemokine response. These cardioprotective effects are consistent with previous data of neuroprotection in similar *Clec4e* transgenic models of ischemic stroke (11,12). In SMCs, PRRs are also functionally expressed and have been shown to induce a proinflammatory phenotype upon activation (23). After activation of PRRs, vascular SMCs contribute to the early inflammatory response by producing proinflammatory mediators that increase leukocyte infiltration and reactive oxygen species production. These proinflammatory effects have direct consequences on tissue perfusion and vascular permeability and could therefore influence acute injury and leukocyte infiltration.

The role of PRRs, and more specifically CLEC4E, on monocytes/macrophages has been investigated in more detail in the setting of atherosclerosis. Multiple studies showed that Clec4e favors a proinflammatory, proatherogenic phenotype of macrophages, contributing to plaque progression (24,25). In the setting of myocardial ischemia, CLEC4E is upregulated in the acute phase of AMI in peripheral blood monocytes and could promote the proinflammatory phenotype of infiltrating monocytes/macrophages, thereby increasing phagocytic activity and degradation properties. Although we did not observe a genotype-dependent difference in monocyte/macrophage infiltration 72 hours after I/R injury (data not shown), we cannot exclude transcriptional differences in the inflammasome or a shift toward a more reparative macrophage phenotype in Clec4e knockout mice.

Taken together, these data show an important role of Clec4e on cardioprotection, but elucidation of the underlying mechanisms requires additional single-cell multi-omics analysis.

Third, we measured a significant increase in *Grk2* expression in circulating neutrophils from *Clec4e*$^{-/-}$ mice 24 hours after I/R injury compared with WT mice. In addition, we observed reduced migration in response to Cxcl2 of neutrophils isolated from *Clec4e*$^{-/-}$ mice and noted significantly reduced baseline Cxcr2 expression levels on isolated *Clec4e*$^{-/-}$ bone marrow cells, confirmed in isolated neutrophils using immunocytochemistry. We also recorded a significant increase in Mip2/Cxcl2 levels in plasma collected 24 hours after I/R injury. Together, these results suggest a significant effect of Clec4e deletion on the CXCR2/CXCL2 chemokine-signaling pathway. The effect of *Clec4e* genetic deletion has been previously reported in a mouse model of polymicrobial septic peritonitis, showing reduced neutrophil infiltration in the peritoneum of *Clec4e*$^{-/-}$ mice and Grk2-mediated desensitization of the CXCR2 receptor (17,18). We hypothesized that a similar mechanism would render neutrophils less responsive for chemokine gradients produced by the ischemic heart (26,27), and our results confirm a comparable effect of Clec4e on chemokine signaling in our setting of sterile inflammation. Neutrophils isolated from healthy WT or *Clec4e*$^{-/-}$ mice did not exhibit a significant difference in baseline *Grk2* expression, suggesting that the effects of Clec4e signaling on Grk2 expression are only induced in response to I/R injury. Interestingly, protein analysis revealed that there is a reduction in baseline Cxcr2 receptor expression in *Clec4e*$^{-/-}$ mice, which could explain why *Clec4e*$^{-/-}$ cells showed reduced migration toward Cxcl2 gradients and reduced neutrophil infiltration in ischemic myocardium. The increase in plasma Mip2/Cxcl2 in Clec4e$^{-/-}$ mice could be a counterregulatory mechanism in response to reduced neutrophil infiltration, as proposed in the setting of polymicrobial septic peritonitis (26). It has been shown that Toll-like receptor-4 signaling, which is closely linked to CLEC4E signaling (27), cross-talks with chemokine signaling via downstream activation of MAP/ERK Kinase Kinase (MEKK) and results in transcriptional downregulation of *Grk2,* promoting leukocyte migration (16) (**Figure 8**). The exact downstream pathway and interactions between *Clec4e* gene function, Grk2, and chemokine signaling require further investigations in follow-up studies.

Fourth, comprehensive cardiac MRI analysis found that *Clec4e*$^{-/-}$ mice have improved LV remodeling after I/R injury. The benefit is most prominent in mice with the largest area at risk and a scar burden of >25% of LV surface area, which we classified as the highest infarct severity score, but it was also detectable in mice with a scar burden >8% and <25% of LV surface area. In contrast, Arumugam et al (12) did not report a statistical difference in LV volumes or LV function after permanent ligation of the LAD in heterozygous *Clec4e*$^{+/-}$ mice. Major differences in experimental model (permanent vs transient ligation), genetic background (partial *Clec4e* deletion vs homozygous deletion), and in cardiac phenotyping (transthoracic echocardiography versus 7-T MRI) likely account for the respective findings.

Fifth, we performed unbiased RNA-sequencing to better understand downstream pathways that mediate the differential remodeling processes in both genotypes and measured 398 significantly differentially expressed genes, predominantly involved in ECM regulation and cell adhesion. Although the ECM serves as an endogenous DAMP to PRR on innate immune cells (28,29), studies have found that PRR signaling also influences collagen production and ECM remodeling (30). Our bioinformatics analysis and validation by qRT-PCR revealed enrichment for genes involved in collagen assembly and biosynthesis but also in cellular oxidant detoxification and neoangiogenesis.

Finally, to explore the translational relevance of our preclinical injury models, we performed exploratory microarray analysis on peripheral blood collected from patients with AMI (7) and confirmed significant upregulation of *CLEC4E*, with the highest expression in patients with STEMI. Subsequent RT-PCR validation in a separate cohort of 138 patients with AMI and 20 patients with CAD revealed a highly significant correlation of *CLEC4E* transcript levels in peripheral blood with peak high-sensitivity TnT cardiac necrosis marker. These data are consistent with our experimental data in porcine and murine I/R injury and extend previous reports on significantly upregulated *CLEC4E* levels in circulating monocytes from patients with CAD and in atherosclerotic plaques (24,25). Others have investigated the potential role of peripheral blood *CLEC4E* expression levels, as a biomarker for CAD severity (31), thereby also validating the gene expression tests for determining CAD risk. In concert, these data highlight the increasing importance and complex interplay of CLEC4E signaling in CAD and ACS.

STUDY LIMITATIONS. First, to reduce sex-related variability, we only considered male C57Bl6/J mice. It has been reported that the estrogen-receptor ß has potential cardioprotective effects (32). Therefore, future I/R experiments in female mice are required. Second, the relatively short follow-up period of 4 weeks in I/R mice limits the possibility of evaluating survival rates and the development of advanced heart failure. Third, a baseline cardiac MRI assessment was not performed for the murine experiments. Fourth, the RNA-sequencing data need further validation and mechanistic studies to prove causality. Finally, further studies are required to elucidate the molecular mechanisms between Clec4e signaling and chemokine signaling.

CONCLUSIONS

Our data in 2 different experimental models of acute myocardial ischemia and in patients with AMI emphasize an important role of the innate immune receptor CLEC4E as a direct activator in the local response to cardiac damage. Observations in *Clec4e* knockout mice also indicate that blocking CLEC4E improves cardiac repair after myocardial I/R injury and point toward a new modifiable target of sterile inflammation during acute myocardial ischemic damage.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: The CLEC4E-receptor plays an important role in myocardial healing after ischemia-reperfusion injury by increasing cardiac damage, inflammation, and LV-remodeling.

TRANSLATIONAL OUTLOOK: Our combined data in mice, pigs, and patients with acute coronary syndromes suggest that targeting CLEC4E might hold a promising strategy in the treatment of ischemic injury.

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KEY WORDS CLEC4E, inflammation, ischemia-reperfusion injury, magnetic resonance imaging, myocardial remodeling

APPENDIX For supplemental Materials and Methods, tables, and figures, please see the online version of this paper.