Cardiac arrhythmias are a primary contributor to sudden cardiac death, a major unmet medical need. Because right ventricular (RV) dysfunction increases the risk for sudden cardiac death, we examined responses to RV stress in mice. Among immune cells accumulated in the RV after pressure overload-induced by pulmonary artery banding, interfering with macrophages caused sudden death from severe arrhythmias. We show that cardiac macrophages crucially maintain cardiac impulse conduction by facilitating myocardial intercellular communication through gap junctions. Amphiregulin (AREG) produced by cardiac macrophages is a key mediator that controls connexin 43 phosphorylation and translocation in cardiomyocytes. Deletion of Areg from macrophages led to disorganization of gap junctions and, in turn, lethal arrhythmias during acute stresses, including RV pressure overload and β-adrenergic receptor stimulation. These results suggest that AREG from cardiac resident macrophages is a critical regulator of cardiac impulse conduction and may be a useful therapeutic target for the prevention of sudden death.

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Heart failure is a growing medical and economic burden worldwide. Researchers and development efforts have focused on left ventricular (LV) dysfunction and left heart failure, but right heart failure has been gaining increasing attention as a serious unmet medical need. Right heart failure associates with adverse outcomes in a variety of heart diseases, including left heart failure, in part by compromising cardiac function and worsening serious arrhythmias. Indeed, right ventricular (RV) dysfunction increases the risk for sudden cardiac death. However, the molecular mechanisms for RV stress responses remain poorly understood.

Recent studies have revealed the various roles played by immune cells in the maintenance of homeostasis and the development of cardiac diseases. For instance, many reports indicate that macrophages promote left ventricular (LV) remodeling and, conversely, are required for proper adaptive responses to stress and healing after myocardial infarction. Other immune cells such as lymphocytes and mast cells also reportedly play important roles in the RV remodeling; however, very little is known about the actions of immune cells during RV stress responses.

Here, we show that cardiac macrophages play an essential role in survival during cardiac stress by maintaining the cardiac electrical conduction through regulating cardiac gap junction formation. These findings may pave the way for a new therapeutic method for sudden death.

**Results**

**Macrophages are vital for survival after right heart burden.**

To assess the functional roles of immune cells during acute RV stress, we imposed pressure overload on the right ventricle by pulmonary artery banding (PAB) (Fig. 1a). Accumulation of various types of immune cells, including macrophages, granulocytes, CD4+ and CD8+ T cells, and B cells, was observed after PAB (Fig. 1b, Supplementary Fig. 1). To gain insight into the functions of these cell populations during acute RV stress, we performed PAB on mice treated with clodronate liposomes, or with anti-Ly6G antibody, which depleted granulocytes; Cd4a+ and Cd8a− mice, which lacked CD4+ and CD8+ T cells, respectively; and Rag2−/− mice, which lacked mature T and B cells. While the interventions affecting granulocytes and T and B cells did not impact survival after PAB, more than half of the clodronate-treated mice died within 4 h after PAB (Fig. 1c). Electrocardiograms (ECGs) showed that, after PAB, 4 of 57% of the clodronate-treated mice died within 4 h after PAB (Fig. 1d and Supplementary Table 1). To further investigate whether AREG mediates the anti-arrhythmic action of macrophages, we next continuously administered AREG to clodronate-treated mice beginning the day prior of PAB. This AREG supplementation prevented the sudden death otherwise seen after PAB (Fig. 2f and Supplementary Table 3). Collectively, these results demonstrate that Areg deficiency confers abnormal cardiac electrical conduction and arrhythmogenicity, even in the steady-state, which leads to severe arrhythmias and sudden death after RV pressure overload.

It has been reported that sympathetic nerve activation triggers and facilitates arrhythmias and is an important mechanism underlying sudden cardiac death, and that isoproterenol induces AV block and lethal arrhythmias in mice with conduction disturbances. This prompted us to test the effects of β-adrenergic receptor stimulation. Isoproterenol administration (5 mg/kg) caused sudden death in ~57% of Areg−/− mice within 2 days (Fig. 2g) and induced severe arrhythmias, including advanced AV block and ventricular fibrillation (VF) (Fig. 2h and Supplementary Fig. 7). All ECGs at the time of death showed VF in the isoproterenol-administered Areg−/− mice. By contrast, WT mice had only infrequent isolated PVCs and did not die.

We further tested whether AREG deficiency promotes ventricular arrhythmogenic substrates by analyzing electrical activation in Langendorff-perfused whole heart preparations using optical mapping. VT/VF was induced through programmed stimulation or burst pacing. While VT was induced in only 1 of 6 WT hearts, all 6 Areg−/− hearts developed VT when subjected to the VT/VF induction protocol (Fig. 2i).

**AREG regulates cardiac gap junction formation.** Because Areg−/− mice exhibited multiple types of arrhythmias, we speculated that they were subjected to global electrical conduction disturbances. Myocardial electrical conduction depends primarily on the intercellular transfer of current through gap junctions composed of connexin protein complexes. We, therefore, performed western blotting to examine the cardiac expression of connexin (Cx) proteins in WT and Areg−/− mice. Although continuous ECG monitoring of freely moving 8- to 12-week-old Areg−/− mice showed sinus arrest or sinoatrial (SA) block, intermittent complete AV block, and premature ventricular contractions (PVCs) in all 6 mice tested (Fig. 2a and Supplementary Table 2). By contrast, no arrhythmias were observed in wild-type (WT) mice (n = 6). When we imposed RV pressure overload on Areg−/− mice with PAB, the mice developed AV block, and >85% of them died within 24 h (Fig. 2b, c and Supplementary Table 3) with no noticeable histological or functional changes in their RVs (Supplementary Figs. 4 and 5). To confirm that macrophage-derived AREG is important for preventing arrhythmias, we transplanted bone marrow (BM) from WT or Areg−/− mice into lethally irradiated WT mice. Six weeks after the BM transplantation (BMT), 99.2% of the cardia-regent macrophages were replaced with BM-derived cells. After PAB, 57% of the chimeric mice receiving Areg−/− BM died within several hours, and >80% died within 7 days. By contrast, no mice receiving WT-BMT died after PAB (Fig. 2d). AV block was recorded in the Areg−/−-BMT mice after PAB (Fig. 2e and Supplementary Table 3). In a publicly available single-cell RNA-seq dataset from heart stromal cells, most Areg-expressing cells were Cd68+, indicating that macrophages are the major immune cell type expressing Areg, although some other cells might express AREG after PAB (Supplementary Fig. 6). Accordingly, the results of Areg−/−-BMT strongly suggest that AREG produced by macrophages is essential for protection against AV block and sudden death after PAB.

To further investigate whether AREG mediates the anti-arrhythmic action of macrophages, we next continuously administered AREG to clodronate-treated mice beginning the day prior of PAB. This AREG supplementation prevented the sudden death otherwise seen after PAB (Fig. 2f and Supplementary Table 3). Collectively, these results demonstrate that Areg deficiency confers abnormal cardiac electrical conduction and arrhythmogenicity, even in the steady-state, which leads to severe arrhythmias and sudden death after RV pressure overload.
there were no apparent differences in the levels of Cx40 and Cx45 or their band positions between WT and Areg−/− mice, Cx43 bands from WT heart samples exhibited band shifts and smears, which were eliminated by treatment with lambda protein phosphatase before loading (Fig. 3a). This indicates that the band shifts and smearing reflect phosphorylation of Cx43. Levels of Cx43 phosphorylation appeared to be much reduced in Areg−/− hearts, and the reduced phosphorylation persisted after PAB. Phosphorylation of Cx43 reportedly affects its localization and function20.

In mice, Cx43 is the primary component of gap junctions in both the atria and ventricles21. Phosphorylation of Cx43 is linked to its translocation and modulates gap junction properties, and dephosphorylation of Cx43 is associated with electrical uncoupling and susceptibility to arrhythmia20,22. We immunohistochemically stained Cx43 in mouse hearts (Fig. 3b). In WT mice, Cx43 was primarily localized to the intercalated discs between myocytes, as previously reported 23. By contrast, the distribution of Cx43 was disorganized within cardiomyocytes in Areg−/− mice, with Cx43 mislocated to the cells’ lateral surfaces.

**Fig. 1** Macrophage depletion causes sudden death after right heart stress. **a** Mouse model of right heart pressure overload (PAB). The pulmonary artery was ligated to increase the RV pressure to 30-35 mmHg from 5-10 mmHg. **b** Flow cytometric analysis of immune cells in the right atrial (RA) and RV free wall in wild-type (WT) mice after PAB. n = 4 in each group. One-way ANOVA followed by Tukey’s post-hoc test. *P < 0.05 vs. pre: steady-state. Box plots show center line as median, box limits as upper and lower quartiles, whiskers as minimum to maximum values. **c** Effects of depleting macrophages (clodronate), granulocytes (Ly6G Ab), CD4+ (CD4KO) or CD8 T+ (CD8KO) cells, or T and B cells (Rag2KO) on survival after PAB. *P < 0.0001 (log-rank test). **d** Representative six-channel ECGs from clodronate-treated mice after PAB. Shown is the representative sequence of arrhythmia progression observed in a mouse. Bars, 400 msec.
Fig. 2 Areg deficiency leads to abnormal cardiac electrical conduction at steady-state and lethal arrhythmias under cardiac stress. a ECGs from freely moving Areg−/− mice recorded using telemetry. Spontaneous AV block, PVCs, and sinus arrest or SA block were observed in Areg−/− mice. Bars indicate 200 msec b Survival rates among WT and Areg−/− mice after PAB. *P < 0.001, log-rank test. c Representative ECG from an Areg−/− mouse showing complete AV block and ventricular arrest after PAB. d, e BM chimeric mice that received WT (WT-BMT) or Areg−/− (Areg−/−-BMT) BM were subjected to PAB. Survival rates after PAB are in d. *P < 0.001, log-rank test. In e, a representative ECG from an Areg−/−-BMT mouse after PAB is shown. f Survival curves for clodronate-treated mice with or without continuous administration of AREG (5 μg/day) from 24 h before PAB. n = 10 in each group. *P < 0.01, log-rank test. g, h WT and Areg−/− mice were intraperitoneally administered isoproterenol (bolus, 5 mg/kg). Survival rates are shown in g. h, representative ECGs from WT and Areg−/− mice are shown. *P < 0.05, log-rank test. i Numbers of Langendorff-perfused hearts that developed VT/VF. *P < 0.01 (χ²-test).

The disorganization was reversed by intraperitoneal administration of recombinant AREG (Fig. 3b). This mislocation of Cx43, called lateralization, is associated with arrhythmogenicity.24,25. One consequence of Cx43 lateralization is the opening of Cx43 hemichannels that are mislocated away from intercalated discs and do not form gap junction channels with adjacent cells. This results in abnormal ion trafficking and electrical conduction.15,26. To determine whether Cx43 lateralization is involved in the life-threatening arrhythmias seen in Areg−/− mice, we utilized Gap19, which selectively inhibits Cx43 hemichannels without affecting normal gap junctional communication.17,27. Intraperitoneal administration of Gap19 to Areg−/− mice clearly reduced the incidence of isoproterenol-induced death (Supplementary Fig. 10). These results suggest that AREG is critically involved in the regulation of Cx43 localization and cardiac gap junction formation, possibly through Cx43 phosphorylation, and that Cx43 lateralization is a cause of life-threatening arrhythmias triggered by cardiac stress in Areg−/− mice.

AREG promotes phosphorylation of Cx43. We next carried out dye transfer assays to further assess the effects of AREG on gap junctional intercellular communication (GJIC) between cultured mouse cardiomyocytes.28. In this assay, a line is scraped into a confluent layer of cardiomyocytes, which enables a membrane-impermeant dye, 5(6)-carboxyfluorescein, to enter the affected cells through a transient tear in their plasma membrane. The dye can then migrate to adjacent cells through gap junctions. We quantified the level of dye transfer based on the distance the dye traveled from the scrape line (Fig. 4a). To assess the effects of cardiac macrophages on GJIC, we co-cultured cardiomyocytes with CD45+CD11b+ Ly6G−/80+ Ly6C−/lo cardiac macrophages. The level of dye...
**Fig. 3** *AREG promotes gap junctional connection between cardiomyocytes through Cx43.*

*a* Western blot analysis of Cx40, Cx43, and Cx45. Representative immunoblots are shown. α-Tubulin was used as a loading control. Note the mobility shift of Cx43 bands in WT mouse hearts.

*b* Immunohistochemical staining of Cx43 (brown) in the myocardium. Nuclei (blue) were counterstained with methylene blue. WT and *Areg*−/− mice were intraperitoneally administered either vehicle (PBS) or recombinant AREG (5 μg), and the hearts were harvested 30 min later. WT and *Areg*−/− mice not receiving AREG were administrated vehicle (PBS) 30 min before sacrifice. The bar indicates 60 μm. The images show representative immunohistochemically stained hearts from WT or *Areg*−/− mice at steady-state.

*c* Fractions of Cx43 localized to the intercalated discs in cardiomyocytes in RVs. *n* = 6 mice in each group. One-way ANOVA followed by Tukey’s post-hoc test. *P* < 0.0001 vs. WT. **P** < 0.0001 vs. *Areg*−/−. Data are presented as mean values ± SEM.
transfer was significantly increased in the presence of macrophages (Fig. 4b).

We next tested the effects of AREG produced by macrophages on GJIC. As indicated by the dye transfer, GJIC between cardiomyocytes cocultured with Areg^-/- macrophages was significantly lower than between cardiomyocytes cocultured with WT macrophages (Fig. 4c). Moreover, recombinant AREG protein similarly increased the level of dye transfer (Fig. 4d).

We then assessed GJIC function by using a cell motion imaging system to analyze the conduction velocity of contraction propagation in cultured cardiomyocytes. Conduction velocities in cardiomyocytes co-cultured with cardiac macrophages were higher than in cardiomyocytes cultured alone (Supplementary Fig. 11). AREG similarly increased conduction velocity in cultured cardiomyocytes. In mouse hearts in vivo, optical mapping showed that electrical propagation was impaired in...
Fig. 4 AREG facilitates gap junctional intercellular communication by stabilizing Cx43 gap junctions. a Schematic of the dye transfer assay. Mouse neonatal cardiomyocytes were cultured with or without cardiac resident macrophages. The cell-impermeable dye was loaded by scraping that caused tearing of the plasma membrane, and the level of dye transfer between cardiomyocytes was evaluated as the distance of dye-stained cells from the scraped edge after 15 min. b, c Mouse neonatal cardiomyocytes were cultured with or without WT or Areg<sup>−/−</sup> cardiac macrophages. Shown are representative images of dye-stained cells spreading from the scrape and the relative extent of dye transfer. Effects of macrophage coculture (b) and the lack of AREG in cocultured macrophages (c) on dye transfer were analyzed. n = 24 and 18, and n = 22 and 23 (images) *<p < 0.0001, two-tailed unpaired Student’s t-test. d Effects of AREG (100 ng/mL) and the indicated signal inhibitors (10 µmol/l, each) on dye transfer. n = 28, 28, 24, 24, and 30; *<p < 0.05, post-hoc Dunnett’s test. e, f A Cx43-GFP plasmid was transfected into HeLa cells, and the cells were treated for 5 h with or without AREG (100 ng/mL) or AG1478 (10 µmol/l), as indicated. In e, formation of Cx43-GFP gap junction plaques (white arrows) at the cell-cell borders is shown. Cell borders are marked by dashed lines in upper panels. Scale bars, 20 µm. In f, plaque size is shown. n = 47, 39, and 36; *<p < 0.01, ***<p < 0.001, post-hoc Tukey’s test comparing cell adhesion between two-cardiomyocytes. g Western blotting of Cx43-GFP transfected HeLa cells treated for 6 h with AREG and 10 µmol/l AG1478 (A), U0126 (U), or FR180204 (F), as indicated. The positions of unphosphorylated and phosphorylated forms of Cx43 are shown, h Schematic model of how amphiregulin derived from cardiac macrophage suppresses arrhythmia. Throughout, box-and-whisker plots represent the median, the first and third quartiles, and the minimum and maximum values.

**Discussion**

Cardiac arrhythmias are a primary contributor to sudden cardiac death in patients with heart disease<sup>35</sup> and preventing sudden cardiac death remains a major unmet medical need. It has been suggested that altered expression of various genes, including genes related to ion channels and gap junctions,<sup>36</sup> contributes to the arrhythmogenicity in heart failure patients. The present study demonstrates that AREG derived from cardiac macrophages maintains electrical impulse conduction by controlling the alignment of Cx43 at gap junctions between cardiomyocytes. Phosphorylation of Cx43 by AREG facilitates intramyocardial conduction by promoting formation of large gap junctions. Previous studies showed that in a normal heart, Cx43 proteins primarily exist in a phosphorylated state and that ischemia induces dephosphorylation.<sup>20</sup> Our finding that gap junctions are disorganized in Areg<sup>−/−</sup> mice suggests that dysregulation of Cx43 phosphorylation is an important mechanism underlying severe arrhythmia and sudden death induced by RV stress. That said, functions other than Cx43 regulation, such as regulatory roles in other processes in cardiac physiology and stress response<sup>5</sup>, may also be involved in AREG’s antiarrhythmic action.

Hulsmans et al. recently reported that macrophages contribute to cardiac conduction in the AV node by directly connecting to cardiomyocytes through Cx43-containing gap junctions.<sup>38</sup> While Cx43 deletion from macrophages prolonged AV conduction, it did not cause advanced AV block, suggesting that additional mechanisms, such as AREG-mediated facilitation of intramyocardial electrical coupling, are critically involved in AV nodal conduction.<sup>14</sup> More importantly, cardiac macrophages exist throughout the myocardium, and our finding that Areg deletion generates substrates for ventricular arrhythmia suggests that macrophages and AREG are required for maintenance of myocardial electrical conduction widely throughout the heart.

In addition to systemic and hematopoietic loss of Areg, acute induction of macrophage damage using clodronate liposomes made mice highly susceptible to severe arrhythmia and sudden death. This indicates that macrophages are required not only to establish gap junctional communication, but also to continuously and dynamically regulate gap junctions, particularly during cardiac stresses, such as acute RV pressure overload. AREG supplementation prevents sudden death, which also highlights the dynamic nature of gap junctional regulation and suggests that cardiac macrophages and AREG are potential therapeutic targets for both long-term and acute treatment of life-threatening...
arrhythmias. Notably, Son et al. reported that Arreg expression is reduced in LV myocardial tissues of patients who suffered sudden cardiac death, suggesting that AREG is also important for preventing severe arrhythmias in humans.

Methods
Animal studies. Male C57BL/6j mice were purchased from CLEA Japan and Jackson Laboratory and maintained on a standard mouse chow diet under sterile barrier conditions, on a 12-1 light-dark cycle with 18°C–23°C and 40–60% humidity. Male Arreg homozygous null (Arreg−/−) mice were backcrossed to C57BL/6j mice over ten generations. Rag2−/−, Cd4−/−, and Cd8a−/− mice were purchased from Taconic (Germantown, NY). The genotypes of all mice were determined using genomic PCR. The isoproterenol challenge entailed bolus administration of isoproterenol (Sigma, USA) intraperitoneally at a constant ECG recording rate of 170. Clodronate liposomes and control liposomes were purchased from LIPOSOMA B. V. A total of 10 µl of clodronate or control liposome solution per gram of mouse were intravenously administered 24 h before PAB. After the first administration of clodronate or control liposomes, the same volume of liposomes was also administered every 7 days for long term depletion, per the manufacturer’s instructions. All protocols for animal experiments were approved by the Animal Care and Use Committee of the University of Tokyo.

Cell culture. Mouse cardiomyocyte cultures were prepared from one- to three-day-old male and female C57BL/6j mice using a neonatal heart dissociation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Cardiac macroporphages were depleted from primary cultures of cardiomyocytes using anti-CD11b mouse beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) with an autoMACS® Pro Separator according to the manufacturer’s instructions. The separated cells were maintained in a 5% CO2 incubator at 37°C on collagen-coated 35-mm culture dishes in high glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco, 11995-065, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, SH30363.01, USA), 100 µU/ml penicillin and 100 µg/ml streptomycin. The cells were used for scrape loading/dye transfer assays when they reached confluence.

Human epithelial cervical carcinoma cells (HeLa cells, ATCC, CCL-2) were cultured in DMEM (Gibco, 11959-065, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, SH30363.01, USA), 100 µU/ml penicillin, and 100 µg/ml streptomycin. The cells were split the day before transfection at 70–90% confluence. The cells were transfected using LipofectAMINE 2000 (Life Technologies, 11668-019, USA) according to the manufacturer’s instructions. A total of 10 µmol/l AG1478 (Wako, 017-20151, Japan), U0126 (Abcam, ab14141, USA) or FR180204 (Sigma, SML0230, USA) was added 1 h before supplementation with the indicated concentrations of recombinant mouse AREG protein (989-AR/CF, R&D systems, Minneapolis, MN, USA). The transfected cells were visualized using confocal microscopy (LSM 5100 Meta, Carl Zeiss, Germany). For analysis of the effects of EGFR ligands on Cx43 phosphorylation (Supplementary Fig. 3), AREG (100 ng/ml), H89 (100 µg/ml, SRP6050, Sigma, USA), or TGF-α (100 ng/ml, 239-A-100, R&D systems, Minneapolis, MN, USA) were added to the cells for 30 min.

Scrape loading/dye transfer assay. Gilc levels were determined using the scrape loading/dye transfer technique as previously reported, with minor modifications. Briefly, cardiomyocytes were grown to confluence in 35-mm culture dishes. For coculture with cardiac-resident macrophages, sorted cardiac CD11b+ F4/80+ cells (“Ly6ClowLy6G-” macrophages) (15,000 cells/per well) were added to cardiomyocytes. After protein concentrations were determined using a BCA protein assay kit (Pierce, 23227, Rockford IL, USA), protein samples were separated using SDS-PAGE and transferred to nitrocellulose membranes (BioRad, 1620112, Hercules CA, USA). In the dephosphorylation experiments, the samples were treated for 1 h with lambda protein phosphatase (U1a, sc-200312A, Santa Cruz Biotechnology, USA). For repeated AREG administrations to mice, 5 µg recombinant mouse AREG protein (989-AR/CF, R&D systems, Minneapolis, MN, USA) dissolved in 50 µl of PBS was subcutaneously administrated to Arreg−/− mice as previously described. For continuous administration of AREG to mice, 5 µg/day of AREG was administered using osmotic minipumps (Alzet, Cupertino, CA, USA).

Immunohistochemistry. Cx43 staining was accomplished using anti-Cx43 antibodies (C6219, Sigma, 1:2000) and detected using a DAB system (R&D Systems, Minneapolis MN, USA). The images were acquired using a FSX1000 (Olympus, Tokyo, Japan). Quantification of Cx43 present at gap junctions was evaluated by measuring the area of Cx43 at gap junctions and total Cx43 using Image J 1.53a software (NIH, USA).

Mouse model preparation. For the BMT experiments, 8-week-old mice were irradiated with a lethal dose (9 Gy). After 24 h, BM cells (1 x 10^6) isolated from donor mice were injected into the recipient mice through the tail vein. The donor BM was harvested in DMEM, filtered through a 100 µm cell strainer (Greiner Bio-One, 542000, Austria), washed in fresh DMEM with 10% PBS, and resuspended in PBS. For the PAB experiments, the surgery was performed largely as previously described. We optimized procedures to minimize perioperative death and gain consistent RV pressure overload. Briefly, 8- to 12-week-old mice were anesthetized with intraperitoneal urethane, after which anesthesia was maintained with 2% isoflurane. After orotracheal intubation, the mice were mechanically ventilated. An incision was made in the second left intercostal space using sterile techniques. The pulmonary artery was isolated, and a suture ligature was placed around the vessel. The suture was tied against a 23-gauge needle that was then rapidly removed. After the chest was closed, the mouse was extubated and allowed to recover from the anesthesia.

Western blotting. Hearts were removed from the experimental mice and flash-frozen in liquid nitrogen. The tissues were minced using a BioMasher® II (Nippi, 300-95441, Tokyo, Japan), after which the cell pellets were lysed with RIPA buffer containing protease inhibitor cocktail (COMPLETE, EDTA-free, Roche, USA). For repeated AREG administrations to mice, 5 µg recombinant mouse AREG protein (989-AR/CF, R&D systems, Minneapolis, MN, USA) dissolved in 50 µl of PBS was subcutaneously administrated to Arreg−/− mice, 3 times a week with Picrosirius red.

Paraffin-embedded fixed heart tissues were cut into 5-µm-thick sections, after which the sections were dewaxed, the antigens were retrieved using microwave antigen retrieval (pH 8.0), and the sections were incubated first with mouse anti-N-cadherin antibody (333900, ThermoFisher, USA, 1:500) and rabbit anti-Cx43 antibody (C6219, Sigma, 1:2000), and then with Alexa-Fluor-488-conjugated anti-mouse IgG-HRP antibodies (Cell Signaling Technology, 7076, USA, 1:5000) were used as secondary antibodies. For western blotting: rabbit anti-Cx43 polyclonal antibodies (AB1726, Merck Millipore, USA, 1:500) were used to detect Cx43, followed by rabbit anti-a-tubulin monoclonal antibodies (T6199, Sigma, USA, 1:5000) and then with Alexa-Fluor-635-conjugated anti-rabbit-IgG (A-31100, ThermoFisher, USA, 1:2000) and Alexa-Fluor-635-conjugated anti-rabbit-IgG (A-31576, ThermoFisher, USA, 1:2000). The heart sections were visualized using confocal microscopy (LSM 5100 Meta, Carl Zeiss, Germany).

Plasmid construction. To construct a green fluorescent protein (GFP)-tagged Cx43 expression plasmid, P132 and Cx43 delta dt GFP were purchased from Cecilia Lo (Addgene Plasmid, USA, #17668 and #17671). P132 was digested with KpnI and BpuI, and Cx43 delta dt GFP was digested with KpnI and XbaI. Oligonucleotides were used to link the full-length Cx43 and the Gfp-containing backbone. After ligation using T4 DNA ligase (Tak, 6023, Japan), competent DH5α Escherichia coli (Tak, 9057, Japan) was transformed with the plasmid. Positive colonies were identified and selected, and the insert size was confirmed by gel electrophoresis. Thereafter, the sequence of the cDNA encoding the chimeric protein was verified. The sequence of the oligonucleotides used in these experiments are shown in Supplemental Table 4.

To investigate intracellular calcium ions in Cx43 are phosphorylated by AREG, we used a QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies, California, USA) according to the manufacturer’s instructions to introduce mutations at the consensus phosphorylation sites for the Akt (S373), PKC (S368), cAMP (S365), CK1 (S325/328/330) and MAPK (S255/262/279/282) kinases. The sequences of the primers used were shown in Supplemental Table 4.

Long-term ECG monitoring. Long-term ECGs were recorded from experimental mice using implanted telemetry devices (Sofron, Tokyo, Japan). Under intraperitoneal anesthesia, a radiofrequency transmitter was inserted into a subcutaneous tissue pocket. The leads were tunneled subcutaneously and fixed to the pectoral muscle in the lead II configuration. The experiments were performed 1 week after the surgical instrumentation. The telemetry data were recorded continuously through a receiver placed under the mouse cage. LabChart pro version 8 (AD Instruments, Australia) was used for beat-to-beat analysis to detect arrhythmias manually. VT/VF were diagnosed based on Lambeth Convention guidelines.

ARTICLE NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-021-22178-0 | www.nature.com/naturecommunications
Optical action potential mapping. We used a high-resolution optical mapping system to examine the electrophysiological properties of the heart. Details of the system and the mapping procedure were described previously.2  In brief, isolated mouse hearts were continuously perfused on a Langendorff apparatus with modified Krebs-Ringer solution equilibrated with 95% O2-5% CO2 (37 °C, pH 7.4). The hearts were then stained with the voltage-sensitive dye 4-[(6-[2-(di-n-butylamino)-6-naphthalenylvinyl]pyridinium (di-4-ANEPPS). We used premature stimulation or burst pacing to induce VT/VF. After rapid stimulation at an S1–S1 interval of 100 ms, a single premature stimulation (S2) was applied, and the sequence was repeated with progressive shortening of the S1–S2 interval. When VT/VF was not induced, burst pacing was applied. In addition, VT/VF induction was also tested in the same protocol under 3 µM isoproterenol.

Conduction velocity (CV) was measured as previously described.4 Briefly, CV was measured during constant stimulation of the center of the LV free wall using a basic cycle length of 150 ms. The longitudinal (L) direction of propagation was determined from the activation map so that it crossed the most widely spaced isochrones. A second line for the transverse (T) direction of propagation was drawn perpendicular to the first line through the most densely spaced isochrone.

Echocardiography. Mice were anesthetized with isoflurane (2–3% for induction; 0.25–2% for maintenance) and set in a supine position. Transthoracic echo-cardiography was recorded using a Vevo 2000 imaging system (VisualSonics). Pulmonary artery peak velocity, right ventricular fractional shortening, and right ventricular wall-thickness were measured as previously reported.4

Flow cytometric analysis and cell sorting. Samples for flow cytometry were prepared as previously described. Briefly, mice were anesthetized, and the heart was exposed and perfused with 10 ml PBS from the left ventricle. Thereafter, the heart tissue was mechanically minced using a scalpel, and the tissue was incubated in Dulbecco’s modified Eagle’s medium (DMEM) containing 1% elastase (Worthington Biochemical) for 120 min at 37°C. During the incubation, the cells in the suspension were dissociated by sequentially passing the extract through 20-, 21- and 23-gauge needles at 30-min intervals. To further dissociate the cells, they were passed through a 23-gauge needle three times and then filtered through a 40-μm cell strainer (BD). The cells were then centrifuged at 300 x g for 5 min, washed with PBS, and resuspended in FACS buffer (PBS supplemented with 1% FBS). After removing erythrocytes in BD PharmLyse solution (BD), the isolated cells were stained with fluorescein-conjugated antibodies and subjected to flow cytometric analysis and cell sorting using a FACS Aria III instrument (BD). Data were acquired on FACSDIVA v8.0.2 (BD) and analyzed using FlowJo (Tree Star). Cells isolated for DNA purification were fixed before staining with antibodies for flow cytometric analysis, as previously reported.4 FACs-seq staining strategies were shown in Supplementary Figure 16.

Antibodies. For flow cytometric analysis, anti-CD45.2-V500(562129, BD bioscience, 1:1000), anti-CD45.2-APC(109814, BioLegend, 1:100), anti-CD45.2-V450(103235, BioLegend, 1:100), anti-CD90-PerCP/Cy5.5(127615, BioLegend, 1:100), anti-B220-PerCP/Cy5.5(30328,BioLegend, 1:100), anti-CD11b-BV421(102151, BioLegend, 1:100), anti-CD64-APC(13906, BioLegend, 1:100), anti-F4/80-PE(123110, BioLegend, 1:100), anti-Ly6c-PE-Cy7(128081, BioLegend, 1:100) were used. For western blotting, anti-Cx40 (AB1726, Merck Millipore, 1:500), anti-Cx45(7076, Cell Signaling Technology, 1:5000) were used. After removing erythrocytes in BD PharmLyse solution (BD), the isolated cells were stained with fluorescein-conjugated antibodies and subjected to flow cytometric analysis and cell sorting using a FACS Aria III instrument (BD). Data were acquired on FACSDIVA v8.0.2 (BD) and analyzed using FlowJo (Tree Star). Cells isolated for RNA purification were fixed before staining with antibodies for flow cytometric analysis, as previously reported.4 FACs-seq staining strategies were shown in Supplementary Figure 16.
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48. The authors declare no competing interests.

49. Additional information Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-22178-0.

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51. Peer review information *Nature Communications* thanks Robin Shaw and the other anonymous, reviewer(s) for their contribution to the peer review of this work.

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