

Complement Receptor C5aR1 Plays an Evolutionarily Conserved Role in Successful Cardiac Regeneration

BACKGROUND: Defining conserved molecular pathways in animal models of successful cardiac regeneration could yield insight into why adult mammals have inadequate cardiac regeneration after injury. Insight into the transcriptomic landscape of early cardiac regeneration from model organisms will shed light on evolutionarily conserved pathways in successful cardiac regeneration.

METHODS: Here we describe a cross-species transcriptomic screen in 3 model organisms for cardiac regeneration: axolotl, neonatal mice, and zebrafish. Apical resection to remove ≈10% to 20% of ventricular mass was carried out in these model organisms. RNA-sequencing analysis was performed on the hearts harvested at 3 time points: 12, 24, and 48 hours after resection. Sham surgery was used as internal control.

RESULTS: Genes associated with inflammatory processes were found to be upregulated in a conserved manner. Complement receptors (activated by complement components, part of the innate immune system) were found to be highly upregulated in all 3 species. This approach revealed induction of gene expression for complement 5a receptor 1 in the regenerating hearts of zebrafish, axolotls, and mice. Inhibition of complement 5a receptor 1 significantly attenuated the cardiomyocyte proliferative response to heart injury in all 3 species. Furthermore, after left ventricular apical resection, the cardiomyocyte proliferative response was diminished in mice with genetic deletion of complement 5a receptor 1.

CONCLUSIONS: These data reveal that activation of complement 5a receptor 1 mediates an evolutionarily conserved response that promotes cardiomyocyte proliferation after cardiac injury and identify complement pathway activation as a common pathway of successful heart regeneration.

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Clinical Perspective

What Is New?

- RNA-sequencing analysis of models of successful cardiac regeneration identified evolutionarily conserved mechanisms that are active in early cardiac regeneration.
- Genes associated with inflammatory pathways are upregulated in all 3 species.
- Complement 5a receptor and complement 3a receptor were highly upregulated in a conserved manner during early cardiac regeneration.
- Inhibition of complement 5a receptor 1 inhibits cardiac regeneration, reducing the number of proliferating cardiomyocytes and increasing scar formation.
- Genetic deletion of complement 5a receptor 1 impairs cardiac regeneration after apical resection.

What Are the Clinical Implications?

- Identification of complement receptors induced in early cardiac regeneration provides insight into how successful heart regeneration can be initiated.
- Understanding how organisms achieve heart regeneration may reveal why adult humans fail to regenerate myocardium.

Although organisms such as zebrafish, axolotl, and newt exhibit cardiac regenerative ability throughout life,^{1–5} mammalian hearts have limited natural regenerative potential with the exception of a narrow regenerative window demonstrated in neonatal mice.⁶ Adult mammals, including humans, fail to regenerate significant myocardium after injury. Instead, the injured muscle is replaced with scar tissue, compromising the contractility of the remaining myocardium when the extent of injury is severe.^{2,7,8} This inability of the adult mammalian heart to regenerate significant amounts of tissue can lead to subsequent heart failure, a leading cause of mortality.^{7,9}

On injury in animals that can regenerate myocardium, some cardiomyocytes undergo dedifferentiation and proliferation to regenerate the lost tissue.⁴ Lineage mapping of newly derived cardiomyocytes in regenerating hearts has shown that new cardiomyocytes are primarily produced via cardiomyocyte cell division in zebrafish^{1,10,11} and neonatal mice.⁶ Furthermore, it has also been shown that cardiomyocyte renewal in adult uninjured mouse hearts occurs through cardiomyocyte division.¹² The evidence that a significant majority of new cardiomyocytes are derived from preexisting cardiomyocytes has focused attention on cardiomyocyte division as a key step in successful cardiac regeneration.¹³

In this study, we leveraged 3 different animal models of successful cardiac regeneration to obtain insights into

early events that may activate the cardiomyocyte cell cycle after cardiac injury. We aimed to define a conserved molecular pathway that plays a critical role early in the heart regenerative response using a cross-species comparative genomic approach. We performed an RNA-sequencing screen in zebrafish (*Danio rerio*), axolotl (*Ambystoma mexicanum*), and neonatal mouse (*Mus musculus*) 12, 24, and 48 hours after cardiac apical resection. With the availability of recent tools to conduct such large-scale and multispecies studies, gene expression profiling in multiple species with enhanced capacity for cardiac regeneration is a powerful method to identify genes and pathways that play critical roles in cardiac regeneration. In addition to the upregulation of cell cycle and inflammatory genes across all 3 species, components of complement signaling were also upregulated in the regenerating hearts. Complement 5a receptor 1 (C5aR1) was one of the most significantly upregulated genes early in the regenerating hearts of all species analyzed.

C5aR1 is a G-protein–coupled receptor activated by complement 5a, a peptide anaphylotoxin generated by the cleavage of full-length complement 5 protein.¹⁴ The complement pathway is a component of the innate immune system that serves to augment the ability of the immune system to identify and initiate the clearance of foreign material.¹⁵ Complement components are synthesized by the liver as zymogens. Activation of the complement cascade by external cues results in sequential proteolytic cleavage of complement proteins into peptides that activate G-protein–coupled receptors.^{15,16} Expression of complement components has previously been noted in other regenerating tissues such as the axolotl and newt limb, mammalian liver, and chick embryonic retina.^{17–20} C5a has been shown to play a crucial role in murine liver regeneration,²¹ and several studies implicate C5a and C5aR in ischemic injury and cardiac dysfunction after sepsis.^{22–24} Here we show that complement signaling is required to initiate cardiomyocyte proliferation during heart regeneration.

METHODS

The data and pipelines from our RNA-sequencing experiments have been deposited with GEO (accession no. GSE108493), and the material was publicly released on February 1, 2018. Additional data and methods pertinent to the findings of this study are available from the corresponding author (R.T.L.) on reasonable request.

Animals

All experiments were conducted in accordance with the Guide for the Use and Care of Laboratory Animals and approved by the Harvard University Institutional Animal Care and Use Committee. Wild-type (WT) CD-1 and C57BL/6 mice were obtained from Charles River Laboratories. C5aR1 knockout mice were obtained from the laboratory of Dr Craig Gerard at Boston Children's Hospital.

For RNA-sequencing experiments, zebrafish of strain WT AB and WT TuAB were produced, maintained, and housed in the laboratory of Dr Calum MacRae at Massachusetts General Hospital. Ventricular apical resection was performed on 9-month-old zebrafish. For inhibition of C5aR1, transgenic zebrafish *Tg(cmlc2:nlsDsRed-Express)^{hsc4}* were used.²⁵ Adult zebrafish used for experiments were grown and maintained in compliance with the Institutional Animal Care and Use Committee of Massachusetts General Hospital.

WT black axolotl were maintained and housed in the laboratory of Dr Jessica Whited at Brigham Regenerative Medicine Center. Subadult axolotls were used for ventricular resection, and leucistic axolotls were used for C5aR1 inhibition studies.

Ventricular Apical Resection

Ventricular apical resection was performed on zebrafish between 4 and 6 months of age as previously described.¹ Axolotl resection surgeries were performed on axolotls between 8 and 12 months of age as previously described.²⁶ Animals were anesthetized in tricaine mesylate (MS-222) for 20 minutes. Lateral thoracotomy was then performed to expose the heart ventrally. Microsurgery scissors were used to resect $\approx 10\%$ to 20% of the ventricle. The control—or sham—animal group underwent the thoracotomy but not apical resection. After a blood clot was formed, a 6-0 prolene suture was used to suture the incision in the thoracic cavity.

Neonatal mouse resection procedures were performed on pups 1 day of age as described previously.^{6,27} These mice were anesthetized on ice for 4 minutes. All mice underwent a lateral thoracotomy. Resected animals received a $\approx 10\%$ resection of the ventricle to expose the left ventricular chamber, and the control surgery animals (sham) received only a thoracotomy.

RNA Isolation and Sequencing

Total RNA from the lower half of the ventricle of resected and sham hearts was isolated using TRIzol at 3 different time points: 12, 24, and 48 hours after surgery. Three biological replicates were used for each time point and condition in each species. For neonatal mice and axolotls, each replicate represents myocardium from a single animal. Because of the smaller size of the zebrafish heart, each biological replicate represents 3 pooled tissue samples. Polyadenylated mRNA was isolated using the Wafergen no. 400047 protocol, and RNA-sequencing libraries were prepared with the IntegenX Directional PrepX mRNA kit as previously described.²⁸ Paired-end (50 base pairs) sequencing was performed using an Illumina HiSeq 2500. Polyadenylated mRNA isolation, library preparation, and sequencing were performed by the Biopolymers Facility at Harvard Medical School.

RNA-Sequencing Data Analysis

Tophat 2 was used to align paired-end sequence reads to the GRCm30 genome for mouse and GRCz10 genome for zebrafish.²⁹ Cufflinks was used to estimate gene expression levels in aligned reads, and the resulting data were processed with Cuffdiff to identify differentially expressed genes between resected and sham groups at each time point and species.³⁰ In contrast to zebrafish and the mouse, the full genome of the axolotl has not been sequenced. Thus, we took an approach

where we first mapped the axolotl RNA-sequencing data to a previously published transcriptome that was constructed using the Trinity program.^{31–33} RNA-Seq by expectation maximization (RSEM) was used to quantify gene expression,³⁴ and differential gene expression analyses were performed using Empirical Bayesian RNA-seq analysis (EBseq).³⁵ For all species, a given gene had to be identified as differentially expressed (false discovery rate [FDR]–adjusted *P* value < 0.05) by the programs described earlier and have a fold change of > 1.2 to be considered in our final analyses. After identifying differentially expressed genes using the criteria described earlier, we constructed 2 datasets for each species that consisted of genes that were either upregulated or downregulated at 12, 24, or 48 hours after surgery. We performed this step to account for species-specific time differences during early heart regeneration.

Orthology and Gene Ontology Analyses

Ensemble BioMart was used to identify the mouse orthologs of differentially expressed zebrafish genes.³⁶ Axolotl genes were annotated using Trinotate as previously described.³³ The Web-based Gene Set Analysis Toolkit (WebGestalt) was used to perform gene ontology analyses on the set of genes that were either upregulated during heart regeneration in both neonatal mice and zebrafish or downregulated during heart regeneration in both neonatal mice and zebrafish at 12, 24, or 48 hours after surgery.^{37,38} *Musculus* was used as the organism of interest, overrepresentation enrichment analysis was used as the method of interest, and gene ontology was used for the functional database. Ensemble gene identifiers were used as inputs, and the genome was used as the reference set for enrichment analysis. Gene ontology terms with an FDR-adjusted *P* value < 0.05 were considered to be statistically significant.

Inhibition of C5aR1 Using PMX205 After Apical Resection

In axolotl, 0.05 mg PMX205^{39,40} (Tocris, #5196) was delivered via intraperitoneal injection for 14 days after resection.

PMX205 (R&D, 5196, 2.5 mg·kg⁻¹) or vehicle control was injected intraperitoneally into adult zebrafish. Injections were initiated 1 hour before ventricular resection and then daily for 10 days after ventricular amputation.

In mice, 0.025 mg PMX205 was administered by a subcutaneous injection 30 minutes before resection, and 0.025 mg was delivered after resection on the day of surgery. For the next 3 days, 0.05 mg PMX205^{39,40} (Tocris, no. 5196) was administered.

Histology, Immunofluorescence, and Western Blotting

Zebrafish hearts were extracted and fixed as described.⁴¹ Hearts were then processed for histological and immunofluorescence analysis as described.⁴² Primary antibodies used were anti proliferating cell nuclear antigen (PCNA, sc-56, 1:50, Santa Cruz Biotechnologies), anti-DsRed (1:200, Clontech), and antitropomyosin (clone CH1, 1:100, Developmental Studies Hybridoma Bank). Alexa (488, 568, 633)–conjugated

Table. List of Antibodies Used for Immunostaining

Primary antibodies	Phospho-histone 3 (rabbit, Millipore), 1:100
	C5aR1 (rabbit, Santa Cruz), 1:100
	Cardiac troponin T (mouse, Abcam), 1:200
	CD31 (rat, Abcam), 1:100
	CD68 (rabbit, Abcam), 1:100
Secondary antibodies	Alexa 488, 568, 647 (Invitrogen), 1:500

antibodies (Invitrogen) were used to detect primary antibody signal. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), and slides were mounted in FluorSave (Millipore). We tried 2 antibodies for C5aR1 localization: SantaCruz sc-56 and Thermo Fisher MA1-81761. We observed the best signal with SantaCruz anti-C5aR1 in our immunostaining experiments.

Hearts were fixed with 4% paraformaldehyde overnight, followed by cryoprotection through a sucrose gradient of 10%, 15%, 20%, and 30% sucrose in PBS. Samples were left in each sucrose solution until the tissue sank to the bottom of the vial and then left in 30% sucrose overnight at 4°C. The following day, samples were embedded in optical cutting temperature compound and sectioned longitudinally (12 μM). Antibodies used for immunostaining are listed in the Table.

Mouse anti C5a (Abcam) was used at a 1:1000 dilution to detect C5a levels in resected and sham hearts 48 hours after resection. In addition, 50 ug tissue lysate was loaded per well in the Western blot. Cell signaling rabbit anti-GAPDH was used at 1:2000 for loading control.

The 5-ethynyl-2-deoxyuridine (EdU) was purchased from Carbosynth, Inc. Primary cardiomyocytes were isolated from WT mice and cultured in fibronectin-coated dishes at a density of 300000 cells per well. Seventy-two hours after plating, cells were treated with varying concentrations of C5a (saline for control) and incubated with medium containing 20 μmol/L EdU for 24 hours. After treatment, cells were fixed and stained with PCM-1 (Abcam), EdU (Sulfo-Cy3 azide), and DAPI and imaged using CellDiscoverer (Zeiss).

Imaging

A total of 3 sections per heart were quantified for each group in zebrafish. Discosoma red fluorescent protein (DsRed⁺)/proliferating cell nuclear antigen⁺ cardiomyocyte nuclei were divided by the total DsRed⁺ cardiomyocytes to generate a proliferation index.

Fluorescently stained sections were imaged with Zeiss LSM 700 (mouse) and Zeiss Axiozoom (Axolotl) (HCBI, Harvard University). A total of 5 sections per heart were quantified for each group in a blinded manner in axolotl and mouse.

Statistical Analysis

All data are presented as mean±SEM. All statistical analyses were performed using Prism 7 software (GraphPad). After normality testing using the Wilk–Shapiro test, the 2-tailed Student's *t* test was used to compare data from individual experimental groups. A value of *P*<0.05 was considered significant.

RESULTS

Transcriptomic Analyses Indicate a Conserved Role for the Inflammatory Response and Metabolic Regulation During Early Neonatal Mouse and Zebrafish Heart Regeneration

We utilized 3 model organisms capable of successful cardiac regeneration: neonatal mice, zebrafish, and axolotls. We resected ≈10% to 20% of the ventricular myocardium in all 3 species, after which RNA-sequencing was carried out on the regenerating hearts of each species at 12, 24, and 48 hours after resection. We undertook this approach to assess differential gene expression at 3 different time points because studies have indicated that cardiac regeneration occurs at different rates across the 3 species; neonatal mice successfully accomplish regeneration in as few as 21 days, whereas zebrafish and axolotl heart regeneration take ≥60 days to replace the lost myocardial tissue.^{6,43} RNA-sequencing on sham-operated hearts at each time point was used as control for data analysis. These internal controls were performed to account for potential systemic effects because of surgical opening of the thoracic cavity and for the rapid growth of the neonatal mouse during the first few days after birth. The zebrafish and mouse genomes are well annotated and enabled feasibility of an RNA-sequencing experiment with multiple samples. Although the axolotl genome is incomplete, a high number of transcriptomes and various annotations exist to assist in analysis.³³

After acquisition of the RNA-sequencing data, we found that 1433 genes were differentially expressed in neonatal mice, 4502 genes were differentially expressed in zebrafish, and 4639 gene contigs were differentially expressed in axolotl heart regeneration across all time points. We undertook a systematic computational approach to refine the dataset to identify genes that are functionally important for heart regeneration across all 3 species (Figure 1A). First, we identified orthologous mouse and zebrafish genes that were consistent in terms of their differential expression (ie, both upregulated or both downregulated) during early regeneration (Figure 1A). We found that 233 genes were commonly upregulated during the first 2 days of heart regeneration (Figure 1B). These genes represented 23.5% of upregulated mouse genes (233/990 genes) and 11.1% of mouse genes (233/2103) with an orthologous relationship to upregulated zebrafish genes. Furthermore, we identified 64 genes common to zebrafish and mice that were downregulated during this early time period (Figure 1C). These genes constitute 12.1% of mouse genes that were downregulated within the first 2 days of heart regeneration (64/527) and 4.1% (64/1572) of genes corresponding to the mouse orthologues of downregulated zebrafish genes.

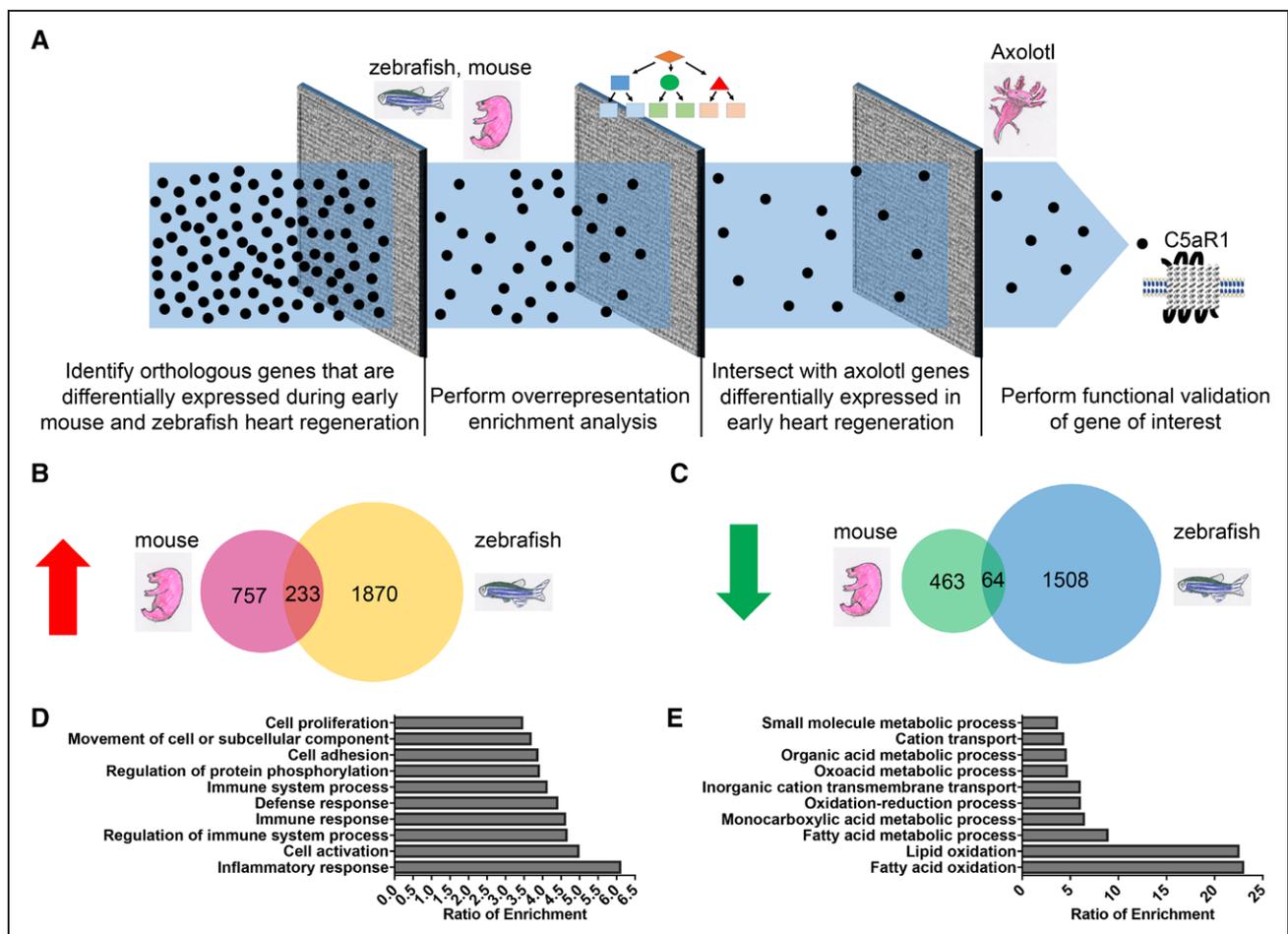


Figure 1. RNA-sequencing analysis pipeline to identify evolutionarily conserved genes involved in early heart regeneration.

Ventricular myocardium was resected, and RNA-sequencing was performed at 12, 24, and 48 hours after injury in neonatal mice, axolotl, and zebrafish. Each time point was internally controlled with sham surgery. **A**, Schematic of transcriptomic approach for identifying genes whose expression was conserved during early heart regeneration. Black dots represent genes, and gray squares represent computational filtration steps. **B**, Intersection of genes that were upregulated at 12, 24, or 48 hours relative to sham controls in neonatal mice and zebrafish. **C**, Intersection of genes that are downregulated at 12, 24, or 48 hours relative to sham controls in neonatal mice and zebrafish. **D**, Gene ontology analysis of commonly upregulated neonatal mouse and zebrafish genes. **E**, Gene ontology analysis of commonly downregulated neonatal mouse and zebrafish genes. C5aR1 indicates complement 5a receptor 1.

Next, we attempted to identify conserved gene expression modules by performing overrepresentation analyses on the common differentially expressed genes (Figure 1A). Using WebGestalt,³⁸ we found that the set of genes commonly upregulated during mouse and zebrafish heart regeneration was significantly enriched for gene ontology terms such as inflammatory response, regulation of immune system process, and immune system process (FDR <0.05; Figure 1D). These data suggest that regulation of the inflammatory response during early heart regeneration is a conserved process across species and is consistent with previous studies demonstrating the functional importance of the early immune response to regeneration.^{44–46} Similarly, we also observed significant enrichment (FDR <0.05) for cell proliferation (Figure 1D), which is consistent with cardiomyocyte division as well as possibly nonmyocytes during heart regeneration.^{6,7,10,11}

Biological processes such as fatty acid oxidation, lipid oxidation, and fatty acid metabolic process had the highest ratio of enrichment among significant gene ontology terms (FDR <0.05) in the set of genes commonly downregulated in neonatal mouse and zebrafish heart regeneration (Figure 1E). These data suggest that fundamental changes in metabolic processes during early heart regeneration are evolutionarily conserved across species.

Cross-Species Gene Expression Profiling Indicates a Conserved Role for Complement Components During Early Heart Regeneration

Motivated by our ability to uncover broader biological processes that are known to be essential for regenera-

tion, we focused our attention on genes belonging to the inflammatory response classification that are commonly upregulated during mammalian and zebrafish heart regeneration (Figure 1D). This particular category comprised 38 genes and had the highest enrichment ratio relative to other gene ontology terms in the upregulated gene set (Figure 1D). We then identified 15 inflammatory response genes that were also upregulated during early axolotl heart regeneration (Figure 2A). These comprised the highest confidence set of upregulated genes during early cardiac regeneration across the 3 species (Figure 2B).

These include C5aR1 and complement component 3a receptor, which serve as the receptors for complement components C5a and C3a, respectively, and have been shown to mediate complement signaling in macrophages (Figure 2A).⁴⁷ The complement cascade is an ancient component of the innate immune system. Recent studies have shown that complement may also mediate cross-talk between the innate and adaptive immune responses.⁴⁸ On binding to their corresponding receptors, complement peptides C5a and C3a act as potent inflammatory mediators that can trigger the degranulation of endothelial cells, mast cells, and phagocytes, and they function as chemoat-

tractants for neutrophils, monocytes, and macrophages.⁴⁹ Complement proteins have been noted in several regenerating tissues such as newt lens, axolotl limb, and mouse liver.^{19,20} It is interesting to note that the expression of C5 and C3 was observed in regenerating limb tissues such as blastema and wound epidermis, suggesting that the complement system could play functional roles in other regenerative contexts beyond heart regeneration.²⁰ Although most of the evidence supporting the expression and role for the complement system in regeneration is mainly observational, Strey et al²¹ showed mechanistic evidence supporting the crucial role of C5a signaling in murine liver regeneration after partial hepatectomy.

The evolutionarily conserved upregulation of complement components observed during early heart regeneration in neonatal mouse, zebrafish, and axolotl, along with studies implicating the complement system in regenerative processes outside of the heart, suggest a role of complement receptors in cardiac regeneration. Of the 2 receptors identified, C5aR1 was upregulated during regeneration (Figure 2A). When normalized to expression levels in sham-operated animals, C5aR1 expression in all 3 species was upregulated significantly, peaking at slightly different time points within the first

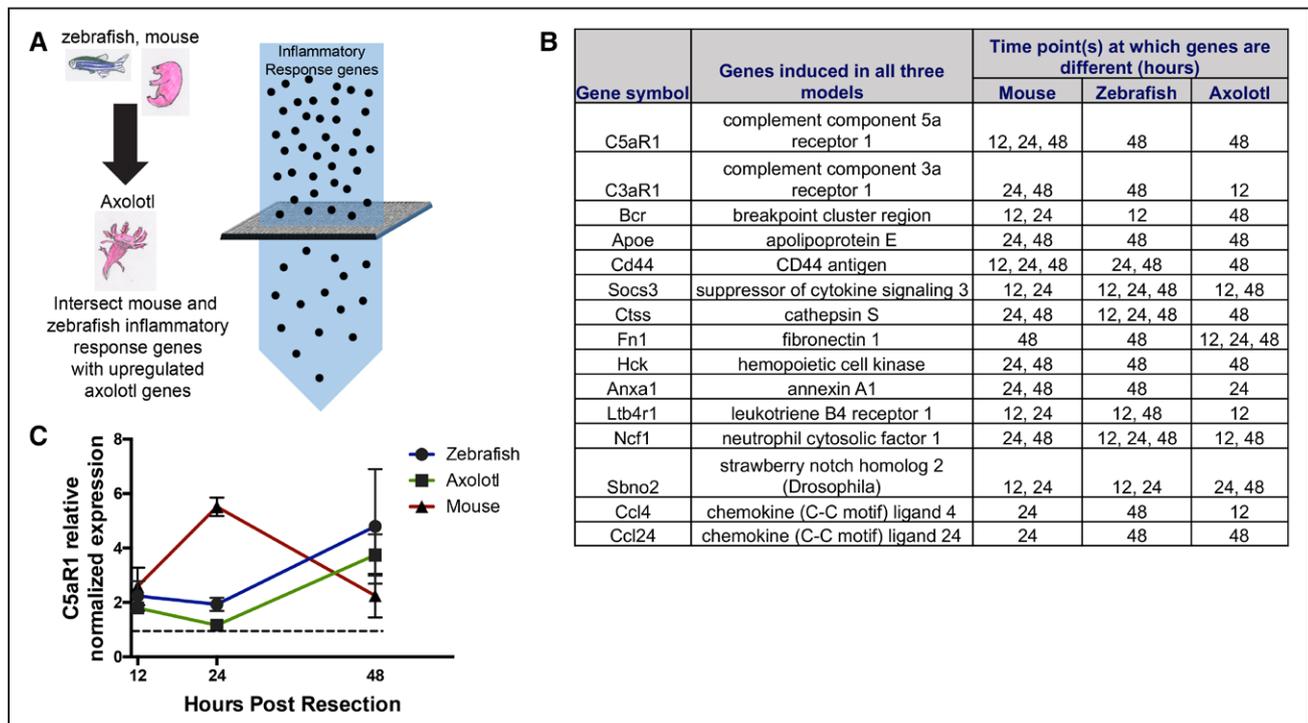


Figure 2. Schematic of analysis pipeline used to identify upregulated inflammatory genes in mouse, zebrafish, and axolotl.

A, Inflammatory response genes including complement components and associated proteins that are upregulated in early cardiac regeneration in an evolutionarily conserved manner. The list is ordered according to fold change; genes with higher fold change in mouse are first, followed by genes in decreasing order. Note that the top 2 genes are receptors for activated complement components, C5aR1 and C3aR1. **B**, C5aR1 is upregulated in early cardiac regeneration in zebrafish, axolotl, and mouse at the time points shown, in hours (**C**), and expression of C5aR1 is normalized to sham levels (dotted line). C3aR1 indicates complement 3a receptor; and C5aR1, complement 5a receptor.

48 hours (Figure 2B). Specifically, we observed the highest fold change in C5aR1 expression at 24 hours after injury in mice and 48 hours after injury in zebrafish and axolotls. We speculate that the time differences in expression may be attributed to species-specific differences in the heart regeneration response and could possibly reflect the different rates at which heart regeneration occurs in these species. Taken together, our analyses suggested that conserved activation of C5aR1 during early heart regeneration could play a role in cardiac regeneration.

Inhibition of C5aR1 Severely Attenuates Cardiomyocyte Proliferation After Apical Resection

Based on our finding that upregulation of complement signaling is an evolutionarily conserved mechanism in early cardiac regeneration, we sought to test the hypothesis that C5aR1 signaling is required for successful cardiac regeneration after apical resection in zebrafish, axolotl, and mice. We utilized a peptide C5aR1 antagonist, PMX205,^{39,40} to inhibit C5aR1 activity across the 3 species. PMX205 is a highly selective C5aR1 antagonist with a half-maximal inhibitory concentration (IC_{50}) of 31 nM.⁵⁰ Although studies have focused on the immunomodulatory role of C5aR1, it is known to be expressed in other tissues including liver, kidney, and heart.¹⁴ We tested the hypothesis that C5aR1 receptor function is required for effective cardiac regeneration by administering PMX205^{39,40} after apical resection in zebrafish, axolotl, and neonatal mice. PMX205 was administered to the animals after cardiac apical resection (vehicle treatment of resected animals and sham surgery with vehicle and PMX205 administration for control). Cardiomyocyte proliferation was quantified by immunostaining for proliferation markers phospho-histone 3 (pH3; mouse and axolotl) and proliferating cell nuclear antigen (zebrafish). The total number of proliferating

cardiomyocytes per field of view was quantified after imaging of immunostained heart sections.

Because of differences in the rate at which cardiac regeneration occurs in the 3 species, different lengths of treatment with PMX205 were used for each species studied. Apical resection was performed on zebrafish (sham for control), after which PMX205 was administered for 10 days at 0.05 mg/zebrafish by intraperitoneal injection. Zebrafish hearts were harvested 14 days after resection and stained with proliferation marker proliferating cell nuclear antigen. A significant reduction in the number of proliferating cardiomyocytes per unit area was observed in resected zebrafish hearts treated with PMX205 (Figure 3A and 3B). Quantification of proliferating cardiomyocytes from the experimental groups showed that PMX205 effected \approx 2-fold reduction in cardiomyocyte proliferative response in zebrafish after apical resection (Figure 3C).

Axolotls received 14 days of PMX205 administration (0.05 mg/kg) after apical resection. Axolotl hearts were harvested after PMX205 treatment and stained for pH3 and the cardiomyocyte marker Myosin heavy chain (MF20) to quantify cardiomyocyte proliferation. Inhibition of C5aR1 significantly decreased cardiomyocyte cell cycle activity after apical resection in axolotl (Figure 4A through 4C), revealed by a reduction of pH3-positive cardiomyocytes per field of view in resected axolotl hearts treated with PMX205. Quantification of the number of proliferating cells per field of view showed a \approx 2-fold reduction in the number of pH3-positive cardiomyocytes in axolotl hearts treated with PMX205 (quantified blindly in Figure 4D).

Because cardiac regeneration in neonatal mice is faster than in axolotl and zebrafish, we assessed the effect of PMX205 treatment for 4 days after apical resection on neonatal mice (P1). Hearts from experimental mice were harvested 7 days after resection, and proliferation was assessed by staining for pH3. We observed a significant reduction of pH3-positive cardiomyocytes

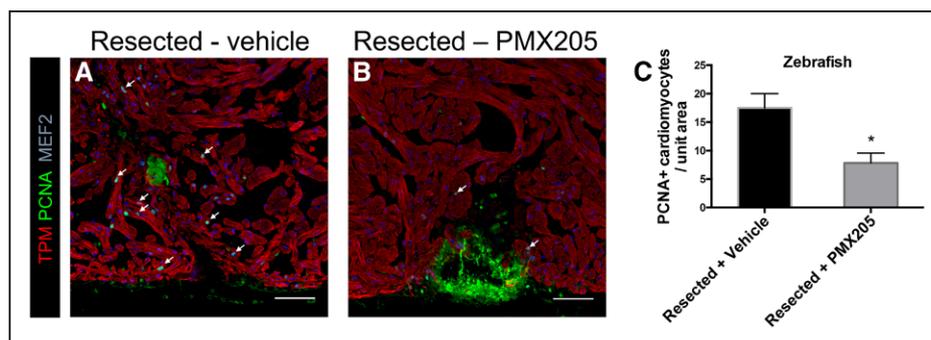


Figure 3. Inhibition of C5aR1 decreases proliferating cardiomyocytes after apical resection in zebrafish.

A, Resected zebrafish treated with vehicle, representative image. **B**, Resected zebrafish treated with PMX205, representative image, with cardiomyocyte marker tropomyosin (TPM; red), proliferating cell nuclear antigen (PCNA) (green), and cardiomyocyte nuclei (MEF2) (blue). Scale bar, 50 μ m. **C**, Quantification of proliferating cardiomyocytes in vehicle- and PMX205-treated hearts of zebrafish. * $P < 0.01$. Vehicle, $n = 5$. PMX205, $n = 8$. C5aR1 indicates complement 5a receptor 1.

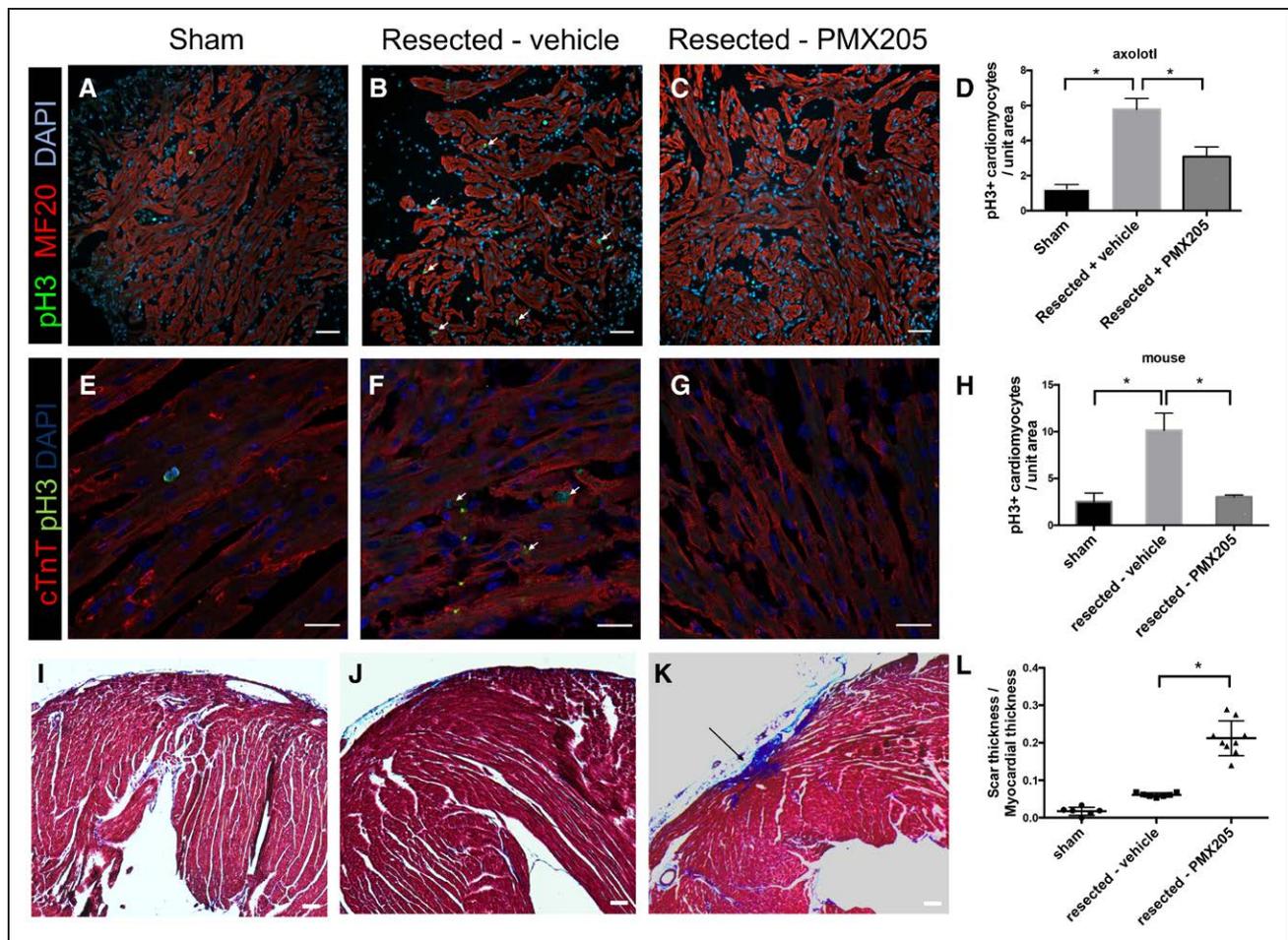


Figure 4. Inhibition of C5aR1 in axolotl and mouse.

Inhibition of C5aR1 results in a reduction of proliferating cardiomyocytes in axolotl hearts. Cardiomyocyte marker myosin heavy chain (MF20) is shown in red, phospho-histone 3 (pH3) is shown in green, and nuclei are in blue. **A**, Sham surgery of axolotl hearts. **B**, Resected vehicle treatment. **C**, Resected PMX205 treatment. Scale bar, 100 μ m. **D**, Quantification of proliferating cardiomyocytes. $*P < 0.05$. $n = 4$. Effect of C5aR1 inhibition on cardiomyocyte proliferation in mouse (**E** through **H**). Cardiac troponin T (cTnT, cardiomyocyte marker) is shown in red, phospho-histone 3 staining in green, and DAPI stained nuclei in blue. **E**, Sham surgery of mouse hearts. **F**, Resected vehicle treatment. **G**, Resected PMX205 treatment. Scale bar, 20 μ m. **H**, Quantification of cardiomyocyte proliferation. $*P < 0.05$. $n = 4$. Effect of C5aR1 inhibition on scar formation after apical resection (**I** through **L**). Masson trichrome images of hearts 21 days after resection show an increase in scar formation in PMX205 treatment. Representative images of sham surgery (**I**), resected vehicle treatment (**J**), and resected PMX205 treatment (**K**). **L**, Quantified scar infiltration. Scale bar, 100 μ m. $*P < 0.05$. $n \geq 6$. C5aR1 indicates complement 5a receptor 1; and DAPI, 4',6-diamidino-2-phenylindole.

in the PMX205-treated animals compared with vehicle treatment, consistent with the reduction of cardiomyocyte proliferation we observe in axolotl and zebrafish (Figure 4E through 4H). Because the mouse model is the most relevant for mammalian heart regeneration among the 3 model organisms used, the effect of C5aR1 inhibition was then assessed at the histological level by quantifying scarring of the murine heart in the different experimental groups 21 days after injury by Masson Trichrome staining. An increase in scarring of the ventricular apex was observed in the group of animals treated with PMX205, further demonstrating that C5aR1 signaling is crucial for the initiation of effective cardiac regeneration (Figure 4I through 4L). Although minimal scarring was observed in resected

hearts treated with vehicle (Figure 4J and 4L), we observed small but consistent scars in the ventricular apex of mice treated with PMX205 after resection (Figure 4K and 4L). To further investigate the role of C5aR1 on cardiomyocyte proliferation, we quantified proliferation in primary cardiomyocytes treated with C5aR1 agonist C5a for a period of 24 hours by measuring EdU incorporation. We did not observe a significant increase in cardiomyocyte proliferation on C5a treatment (Figure I in the online-only Data Supplement). This finding indicates that the proliferative effect elicited by C5a–C5aR1 signaling is potentially invoked after cardiac injury. Overall, our results across the 3 species support the hypothesis that C5aR1 plays an important role in heart regeneration and is essential for the initiation of

a cardiomyocyte proliferative response required for successful heart regeneration. Our findings also indicate that 1 possible function of the C5aR1 pathway is to inhibit collagen deposition and scarring in mammalian heart regeneration.

C5aR1 Is Primarily Expressed in Cardiomyocytes After Apical Resection

The observed upregulation of C5aR1 expression in the early regenerating myocardium could be a result of immune infiltration of the heart after injury as C5aR1 is expressed in myeloid cells.⁵¹ Macrophages play an essential role in cardiac regeneration, and depletion of macrophages in neonatal mice results in incomplete regeneration.⁴⁶ However, despite extensive scarring and inhibition of microvasculature formation in the regenerating heart, macrophage depletion does not appear to cause a decrease in cellular proliferation after cardiac injury.⁴⁶ This finding suggests that decreased cardiomyocyte proliferation on C5aR1 inhibition may not be a result of the contribution of C5aR1 from macrophages. C5aR1 expression has been noted in cardiomyocytes^{24,52,53} as well as endothelial cells, where the C5a component of complement has been shown to activate the endothelium.^{54–56} C5aR1 expression has been shown to be upregulated in ischemic cardiomyocytes in adult mice after ischemia/reperfusion injury.²⁴ To identify the cell types expressing C5aR1 in the neonatal mouse heart during regeneration, we conducted extensive immunostaining analyses in the neonatal mouse heart after apical resection (sham for control). We found that C5aR1 was primarily expressed in cardiomyocytes (Figure 5A, seen by colocalization with cardiac troponin T), 48 hours after resection in neonatal mice. Hearts from littermate sham controls did not have expression of C5aR1 (Figure 5B). We validated the antibody by staining heart sections from C5aR1 neonates 48 hours after resection. We do not observe any signal from the antibody in C5aR1 knockout sham and resected hearts (Figure II in the online-only Data Supplement). Furthermore, we also observed colocalization of C5aR1 with the endothelial marker CD31 in the injured hearts (Figure 5C). However, we did not observe localization of C5aR1 staining with the macrophage marker CD68 in injured neonatal hearts (Figure 5D). In-depth immunostaining and imaging analyses of C5aR1 expression pattern in the injured neonatal heart show expression of C5aR1 in the injured zone at the apex (site of resection; Figure IIIA in the online-only Data Supplement), whereas no significant expression was observed in the border and remote zones (Figure IIIB and IIIC in the online-only Data Supplement, respectively). Expression of C5aR1 in cardiomyocytes after cardiac injury circumstantially supports the concept that C5aR1 plays an important role in cardiomyocyte proliferation after injury and sug-

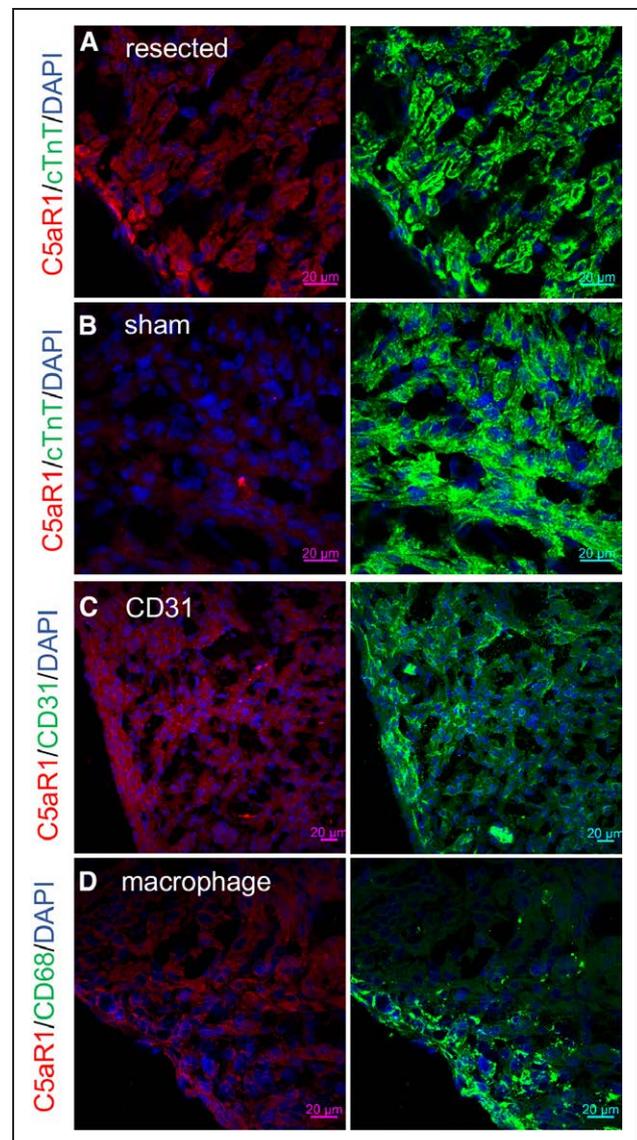


Figure 5. Localization of C5aR1 to cardiomyocytes and endothelial cells after apical resection in neonatal mice.

A, Localization of C5aR1 to cardiomyocytes and endothelial cells after apical resection in neonatal mice. C5aR1 (red) localizes with cardiomyocyte marker troponin T (green) in neonatal mouse hearts 48 hours after apical resection (DAPI stained nuclei in blue). Scale bar, 20 μ m. **B**, C5aR1 upregulation is absent in hearts from littermate sham controls. **C**, C5aR1 (red) expression localizes with endothelial marker CD-31 (green). Scale bar, 20 μ m. **D**, C5aR1 (red) does not localize with macrophage marker CD68 (green) in resected hearts. Scale bar, 20 μ m. C5aR1 indicates complement 5a receptor 1; cTnT, cardiac troponin T; and DAPI, 4',6-diamidino-2-phenylindole.

gests that either cardiomyocytes or cardiac endothelial cells—or both—are cells likely to be receiving the C5a signal during heart regeneration.

A majority of the complement components are secreted proteins, synthesized mainly by the liver as zy-

mogens. Complement component 5, on activation by complement 5 convertase, is cleaved to form C5a and C5b in target tissues. Therefore, we carried out Western blot analysis of neonatal murine hearts 48 hours after injury to detect activation of C5 and cleavage to C5a (sham for control). We observed a significant increase in C5a production in the injured heart 48 hours after resection in mice, in comparison with sham hearts of littermate animals (Figure IV in the online-only Data Supplement) ($n=3$ [all groups]; $*P<0.05$). This increase of C5a levels on cardiac resection accompanied the up-regulation of C5aR1 observed in our RNA-sequencing screen, supporting a role for C5a–C5aR1 signaling in early cardiac regeneration. These data also give further confidence in the approach of pursuing the consequences of genetic ablation of the C5aR1 receptor in the process of heart regeneration.

Genetic Deletion of C5aR1 Abolishes Cardiomyocyte Proliferation After Apical Resection

To further evaluate the role of C5aR1 in cardiac regeneration, a murine global genetic deletion model of C5aR1 was used. Cardiomyocyte proliferation was assessed in C5aR1 knockout mice and WT mice after apical resection. Our hypothesis was that the cardiomyocyte proliferative response 7 days after apical resection would be reduced in C5aR1 knockout mice in comparison with C5aR1 WT mice. Consistent with the regenerative response observed by Porrello et al⁶ in neonatal mice, the littermate WT mice had an increase in the fraction of proliferating cardiomyocytes 7 days after resection (Figure 6A through 6E), whereas in the C5aR1 knockout mice, no significant induction of cardiomyocyte proliferation was observed. This finding is consistent with the results of the receptor inhibition experiments, showing an essential role of C5aR1 in cardiomyocyte proliferation during regeneration.

Furthermore, we analyzed scarring in the hearts of C5aR1 knockout and littermate WT mice 21 days after resection to investigate the effect of C5aR1 in cardiac regeneration at a longer time point. We observed moderate scarring of the apex 21 days after resection in C5aR1 knockout mice, whereas no significant differences in scarring were observed after resection in matched WT control mice (Figure 6F through 6J). Although we did not observe a complete inhibition of apical regeneration of the hearts of C5aR1 knockout mice, an increase in fibrosis observed indicates that the overall regenerative process is partially hampered on loss of C5aR1 (Figure 6F through 6J). This increase of fibrosis was not observed in littermate WT hearts after resection, further supporting a role for C5aR1 in effective cardiac regeneration and minimization of fibrosis after an injury.

Expression of C5aR1 has been reported mainly in myeloid cells and macrophages.⁵¹ Therefore, we quantified macrophage infiltration after apical resection in C5aR1 WT and knockout hearts 48 hours after resection by immunostaining. Although we saw a significant increase in the macrophage infiltration of the injured zone in C5aR1 WT mice 48 hours after resection, we did not see any significant difference in macrophage infiltration between C5aR1 knockout sham and resected hearts (Figure V in the online-only Data Supplement). Because we observed localization of C5aR1 with the endothelial marker CD31, we analyzed the number of blood vessels in the apex 7 days after resection in C5aR1 knockout and WT mice. The total number of blood vessels per field of view was quantified. We did not see a significant difference in the number of blood vessels between sham and resected hearts of both C5aR1 knockout and WT mice (Figure VI in the online-only Data Supplement). Therefore, further downstream experiments need to be carried out with endothelial cell-specific deletion for estimating the effect of C5aR1 on angiogenesis after cardiac injury.

DISCUSSION

Achieving cardiac regeneration in adult mammals is an important goal for cardiovascular research. Although it is now known that adult mammalian cardiomyocytes can undergo cell proliferation to generate new cardiomyocytes, the rate of proliferation is insufficient to replace lost cardiomyocytes and restore function after a cardiac injury.^{57,58} Previous work has established multiple models of successful heart regeneration, including neonatal mice, zebrafish, and axolotl.^{1–4} Over the past decade, studies of cardiac regeneration in different experimental models have shed light on several shared mechanisms across the species, including stimulation of an essential immune response, a role for nerves, and a critical role of cardiomyocyte division.^{4,28,46} However, we still do not understand the barriers to heart regeneration that lead to extensive scarring and eventual heart failure in humans who have major cardiac injuries. This study leveraged similarities among model organisms that have substantial heart regenerative capacity to define a regenerative molecular pathway in the heart across species. The use of 3 unique model systems to identify the transcriptomic landscape of early cardiac regeneration provided insight into an evolutionarily conserved pathway underlying regeneration.

Our observation that C5aR1 is important for effective cardiac regeneration in an evolutionarily conserved manner fits with the existing body of literature supporting a role for the complement system in mediating regenerative responses.¹⁹ Although the role of macrophages in cardiac regeneration has been previ-

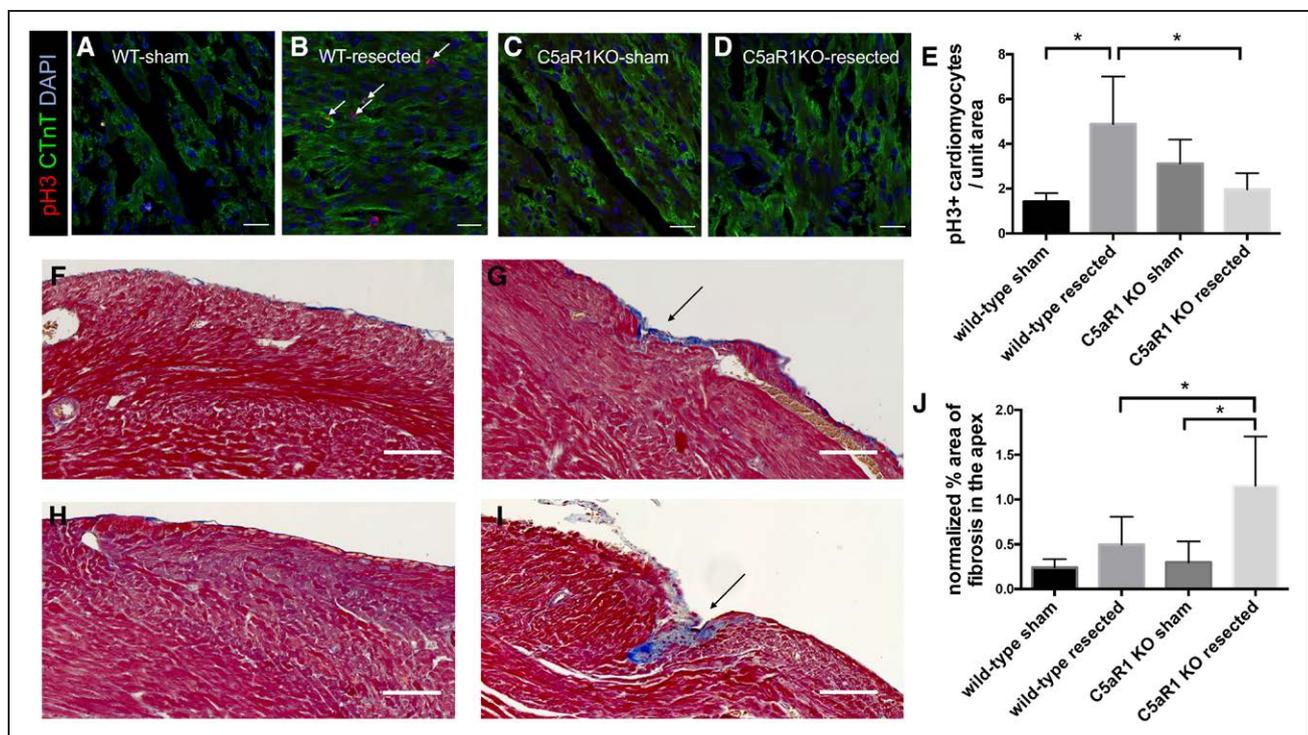


Figure 6. Mice lacking C5aR1 have a reduction in cardiomyocyte proliferation after apical resection compared with wild-type (WT) littermate controls.

A through **D**, Representative images from C5aR1 WT sham surgery (**A**), C5aR1 WT resected (**B**), C5aR1 knockout (KO) sham surgery (**C**), and C5aR1 KO resected (**D**). Cardiac troponin T (green), phospho-histone 3 (red), and nuclei (blue). Scale bar, 20 μ m. **E**, Quantification of proliferating cardiomyocytes shows the absence of proliferative response on resection in C5aR1 KO hearts. $*P < 0.05$. $n \geq 4$. **F** through **J**, Measurement of fibrosis 21 days after apical resection. Representative Masson Trichrome-stained images from C5aR1 WT sham surgery (**F**), C5aR1 WT resected (**G**), C5aR1 KO sham surgery (**H**), and C5aR1 KO resected (**I**). Scale bars, 100 μ m. **J**, Quantification of fibrosis in cardiac apex after resection. $*P < 0.05$. $n = 8$. C5aR1 indicates complement 5a receptor 1; cTnT, cardiac troponin T; and DAPI, 4',6-diamidino-2-phenylindole.

ously examined,⁴⁶ the role played by the immune system in initiating the cardiac regenerative response is not clearly understood. C5a and its receptor C5aR1 have been previously studied in the adult murine heart after septic shock, in ischemia reperfusion, and in hypertension.^{24,52,53,59,60} Activation of C5aR1 because of excessive C5a generation in sepsis has been shown to contribute to cardiac dysfunction because excessive C5a results in increased ROS and impaired Ca^{2+} handling in the cardiomyocytes.^{52,53} However, it has also been shown that C5a-C5aR1 signaling decreases hypertension-induced fibrosis in an angiotensin II-induced hypertension model.⁶¹ Our finding that C5aR1 knockout mice have increased scarring of the heart after apical resection agrees with the observations made by Weiss et al,⁶¹ who found that loss of C5aR1 results in increased cardiac fibrosis on stress or cardiac injury. Furthermore, C5aR1 has been shown to be upregulated in the adult mouse heart; after ischemia/reperfusion, C5aR1 upregulation was evident in ischemic cardiomyocytes.²⁴ However, the role of C5aR1 and complement signaling in initiating cardiomyocyte proliferation and cardiac regeneration has not been investigated.

After apical resection, we observe an upregulation of C5aR1 expression in cardiomyocytes and endothelial cells in the neonatal murine heart (Figure 5). This finding suggests a role for nonmyeloid C5aR1 in heart regeneration. Although we did not observe localization of C5aR1 with the macrophage marker, differences in macrophage infiltration of the injured zone between C5aR1 knockout and WT mice necessitate further downstream studies into the interplay of immune cells and complement components in early cardiac regeneration. It should be noted that differences in macrophage infiltration were not quantified by flow cytometry. Cell-specific conditional C5aR1 deletion models in cardiomyocytes, endothelial cells, and myeloid cells need to be examined in future experiments to define the cell types expressing C5aR1 that contribute to cardiomyocyte proliferative response after injury. Furthermore, the intracellular signaling mechanisms by which cardiomyocyte division is initiated after an injury by C5a signaling in the adult heart may provide new insights. Although we did not observe a significant increase in cardiomyocyte proliferation on treatment with C5a in vitro, it is possible that the effect of C5a-C5aR1 signal-

ing on cardiomyocyte proliferation could be mediated by paracrine signals from nonmyocytes.

A pilot experiment was conducted to analyze the therapeutic potential of C5aR1 activation in adult murine cardiac regeneration after an ischemia/reperfusion injury. A pilot dose of C5a was administered for 4 days after ischemia/reperfusion. We observed a marginal, nonsignificant increase in cardiomyocyte proliferation in the adult mouse heart after ischemia/reperfusion and treatment with C5a. Future analyses of the mechanistic processes underlying the proliferative effect seen by C5aR1 activation need to be conducted. Additionally, elucidation of the mechanisms that lead to the transcriptional upregulation of the receptor gene, *C5aR1*, after injury should provide important details for how the system is regulated. Finally, we have not yet defined the dynamics of the entire complement cascade in these models in comparison with myocardium that fails to regenerate, such as in adult mammals, and these studies might reveal that complement activation is different or identical in these settings.

Currently, treatment for myocardial infarction focuses on reperfusion and limiting ventricular remodeling.⁶⁰ Therapy targeted at replenishing lost cardiomyocytes has not entered the clinical arena. Our findings uncover a molecular pathway involving the interplay of the complement system and cardiac regeneration. We suggest that defining the molecular events of successful heart regeneration could eventually allow us to understand what events are absent in myocardium that cannot regenerate.

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Author Contributions: Drs Natarajan, Uygur, Bryant, Gerard, MacRae, C.E. Burns, C.G. Burns, Whited, and Lee participated in study design and supervision of the project. Drs Bryant and Uygur performed zebrafish and axolotl experiments in the MacRae and Whited laboratories. Dr Gonzalez-Rosa and M. Sharpe performed zebrafish experiments in the Burns' laboratory. Drs Natarajan and Uygur and Y. Abbas, N.N. Ho, and L.H. Cocco-Delgado performed mouse experiments in the Lee laboratory. Dr Bryant and N.N. Ho conducted analyses on the RNA-sequencing data for cross-species comparison of gene expression. All authors reviewed and approved the final manuscript, which was drafted by Drs Natarajan and Lee.

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Disclosures

None.

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