Natural IgM Blockade Limits Infarct Expansion and Left Ventricular Dysfunction in a Swine Myocardial Infarct Model

Smita Sihag, MD*; Michael S. Haas, PhD*; Karen M. Kim, MD; J. Luis Guerrero, BS; Jonathan Beaudoin, MD; Elisabeth M. Alicot, PharmD; Franziska Schuerpf, BS; James D. Gottschall, BS; Robyn J. Puro, PhD; Joren C. Madsen, MD; David H. Sachs, MD; Walter Newman, PhD; Michael C. Carroll, PhD; James S. Allan, MD

Background—Acute coronary syndrome is the leading cause of mortality worldwide. However, treatment of acute coronary occlusion inevitably results in ischemia-reperfusion injury. Circulating natural IgM has been shown to play a significant role in mouse models of ischemia-reperfusion injury. A highly conserved self-antigen, nonmuscle myosin heavy chain II, has been identified as a target of pathogenic IgM. We hypothesized that a monoclonal antibody (m21G6) directed against nonmuscle myosin heavy chain II may inhibit IgM binding and reduce injury in a preclinical model of myocardial infarction. Thus, our objective was to evaluate the efficacy of intravenous m21G6 treatment in limiting infarct expansion, troponin release, and left ventricular dysfunction in a swine myocardial infarction model.

Methods and Results—Massachusetts General Hospital miniature swine underwent occlusion of the midleft anterior descending coronary artery for 60 minutes, followed by 1 hour, 5-day, or 21-day reperfusion. Specificity and localization of m21G6 to injured myocardium were confirmed using fluorescently labeled m21G6. Treatment with m21G6 before reperfusion resulted in a 49% reduction in infarct size (P<0.005) and a 61% reduction in troponin-T levels (P<0.05) in comparison with saline controls at 5-day reperfusion. Furthermore, m21G6-treated animals recovered 85.4% of their baseline left ventricular function as measured by 2-dimensional transthoracic echocardiography in contrast to 67.1% in controls at 21-day reperfusion (P<0.05).

Conclusions—Treatment with m21G6 significantly reduced infarct size and troponin-T release, and led to marked preservation of cardiac function in our study. Overall, these findings suggest that pathogenic IgM blockade represents a valid therapeutic strategy in mitigating myocardial ischemia-reperfusion injury. (Circ Cardiovasc Interv. 2016;9:e002547. DOI: 10.1161/CIRCINTERVENTIONS.115.002547.)

Key Words: immunology ■ inflammation ■ left ventricular function ■ myocardial infarction ■ troponin T

R evascularization therapy for acute coronary syndrome, that is, percutaneous coronary intervention or coronary artery bypass grafting, improves overall survival,1 but does not fully prevent left ventricular dysfunction and clinical heart failure after myocardial infarction (MI).2–4 Despite numerous attempts to pharmacologically reduce myocyte injury after reperfusion, only cyclosporine has shown some promise in reducing infarct size in the clinic.5 Thus, the opportunity exists for a novel therapeutic approach that is adjunctive to revascularization therapy to limit infarct size, preserve cardiac function, and ultimately improve long-term outcomes.6 Here, we describe the first use of a monoclonal antibody (Mab) directed against a novel target exposed early in the acute phase of reperfusion. We hypothesize that the target, a conserved epitope of nonmuscle myosin heavy chain II (NMHC-II), triggers an inflammatory cascade unless its recognition by natural IgM is competitively blocked by treatment with m21G6 Mab.7

Previous experiments using complement or antibody-deficient mice have implicated endogenous IgM antibodies as significant contributors to the pathogenesis of ischemia-reperfusion (I/R) injury.8,9 These studies showed that exposure of neoepitopes on the surface of ischemic tissues provides a binding site for a distinct fraction of circulating IgM.10–12 This fraction, or pathogenic IgM clone (IgM CM-22), recognizes an epitope on NMHC-II that is exposed after reperfusion of ischemic myocardium, intestine, or skeletal muscle.
WHAT IS KNOWN

• Revascularization therapy for ST-segment–elevation myocardial infarction improves overall survival, but still results in left ventricular dysfunction and clinical heart failure in a substantial proportion of patients.
• Lethal reperfusion injury accounts for a considerable component of myocardial infarct size and represents a target for therapeutic intervention.
• Despite numerous attempts to reduce myocyte injury after reperfusion, however, only cyclosporine in 1 study has shown some promise in reducing infarct size in the clinic, thus the need for novel approaches.

WHAT THE STUDY ADDS

• The study describes the first use of a monoclonal antibody (m21G6) directed against a novel cytoplasmic target exposed early in the acute phase of reperfusion in a swine model.
• The target, a conserved epitope of nonmuscle myosin heavy chain-II, triggers an inflammatory cascade unless its recognition by natural IgM is competitively blocked with m21G6.
• Treatment with m21G6 significantly reduces infarct size, troponin-T release and at day 21 post reperfusion, preserves cardiac function.

Methods

Animals and Experimental Design

Thirty Massachusetts General Hospital miniature swine underwent occlusion of the midleft anterior descending (LAD) coronary artery for 60 minutes using a fluoroscopically guided percutaneous balloon catheter approach. Either normal saline or m21G6 (2 mg/kg) was administered intravenously via an internal jugular central venous catheter just before balloon deflation and reperfusion. Acquisition and analysis of all infarct-related and imaging data were performed by investigators blinded to the original treatment.

All animal care and procedures were in compliance with Principles of Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health, and protocols were directly approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee.

Surgical Procedures

All animals were loaded with oral 400 mg twice daily of amiodarone >5 days before inducing myocardial ischemia to reduce the incidence of fatal arrhythmias postballoon occlusion. Animals were sedated with 2 mg/kg Telazol (tiletamine/zolazepam, 1:1) intramuscular before central line placement and 10 mg/kg IV ketamine before all cardiac catheterization and imaging procedures. In addition, animals received 1 g IV cefazolin for preprocedural antibiotic prophylaxis. General anesthesia was maintained with 1% to 2% inhaled isoflurane. Postprocedural pain was treated with 0.03 mg/kg IV buprenorphine or a 25 mcg/h fentanyl patch transdermally. Cardiac harvest was then performed after median sternotomy, aortic cannulation, and cross clamp, with infusion of cold crystalloid cardioplegia (Data Supplement for detailed description).

Echocardiography

For animals that were followed for 3 weeks, 2-dimensional transthoracic echocardiography was performed at baseline, 1 hour, 7, and 21 days post reperfusion. Images were obtained using a high-frequency 2D probe on an iE33 xMATRIX Echocardiography System (Phillips Healthcare Inc, Andover, MA). All animals were in normal sinus rhythm during image collection. Left ventricular end-diastolic (LVED) and left ventricular end-systolic dimensions and volumes were obtained and left ventricular ejection fraction (LVEF) was calculated by averaging values of both Dumesnil and modified Quinones methods.10-12 LVED wall thickness measurements were recorded at 2 locations within the myocardial infarct zone, and the mean was compared with a remote region of noninfarcted myocardium.

Area at Risk and Infarct Size Determination

At 5 days post reperfusion, the hearts were excised and processed for area at risk (AAR) and infarct size measurements (Figures I and II in the Data Supplement) as previously described.7

Serum Hematology and Chemistry Measurements

Serum samples from swine were collected at several time points (4, 8, 24, 48, 72, 96, and 120 hours) and cardiac troponin-T (cTnT), complete blood count, and serum chemistries were analyzed at Massachusetts General Hospital Core Chemistry Laboratory using standard methodology. C-reactive protein (CRP) levels were analyzed using ELISA.

Production and Labeling of m21G6

Production of m21G6 antibody was as previously described.7 For the experiments in Figure 1, the final purified m21G6 was labeled with Alexa 568 dye (Life Technologies Corp, Carlsbad, CA; see Data Supplement for detailed description).
Immunohistochemistry

Punch biopsies of swine heart were harvested as described above, embedded in Optimal cutting temperature compound, and frozen in a dry ice-isopentane bath as previously described1 (see Data Supplement for details).

Exclusion Criteria

Animals were excluded from our study only if complete reperfusion of LAD was unable to be reestablished or if death from an intractable ventricular arrhythmia occurred. No animals were excluded because of incomplete reperfusion; however, 5 animals died due to fatal ventricular arrhythmia, and thus an overall mortality rate of 16.7% is reported.

Statistical Analysis

All continuous data are expressed as means±SEM. The data in Figure 2A and 2B was analyzed using an unequal-variance t test (Welch t test) and by 2-sample Student’s t test in Figure 2C and 2D. Two-factor ANOVA was used to test the effects of treatment (levels: saline or 21G6), day (levels: 5 days or 21 days) and treatment×day on area under the curve cTnT and %I/AAR, transformed by natural logarithm. For post hoc comparisons, Tukey Honestly Significant Difference (HSD) test was used (α=0.05). Where appropriate, a t test was used to compare means between treatment groups. A 2-way repeated measure ANOVA with Sidak multiple comparisons test was used to compare cardiac functional data between and within treatment groups at baseline, 7, and 21-day reperfusion. Two-tailed P values are reported throughout with a significance threshold of P<0.05. All statistical analyses were performed with GraphPad Prism 6.0 software (GraphPad Inc, San Diego, CA), or using the Statistics toolbox in Matlab R2012a (The Mathworks, Natick, MA).

Results

The 12 amino acid N2 sequence on the NHMC-IIB molecule is identical and conserved across multiple species, including mice, swine, and humans, and represents the binding site of m21G6.13 Thus, to confirm whether ischemia induces neoepitope expression in the AAR, fluorescently labeled m21G6 was infused during a 10-minute period, beginning immediately before reperfusion. These swine were subjected to 60-minute mid-LAD occlusion, and followed for 1-hour reperfusion before cardiac arrest and harvest. Punch biopsies were taken from several cross-sections of both ischemic and nonischemic left ventricle and stained with fluorescently labeled CD31 to visualize the vascular endothelium. We observed distinct colocalization of bound m21G6 and CD31, with a high degree of specificity of bound m21G6 to injured myocardium of the left ventricle. A fraction of bound m21G6 was not directly associated with CD31 or vascular endothelium. Representative images in Figure 1 obtained by confocal microscopy depict m21G6 binding preferentially to injured (ie, ischemic) myocardium of the LV when compared with noninjured (ie, nonischemic) myocardium of the LV. Binding of m21G6 was absent in nonischemic regions of the LV, indicating that m21G6 binding is, in fact, specific to injured, reperfused tissues where neoepitope expression is expected to be present.

We next examined the effect of m21G6 treatment on cTnT and infarct size after 60-minute LAD occlusion and 5-day reperfusion. cTnT levels were measured at baseline, every 8 hours for the first 24 hours, and then once daily for the 5 days reperfusion period. Differences in both peak (most frequently at t=8 hours) and integrated cTnT values were then compared between saline- and m21G6-treated animals. Treatment with m21G6 led to a 61% reduction in peak mean cTnT in comparison with saline controls (3.3±0.6 ng/mL versus 1.3±0.13 ng/mL, respectively; P<0.05; Figure 2A). Integrated cTnT levels, moreover, were reduced by 47% (70.9±7.1 ng2/mL2 versus 34.7±7.1 ng2/mL2; P<0.01; Figure 2B). Serum CRP levels were also analyzed via ELISA of serum samples collected at the same time points listed above, and treatment with m21G6 was similarly associated with a significant reduction in CRP release post reperfusion. Animals treated with m21G6 experienced a ~2-fold increase in CRP level from baseline to peak value postreperfusion, whereas those treated with saline experienced nearly a 5-fold increase in CRP level (P=0.039; Figure III in the Data Supplement).

Although cTnT and CRP levels are widespread metrics for cardiomyocyte injury and inflammation, our primary end point for determining the efficacy of m21G6 in mitigating reperfusion injury was infarct size as a function of the AAR (%I/AAR) measured at 5 days post reperfusion. Specifically, treatment with m21G6 reduced %I/AAR by 51%, from 48.8±2.5% in control animals to 25.0±4.6% in treated animals (P<0.005; Figure 2D). As expected, there was no significant difference with respect to the AAR between treatment groups (Figure 2C), thus validating the reproducibility of ischemic territory in our model. Infarct size as a function of total area of the LV was also significantly reduced in m21G6-treated animals when compared with the saline controls (11.0±1.6% versus 20.2±1.7%, respectively; P<0.001).
To evaluate the effect of m21G6 on clinically relevant functional cardiac parameters, n=3 animals in each treatment group were followed for 3 weeks after reperfusion. Of note, treatment with m21G6 similarly led to a significant reduction in both peak and integrated troponin release during the initial 5 days post reperfusion (Table; Figure IV in the Data Supplement). In addition, treatment with m21G6 resulted in a 36% reduction in infarct size at 21 days post reperfusion compared with the saline controls (10.5±2.0% versus 27.7±0.7%, respectively; Table; Figure V in the Data Supplement), despite no significant difference in AAR/LV measurements (Table).

Hemodynamic and functional data were recorded at baseline, 1 hour, 7, and 21 days post reperfusion. Resting hemodynamics were similar between the saline- and m21G6-treated groups at baseline and 1 hour post reperfusion. Saline-treated animals had a higher resting heart rate, but a similar stroke volume to m21G6-treated animals at 21 days post reperfusion (Table I in the Data Supplement). Two-dimensional trans-thoracic echocardiography revealed that in the saline-treated group, LVEF was depressed at 7 days (45.6±4.2% versus 57.4±2.4%; P<0.05) and 21 days post reperfusion when compared with baseline (38.5±2.4% versus 57.4±2.4%; P<0.01). In contrast, LVEF was unchanged in the m21G6-treated group at both 7 and 21 days post reperfusion (Figure 3A). Animals in both treatment groups had comparable left ventricular function within the normal range at baseline, and similarly, both saline- and m21G6-treated groups subsequently experienced an acute decline in LVEF at 1 hour post reperfusion (43.3±4.2% versus 46.8±3.3%, respectively; data not shown). At 21 days post reperfusion, however, LVEF in the m21G6-treated animals was significantly improved in comparison with saline controls (50.5±3.1% versus 38.5±2.4%; P<0.05; Figure 3A).

We further investigated the effect of treatment with m21G6 on ventricular remodeling by measuring LV wall thickness in the infarct zone, as well as LV end-diastolic volumes and dimensions. At baseline and 21 days post reperfusion, end-diastolic LV wall thickness measurements were obtained from the region of the infarct zone, as well as an unaffected region of the LV free wall. In the saline-treated group, LV wall thickness in the infarct zone was significantly reduced at 21 days post reperfusion compared with baseline (5.0±0.2 mm versus 7.7±0.3 mm; P<0.05; Figure 3B), whereas no significant decrease in LV wall thickness was observed in the m21G6-treated group (7.3±0.2 mm versus 7.7±0.3 mm; P=0.61). At 21 days, there was a significant difference in LV wall thickness between saline- and m21G6-treated animals (P<0.01). Moreover, there was no difference between treatment groups in terms of LV wall thickness measurements taken from noninfarcted regions of the LV free wall.

Table. Comparison of the Effect of m21G6 on Serum cTnT and Infarct Size at 5- and 21-Day Reperfusion in Swine MI Model

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>m21G6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak cTnT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC TnT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>%I/AAR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>%I/AAR/LV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>%I/AAR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>%I/AAR/LV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D 5</td>
<td>3.3±0.6</td>
<td>134.7±14.7</td>
</tr>
<tr>
<td>%I/AAR</td>
<td>48.8±2.5</td>
<td>41.7±2.7</td>
</tr>
<tr>
<td>%I/AAR/LV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D 21</td>
<td>3.1±0.5</td>
<td>163.2±21.9</td>
</tr>
<tr>
<td>%I/AAR</td>
<td>27.7±0.7</td>
<td>52.0±5.2</td>
</tr>
<tr>
<td>%I/AAR/LV</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Swine were subjected to 1-hour LAD occlusion and either 5-day or 21-day reperfusion. Before reperfusion, either saline or m21G6 (2 mg/kg) was injected intravenously. Data are presented as mean±SEM and analyzed by 2-factor ANOVA testing effects of treatment and day. For post hoc comparisons, Tukey Honestly Significant Difference (HSD) was used. AAR indicates area at risk; AUC, area under the curve; cTnT, cardiac Troponin-T; LAD, left anterior descending; LV, left ventricle; and MI, myocardial infarction.
*P<0.05 vs 5-day saline.
†P<0.05 vs 21-day saline.
Tic intervention.6,20 In our study, we developed a consistent myocardial infarct size and represents a target for therapeutic reperfusion injury, accounts for a considerable component of adverse ventricular remodeling in comparison with long-term improvement in LV systolic function. This phenomenon, referred to as lethal reperfusion injury, during which administration of therapies for reperfusion injury are feasible. Using this model, we showed that a single systemic inflammation, thrombosis, and adverse myocardial I/R injury as well as other I/R models.7,24–29 Our results demonstrate that m21G6-treated animals at 21 days post reperfusion, this difference did not achieve statistical significance. At 21 days reperfusion in the saline-treated group, a significant increase was observed in LVED dimension compared with baseline (35.3±1.3 mm versus 28.7±2.0 mm; P<0.01). There was also a significant increase in LVED dimension in the saline-treated group at 21 days post reperfusion when compared with baseline (38.0±8.7 mL versus 32.3±1.6 mL; P=0.59). Despite the 30% increase in LV wall thickness, an increase was observed in LVED dimension in the saline group (35.3±1.3 mm versus 29.3±1.8 mm; P<0.05; Figure 3C). In contrast, there was no significant change in end-diastolic volumes among those animals treated with m21G6 at 21 days post reperfusion when compared with baseline (56.4±4.2 mL versus 31.8±5.4 mL; P<0.05; Figure 3C). In the saline group, a significant increase in LV wall thickness at 21 days post reperfusion in comparison with saline-treated controls.

**Discussion**

Although it is well established that revascularization therapy results in activation and migration of numerous acute inflammatory cells in the infarct zone, including neutrophils, monocytes, and macrophages, which release cytokines and mediators of inflammation, thrombosis, and adverse myocardial remodeling with improved recovery of systolic function.

The literature supports that MI followed by revascularization results in activation and migration of numerous acute inflammatory cells in the infarct zone, including neutrophils, monocytes, and macrophages, which release cytokines and mediators of inflammation, thrombosis, and adverse myocardial remodeling with improved recovery of systolic function.

In summary, these data demonstrate that m21G6-treated animals exhibit a substantial reduction in myocardial injury and preservation of cardiac function at 21 days reperfusion, along with evidence of less adverse structural ventricular remodeling in comparison with saline-treated controls.
NMHC-II expressed on reperfused tissues provides a binding site for natural IgM (Figure 4). Blockade of this pathway by specifically inhibiting the binding of pathogenic IgM to the N2 epitope represents a novel and unexploited pathway for therapeutic intervention in the treatment of I/R injury. As we have demonstrated, m21G6 binding to exposed N2 epitopes on NMHC-II after reperfusion prevents binding of circulating pathogenic IgM binding and subsequent complement activation, thereby moderating innate immune system activation (Figure 4). Remarkably, a single dose administration of m21G6 at 2 mg/kg, with a serum half-life in swine of ≈51 hours, was sufficient to dampen this acute inflammatory signaling pathway and subsequently decrease infarct size at 5 days post reperfusion and improve cardiac function at 21 days post reperfusion. Further investigation (data not shown) suggests similar efficacy with the humanized m21G6 in this model, thereby validating this approach for clinical evaluation. Given the prolonged half-life of m21G6, an extended treatment window may reveal similar efficacy and provide greater flexibility in administration after acute MI.

Nonmuscle myosin has been implicated in smooth muscle proliferation associated with atherosclerosis and hypertension but has not been previously identified as a potential target in myocardial reperfusion injury. The precise mechanism whereby the cytoplasmic N2 epitope becomes accessible to circulating IgM has yet to be elucidated. However, in non-myocardial tissues, it is of interest that during apoptosis in a human Jurkat T-cell line, NMHC-A is cleaved in a caspase-specific manner. It has also been reported in Jurkat cells that NMHC-IIA is cleaved and exposed during apoptosis where it is recognized by a subset of chronic lymphocytic leukemia IgM antibodies. Thus, apoptotic cells of any lineage within the infarct zone could provide a nexus for a damaging inflammatory cascade and may contribute to myocardial cell death in the setting of MI.

IgM with injury-inducing specificity for the N2 epitope on NMHC-II is present in all species that have been investigated, including humans, rats, mice, and swine (data not shown), and the N2 binding region is 100% conserved across all of these species. In this study, fluorescently labeled m21G6 exhibits a high degree of binding specificity to ischemic LV tissue, whereas undetectable in nonischemic LV tissues (Figure 4). It is also important to note that there were no m21G6 treatment–related adverse effects on other organs as determined by histological evaluation of the liver, lung, kidney, spleen, or mediastinal lymph nodes in any of the treatment groups (ie, 1 hour, 5, and 21 days post reperfusion). Serum hematology, chemistries, and liver enzymes were also within normal ranges at all time points and across all treatment groups (data not shown).

Although revascularization therapy (ie, percutaneous coronary intervention or coronary artery bypass grafting) dramatically improves overall survival, the full benefits of revascularization are tempered by I/R injury, as some estimates indicate that I/R injury may account for as much as 50% of the final myocardial infarct size according to several animal studies. This is supported by our murine model in which RAG-1−/− mice, which are antibody deficient, were reconstituted with pathogenic IgM and subjected to I/R. Infarct sizes (%I/AAR) were significantly increased in RAG-1−/− mice reconstituted with pathogenic mouse IgM as well as human IgM purified from 10 separate individuals when compared with the RAG-1−/− saline controls (data not shown). We have also established in our murine model that m21G6 is capable of blocking human pathogenic IgM and reducing infarct size and cTn release which not only suggests that the specificity of m21G6 is conserved across species but also that a humanized version of m21G6 may have therapeutic potential. These data demonstrate that circulating pathogenic IgM is specific for the N2 epitope and capable of restoring myocardial I/R injury to a level similarly seen in C57BL/6 mice. However, because neither the RAG-1−/− mice nor the swine used in this study were not completely protected from injury, other mechanisms of injury are likely also involved and may include, for example, mitochondrial permeability pore opening, the Reperfusion Injury Salvage Kinase pathway, as well as other intrinsic and extrinsic pathways. In dose–response curves in C57BL/6 mice with normal antibody levels, as well as in this...
Swine study, we observe a maximal reduction in infarct size because of reperfusion injury to be $\approx 50\%$.

Overall, we have shown that m21G6 has a strong therapeutic effect compared with saline controls in a large animal model by mitigating myocardial necrosis and infarct size, while also enhancing recovery of cardiac function and inhibiting adverse remodeling. Although numerous preclinical studies have identified therapeutic strategies for targeting myocardial I/R injury, many of these therapies have had disappointing results in human clinical trials.\textsuperscript{36} We recognize that preclinical large animal studies, such as ours, do not capture the complex pathophysiological context of human disease, and thus have limited potential for translation to the bedside. However, our experimental model does adhere to current guidelines for the preclinical assessment of novel cardioprotective agents, including timing of drug administration, duration of both ischemia and reperfusion, and evaluation of the appropriate acute and long-term study end points.\textsuperscript{35,36}

Furthermore, the data presented here as well as in our rodent model suggest that this therapeutic approach may have potential for successful clinical translation for multiple reasons: (1) the novel pathway of injury, the presence of pathogenic IgM, and the N2 epitope-binding sequence are all conserved across multiple species, including humans; (2) experimental models performed previously with rodents, and here with large animals, have demonstrated a robust effect of m21G6 in reducing I/R injury; and (3) the therapeutic window may extend for $\leq 90$ minutes post reperfusion.\textsuperscript{7} Therefore, we think that further development and clinical investigation of the humanized m21G6 is warranted.

Acknowledgments

We would like to thank Scott Lilly for his critical discussions and data analysis, Ken Longo for his statistical expertise and data analysis, and Balthasar A. Heesters for his technical assistance in fluorescent image preparation.

Sources of Funding

This work was partly funded by grants from the National Institutes of Health (NIH; HL084821 for E.M. Alicot) as well as support from DeclImmune Therapeutics, Inc. Additional NIH funding included P01HL018646 (Dr. Allan and Madsen) as well as CO6RR020135-01 for construction of the facility used for the production and maintenance of miniature swine.

Disclosures

E.M. Alicot and Drs Haas, Puro, Carroll, Newman have financial interests in DeclImmune Therapeutics, Inc.

References


23. Pietila KO, Harinioen AP, Jokinnity J, Pajernak AI. Serum C-reactive protein concentration in acute myocardial infarction and its relationship to


Natural IgM Blockade Limits Infarct Expansion and Left Ventricular Dysfunction in a Swine Myocardial Infarct Model


_Circ Cardiovasc Interv_. 2016;9:e002547
doi: 10.1161/CIRCINTERVENTIONS.115.002547

_Circulation: Cardiovascular Interventions_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 1941-7640. Online ISSN: 1941-7632

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circinterventions.ahajournals.org/content/9/1/e002547

Data Supplement (unedited) at:
http://circinterventions.ahajournals.org/content/suppl/2015/12/15/CIRCINTERVENTIONS.115.002547.DC1

**Permissions:** Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation: Cardiovascular Interventions_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

**Reprints:** Information about reprints can be found online at:
http://www.lww.com/reprints

**Subscriptions:** Information about subscribing to _Circulation: Cardiovascular Interventions_ is online at:
http://circinterventions.ahajournals.org//subscriptions/
Supplemental Methods

Surgical Procedures

All animals were loaded with oral 400mg twice daily of amiodarone over 5d prior to inducing myocardial ischemia to reduce the incidence of fatal arrhythmias post-balloon occlusion. Animals were sedated with 2mg/kg Telazol® (tiletamine/zolazepam, 1:1) IM prior to central line placement and 10mg/kg ketamine IV prior to all cardiac catheterization and imaging procedures. Additionally, animals received 1g cefazolin IV for pre-procedural antibiotic prophylaxis. General anesthesia was maintained with 1-2% inhaled isofluorane. Post-procedural pain was treated with 0.03mg/kg buprenorphine IV and/or a 25mcg/h fentanyl patch transdermally. In order to facilitate blood collection and administration of experimental agents and medications, a tunneled silastic central venous catheter was placed into the external jugular vein in a sterile fashion 2-5d prior to induction of myocardial ischemia. Lines were cleaned with alcohol and flushed daily with 5mL of heparinized saline (100 units/mL).

During the induction of myocardial ischemia, arterial blood pressure, heart rate and electrocardiogram (ECG) tracing, oxygen saturation, and end-tidal carbon dioxide were continuously monitored and recorded every 15-20min. Core body temperature was maintained at 37-38°C with a Bair hugger warming blanket (Arizant Healthcare Inc., St. Paul, MN). A continuous IV infusion of amiodarone 1mg/min was maintained throughout the procedure to prevent arrhythmia. The right femoral artery was accessed percutaneously and cannulated with a 6Fr or 7Fr introducer sheath. A 6Fr 100cm H-STICK guiding catheter (Cordis Corp., Bridgewater, NJ) was inserted and advanced into the right and left main coronary artery under fluoroscopic imaging to perform selective right and left coronary angiography. Following systemic heparinization (10 u/kg), an Apex PTCA 8x2.5mm Balloon Angioplasty Catheter (Boston Scientific Corp., Natick, MA) was then advanced into the mid-segment of the LAD and inflated to 4-6 mmHg, typically distal to the second diagonal branch. Evidence of occlusion (TIMI 0 flow) was confirmed by angiography and ST elevations in anterior leads of the ECG
tracing (Figure 1 in the Data Supplement). Complete reperfusion (TIMI 3 flow) was confirmed by angiography. Ventricular ectopy was treated with Lidocaine 10-20mg IV boluses as needed. The model carried a mortality rate of 16.7% due to intractable fatal ventricular arrhythmia within the ischemic period. Whole blood collection for complete blood counts (CBCs) and serum analysis of cardiac markers, metabolic parameters, and presence of experimental compounds were performed at baseline, 8h, 16h, 24h, and then once daily for 5d reperfusion in both the 5d and 21d treatment groups.

At the termination of the experiment, sharp tissue biopsies of lung, liver, spleen, and kidney were collected and stored in formalin for histopathologic analysis. Cardiac harvest was then performed following median sternotomy, aortic cannulation and cross clamp, followed by infusion of cold cardioplegia.

**Area at Risk and Infarct Size Determination**

Following cardioplegic arrest and prior to cardiac harvest, the LAD was ligated with a 2-0 silk tie at the precise point of balloon occlusion. Evans blue dye (4%) was infused through the aortic cannula, and thus the unstained region of the left ventricle (LV) defined the area at risk (AAR). Hearts were then explanted and sliced into 4-5 sections of equal thickness starting from the apex. Sections were weighed and then incubated in 1% triphenyltetrazolium chloride (TTC) for 37°C for 15 min, which stains viable tissue in red and infracted tissue in white (Figure 2 in the Data Supplement). Sections were then photographed on both sides using a Nikon Coolpix 8800 digital camera (Nikon Inc., Melville, NY). Infarct size was then determined by computerized planimetry using Adobe Photoshop Software, Version 7 (Adobe Systems Inc., San Jose, CA) as previously described.¹ Infarct size was calculated as a percentage of the AAR as well as the total area of the LV.

**Production and Labeling of m21G6**

Production of m21G6 antibody was as previously described.¹ Briefly, hybridoma cells were grown in roller bottles for 15d in IMDM media (Mediatech Inc., Herndon, VA) supplemented with
Primatone, Kanamycin, 2% low Immunoglobin Fetal Calf Serum (Life Technologies Corp., Carlsbad, CA) and 2-mercaptoethanol. The antibody was purified using 5ml HiTrap Protein G column (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) and labeled with Alexa 568 dye (Life Technologies Corp., Carlsbad, CA). The final purified m21G6 was tested for endotoxin and determined to be 0.015 EU/mg (Associates of Cape Cod Inc., Falmouth, MA).

**m21G6 Half-life Determination in Swine**

Serum samples from swine injected with fluorescent-labeled Mab (21G6 Alexa 568) were collected at different time points (30min, 4h, 8h, 24h, 48h, 72h, 96h and 120h). Circulating levels of mouse anti-non muscle myosin (21G6) were detected by ELISA. Plates were coated with goat anti-mouse IgG (Southern Biotech Inc., Birmingham, AL) and two-fold dilutions of swine serum were incubated for 1h and detected with a goat anti-mouse IgG1 alkaline phosphatase (Southern Biotech Inc., Birmingham, AL). A standard curve was plotted using serial dilutions of purified m21G6.

**Immunohistochemistry**

Punch biopsies of swine heart were harvested as described above, embedded in Optimal cutting temperature compound (OCT), and frozen in a dry ice-isopentane bath. Sections were cut, fixed in acetone for 10min, blocked with phosphate buffered saline (PBS), 1% bovine serum albumin (BSA), and 0.5% Tween 20 and incubated with mouse anti-pig CD31 FITC (MCA1746 AbD Serotec/Bio-Rad Laboratories Inc., Raleigh, NC). Images were obtained with an Olympus Fluoview FV1000 Confocal System (Olympus America Inc., Center Valley, PA). For all treatment groups, punch biopsies were taken from the liver, lung, kidney, spleen, and thoracic lymph nodes and embedded in OCT and frozen as well as fixed by immersion in 10% neutral buffered formalin. Hematoxylin and eosin (H&E) staining was performed on all biopsied tissue.

**Power Calculations**

The sample size calculation for the 21d functional arm of this study including all time points (i.e. 1h, 7d, and 21d) was determined by a power analysis of the %Infarct/AAR data in the 5d study.
We assumed a common standard deviation of 9.2 and calculated 80% power for differences in group means of 26.77 (n=3 per group) or 21.53 (n=4 per group). Non-survival experiments were concluded once statistical significance was achieved to prevent the unnecessary use of research animals in accordance with the MGH Institutional Animal Care and Use Committee Regulations.
Supplemental Tables
## Supp. Table 1

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>m21G6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline 1h reperfusion 21d reperfusion</td>
<td>Baseline 1h reperfusion 21d reperfusion</td>
</tr>
<tr>
<td><strong>HR (beats/min)</strong></td>
<td>86.7 ± 5.9 99.0 ± 4.4&lt;sup&gt;a&lt;/sup&gt; 88.0 ± 1.2</td>
<td>85.3 ± 4.8 95.3 ± 3.8 a 81.3 ± 1.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>SBP (mmHg)</strong></td>
<td>132.7 ± 9.8 93.3 ± 2.3&lt;sup&gt;a&lt;/sup&gt; 99.7 ± 3.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>99.7 ± 3.2&lt;sup&gt;a&lt;/sup&gt; 111.0 ± 7.1&lt;sup&gt;a&lt;/sup&gt; 96.7 ± 3.4 105.7 ± 5.2</td>
</tr>
<tr>
<td><strong>DBP (mmHg)</strong></td>
<td>85.0 ± 10.2 63.7 ± 5.8&lt;sup&gt;a&lt;/sup&gt; 67.0 ± 5.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>67.7 ± 2.0 66.0 ± 2.1 67.7 ± 2.0 66.0 ± 2.1 70.7 ± 4.2</td>
</tr>
<tr>
<td><strong>SV (mL)</strong></td>
<td>19.9 ± 4.0 16.8 ± 4.8</td>
<td>20.0 ± 2.1 21.7 ± 0.5 20.8 ± 0.4 19.6 ± 1.8</td>
</tr>
<tr>
<td><strong>CO (mL/min)</strong></td>
<td>1678.3 ± 268.6 1635.0 ± 451.7</td>
<td>1761.3 ± 189.4 1850.1 ± 120.0 1979.9 ± 91.3 1586.3 ± 115.7</td>
</tr>
</tbody>
</table>

### Suppl. Table 1. Hemodynamic status and LV function. Summary of hemodynamic parameters and 2D TTE left ventricular (LV) functional parameters at baseline (before 1h occlusion), 1h reperfusion, and 21d reperfusion. Data is presented as mean ± SEM and analyzed by two-way repeated measures ANOVA. For post-hoc comparisons, Tukey’s HSD test was used (alpha=0.05).<sup>a</sup>, p<0.05 vs BL saline.<sup>b</sup>, p<0.05 vs 1h m21G6. HR = heart rate; SBP = systolic blood pressure; DBP = diastolic blood pressure; SV = stroke volume; CO = cardiac output.
Supplemental Figures and Figure Legends
Suppl. Figure 1. Representative angiographic images and TTC-stained heart sections in swine. (A). Angiograms of swine cardiac vasculature were taken prior balloon catheter occlusion of the LAD and at regular intervals throughout occlusion. (B). Swine were subjected to 1h LAD occlusion and 5d reperfusion. Following 5d reperfusion, myocardial sections were staining with Evan’s blue and TTC and myocardial infarct size expressed as a percentage of the area at risk.
Suppl. Figure 2. Representative example of TTC-stained swine heart sections. Swine were subjected to 1h LAD occlusion and 5d reperfusion. Hearts were then harvested and perfused with Evan’s blue and stained with TTC as described in Methods. (A). Evan’s blue perfused heart prior to sectioning. (B). Evan’s blue perfused hearts post-sectioning. (C). TTC-stained heart sections. Roman numerals correspond to respective sections in (B) and (C).
Suppl. Figure 3. Effect of m21G6 on CRP levels. Swine were subjected to 1h LAD occlusion and 5d reperfusion. Prior to reperfusion either saline or m21G6 (2mg/kg) was injected IV. Serum samples were taken at several time points as described in text and analyzed for serum CRP levels. Data is represented as the fold difference between baseline and peak CRP levels in each animal. *, p<0.01. N=10 and N=5 for saline and m21G6, respectively.
Suppl. Figure 4. Effect of m21G6 on cTnT (AUC) at 5d and 21d reperfusion. Swine were subjected to 1h LAD occlusion and either 5d or 21d reperfusion. Prior to reperfusion either saline or m21G6 (2 mg/kg) was injected IV. Serum samples were taken at several time points as described in text and analyzed for cTnT (AUC). (A) cTnT (AUC) measurements at 5d and 21d. (B) Two-way ANOVA with interaction summary. Each symbol represents data from one animal. Tx=Treatment. #, p<0.05.
Suppl. Figure 5. Effect of m21G6 on infarct size at 5d and 21d reperfusion. Swine were subjected to 1h LAD occlusion and either 5d or 21d reperfusion. Prior to reperfusion either saline or m21G6 (2 mg/kg) was injected IV. Following 5d or 21d reperfusion, myocardial sections were staining with Evan’s blue and TTC and myocardial infarct size expressed as a percentage of area at risk (AAR). (A) %I/AAR measurements at 5d and 21d. (B) Two-way ANOVA with interaction summary. Each symbol represents data from one animal. Tx=Treatment. #, p<0.05.
Suppl. Figure 1. Representative angiographic images and TTC-stained heart sections in swine. (A). Angiograms of swine cardiac vasculature were taken prior balloon catheter occlusion of the LAD and at regular intervals throughout occlusion. (B). Swine were subjected to 1h LAD occlusion and 5d reperfusion. Following 5d reperfusion, myocardial sections were staining with Evan’s blue and TTC and myocardial infarct size expressed as a percentage of the AAR.

Suppl. Figure 2. Representative example of TTC-stained swine heart sections. Swine were subjected to 1h LAD occlusion and 5d reperfusion. Hearts were then harvested and perfused with Evan’s blue and stained with TTC as described in Methods. (A). Evan’s blue perfused heart prior to sectioning. (B). Evan’s blue perfused hearts post-sectioning. (C). TTC-stained heart sections. Roman numerals correspond to respective sections in (B) and (C).

Suppl. Figure 3. Effect of m21G6 on CRP levels. Swine were subjected to 1h LAD occlusion and 5d reperfusion. Prior to reperfusion either saline or m21G6 (2mg/kg) was injected IV. Serum samples were taken at several time points as described in text and analyzed for serum CRP levels. Data is represented as the fold difference between baseline and peak CRP levels in each animal. *, p<0.01. N=10 and N=5 for saline and m21G6, respectively.

Suppl. Figure 4. Effect of m21G6 on cTnT (AUC) at 5d and 21d reperfusion. Swine were subjected to 1h LAD occlusion and either 5d or 21d reperfusion. Prior to reperfusion either saline or m21G6 (2 mg/kg) was injected IV. Serum samples were taken at several time points as described in text and analyzed for cTnT (AUC). (A) cTnT (AUC) measurements at 5d and 21d. (B) Two-way ANOVA with interaction summary. Each symbol represents data from one animal. Tx=Treatment. #, p<0.05.

Suppl. Figure 5. Effect of m21G6 on infarct size at 5d and 21d reperfusion. Swine were subjected to 1h LAD occlusion and either 5d or 21d reperfusion. Prior to reperfusion either saline or m21G6 (2 mg/kg) was injected IV. Following 5d or 21d reperfusion, myocardial sections were staining with Evan’s blue and TTC and myocardial infarct size expressed as a
percentage of area at risk (AAR). (A) %I/AAR measurements at 5d and 21d. (B) Two-way ANOVA with interaction summary. Each symbol represents data from one animal.

Tx=Treatment. #, p<0.05.
Supplemental References