ONLINE DATA SUPPLEMENT

Blockade of self-reactive IgM significantly reduces injury in a murine model of acute myocardial infarction

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SUPPLEMENTARY MATERIALS

Supplementary Methods

Myocardial model of ischemia-reperfusion injury. Eight to ten week old mice were anesthetized with a continual administration of 2% isoflurane gas through a nose cone. A midline cervical skin incision was made along with reflection of the muscles overlying the trachea to enable visualization and facilitate intubation. A medial sternotomy was then performed which allowed for the identification of the left anterior descending (LAD) coronary artery. An 8-0 silk suture was passed underneath the LAD, through a 1.0 mm section of PE-10 tubing, and then through the chest wall. The chest wall was then closed and the mouse was allowed to recover for one week. After one week, the midsternal skin incision was reopened and the 8-0 silk suture was pulled to acutely occlude the LAD, which was verified by visual inspection of the coronary bed as well as distinguished changes in the electrocardiographic tracing. After 1 h of occlusion, blood flow was restored for 24 h at which point serum samples were taken and the necessary tissues harvested for analysis. Throughout the procedure, body temperature and heart rate were continually monitored and maintained within the normal range.

TTC staining and evaluation. To determine infarct size, mice hearts were harvested 24 h post-occlusion. The ascending aorta was cannulated with a 22-gauge tubing adaptor. The LAD was tied off and 1% Evan's Blue dye was perfused into the aorta and coronary arteries. The heart was then sectioned transversely into four pieces with one piece including the ligated LAD. Each ventricular section was incubated with 1.0% triphenyltetrazolium chloride (TTC) for 15 min at 37°C. Viable myocardium appears red and infarcted tissue appears white following TTC staining. The right ventricle was then

excised and the left ventricle was weighed. Both basal and apical sides were photographed and the area of infarction was determined by computerized planimetry. The hearts were photographed with a Nikon Coolpix 8800 digital camera (Nikon USA). Two epi-illuminated halogen lamps provided the main lighting and a light box where the hearts rested on provided a uniform white background to minimize any shadowing effect. All images were recorded at 8 MegaPixels and then downloaded to the computer for analysis. All images were analyzed with Photoshop software, Version 7 (Adobe System, San Jose, CA) based on procedures described before[1]. Due to subtle color differences between regions of positive and negative staining, each image was enhanced with pseudo-colors to clearly delineate regions that were infarcted (unstained off white – enhanced to yellow), not infarcted but at risk (red), and those not at risk (blue). The relatively uniform white background was assigned a dark green color to contrast those colors assigned to represent the heart. To improve the accuracy and reproducibility of such color enhancements, images were first normalized to bring out details from the shadow regions. Then, separate thresholds were applied to the red and green channels until the three distinct regions of yellow, red, and blue are delineated that would correspond closely to the regions of infarcted, at risk, and not at risk, respectively. Threshold settings that would consistently yield a fair representation of the delineated regions mentioned above were recorded into a macro for batch processing.

Once pseudo-colors were assigned to the images, pixel count from each region was read directly from the histogram. The % infarct/AAR was determined for both the apical and basal sides of each piece. The % infarct of the left ventricle was calculated by dividing the number of yellow pixels by the total number of pixels. The % AAR of the

left ventricle was determined by dividing the yellow and red pixels by the total number of pixels. The % infarct and the % AAR of the left ventricle were divided and normalized for the weight of the sample. The % infarct/AAR for the apical and basal sides of each piece was then averaged. This was done for each heart slice and finally all slices were averaged to give the % infarct/AAR for each heart.

Masson Trichrome quantitation. For each sample, five images in the left ventricle and two images in the right ventricle were taken with the 20X objective on a Leica DMLB digital imaging system (Leica Microsystems, Bannockburn, IL) and further analyzed with Photoshop software (Version 7, Adobe Systems Inc., San Jose, CA) as previously described[1]. However, the procedure was significantly modified so that it could be efficiently applied to bright field images such as those used in this study. It was necessary to enhance the contrast of the stained fibers to get the most accurate quantification. Working in a RGB color space, the dynamic range of the images was first maximized by applying the "auto level" function. Using the "curve" function, the "Look Up Table" was then reversed resulting in images bearing a negative contrast. A threshold was applied to the red channel (which now holds the best contrast information of the stained blue fibers) to delineate regions of the stained fibers. Original "un-enhanced" images were used as comparison to ensure that such delineated regions were fairly representing the stained collagen regions. For reproducibility, these steps were recorded into a macro to batch process all images. Pixel count from each region was carried out as previously described[1].

Immunohistochemistry and histopathology. Cryosections fixed with cold acetone were blocked with 10% Fc-block in PBS/BSA/Tween 20. For C57BL/6 mice,

cryosections were incubated with biotinylated goat anti-mouse IgM (Southern Biotechnology, Birmingham, AL) for 2 h at room temperature (RT) followed by streptavidin Alexa 568 conjugate (Invitrogen, Carlsbad, CA) for 1 h at RT. For RAG-1$^{-/-}$ mice, cryosections were incubated with goat anti-human IgM (Southern Biotechnology, Birmingham, AL) for 2 h at RT followed by Alexa 594 rabbit anti-goat IgG (Invitrogen, Carlsbad, CA) for 2 h at RT. C3d was visualized by polyclonal rabbit anti-human C3d antibody (DAKO, Carpinteria, CA) labeled with FITC (Sigma, St. Louis, MO). All sections were mounted with aqueous mounting medium (Biomedia Corp., Foster City, CA). Fluorescent images in the area at risk and non-risk area were taken with a Zeiss/BioRad Radiance 2000MP Confocal/Multiphoton Imaging system (Carl Zeiss MicroImaging, Peabody, MA). Quantification of IgM and C3d staining was performed for each sample by taking five images in the left ventricle and two images in the right ventricle with the 20X UPlanapo N.A. 0.7 objective and further analyzed with Photoshop software (Version CS2, Adobe Systems Inc., San Jose, CA) as previously described[1].

Supplementary Figures

Figure S1. (A) Representative pictures from NS and N2-treated hearts subjected to I/R and stained with TTC. (B) Quantitation of infarct size and area at risk (AAR) as a percentage of the left ventricle (LV) in NS-injected C57BL/6 mice subjected to I/R. (#, $p<0.05$).

Figure S2. Neutrophil, macrophage, and monocyte populations in the heart characterized by Gr-1 and 7/4 staining. Hearts from mock, NS and N2 peptide treated animals were homogenized into single cell suspensions, stained, and analyzed by flow cytometry for CD11b, Gr-1, 7/4 expression (A) Representative FACS plots show 3 distinct populations by CD11b/Gr-1 staining: monocytes and macrophages ($CD11b^{+}/Gr\text{-}1^{lo}$ and $CD11b^{+}/Gr\text{-}1^{int}$, respectively) as well as infiltrating neutrophils ($CD11b^{+}/Gr\text{-}1^{hi}$). (B) Unlike monocyte or macrophage populations, neutrophils ($CD11b^{+}/Gr\text{-}1^{hi}$) in myocardium show high expression of the 7/4 antigen as defined by histogram analysis.

Figure S3. Effect of N2 treatment on collagen deposition in C57BL/6 mice. (A) C57BL/6 mice were were injected i.v. with NS or N2 peptide and subjected to I/R. Hearts were collected after 21 d reperfusion and cryosections were stained with MT to evaluate collagen content. Representative composite MT-stained cryosections from either NS or N2-treated C57BL/6 mice are shown.

Figure S4. Functional evaluation of anti-C5 antibody in cardiac ischemia/reperfusion injury. (A) Inhibition of complement by anti-C5 monoclonal antibody. Anti-C5 blocked serum complement activity in a dose-dependent manner in 10% serum hemolytic assay. (B) Effect of serum troponin-I levels in C57BL/6 mice treated with anti-C5. NS or anti-C5 (0.5 mg) was injected i.v. into C57BL/6 mice prior I/R. Serum troponin-I levels were

measured after 24 h reperfusion. (0.5 mg is equivalent to 50 μg/ml shown in (A)). (#, $p<0.05$).

Supplementary References

1. Leung H. Using photoshop for measurement and analysis. *Bulletin Micro Soc Canada* 2003;**31**:19-22.

Figure S1

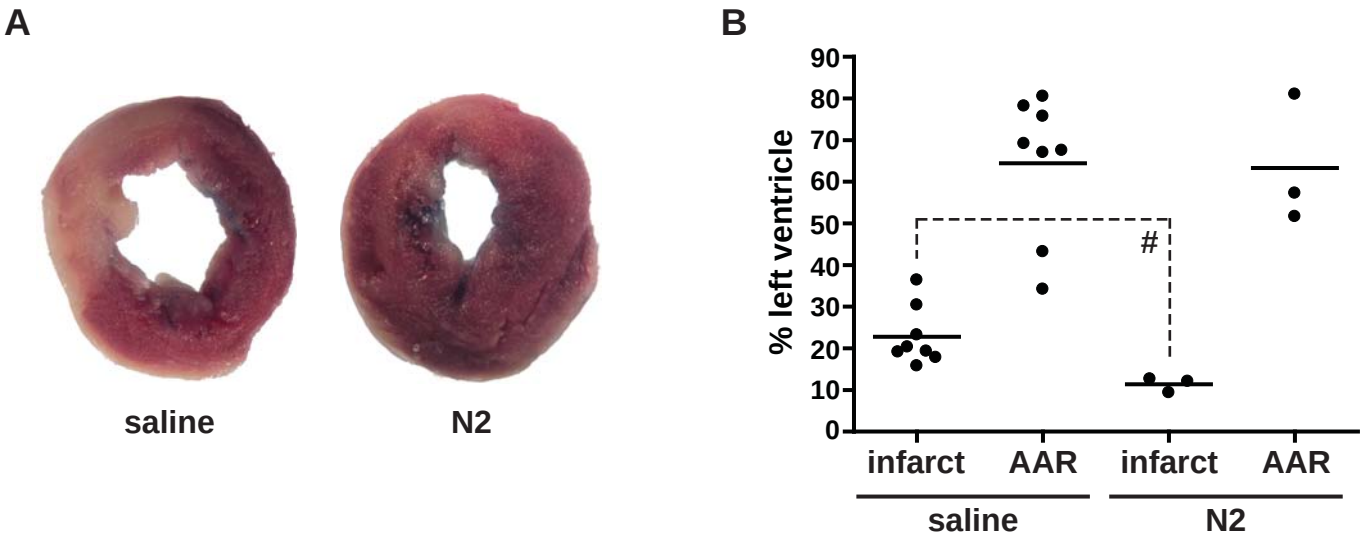

A
saline
N2
B
% left ventricle
90
80
70
60
50
40
30
20
10
0
#
infarct
AAR
infarct
AAR
saline
N2

Figure S2

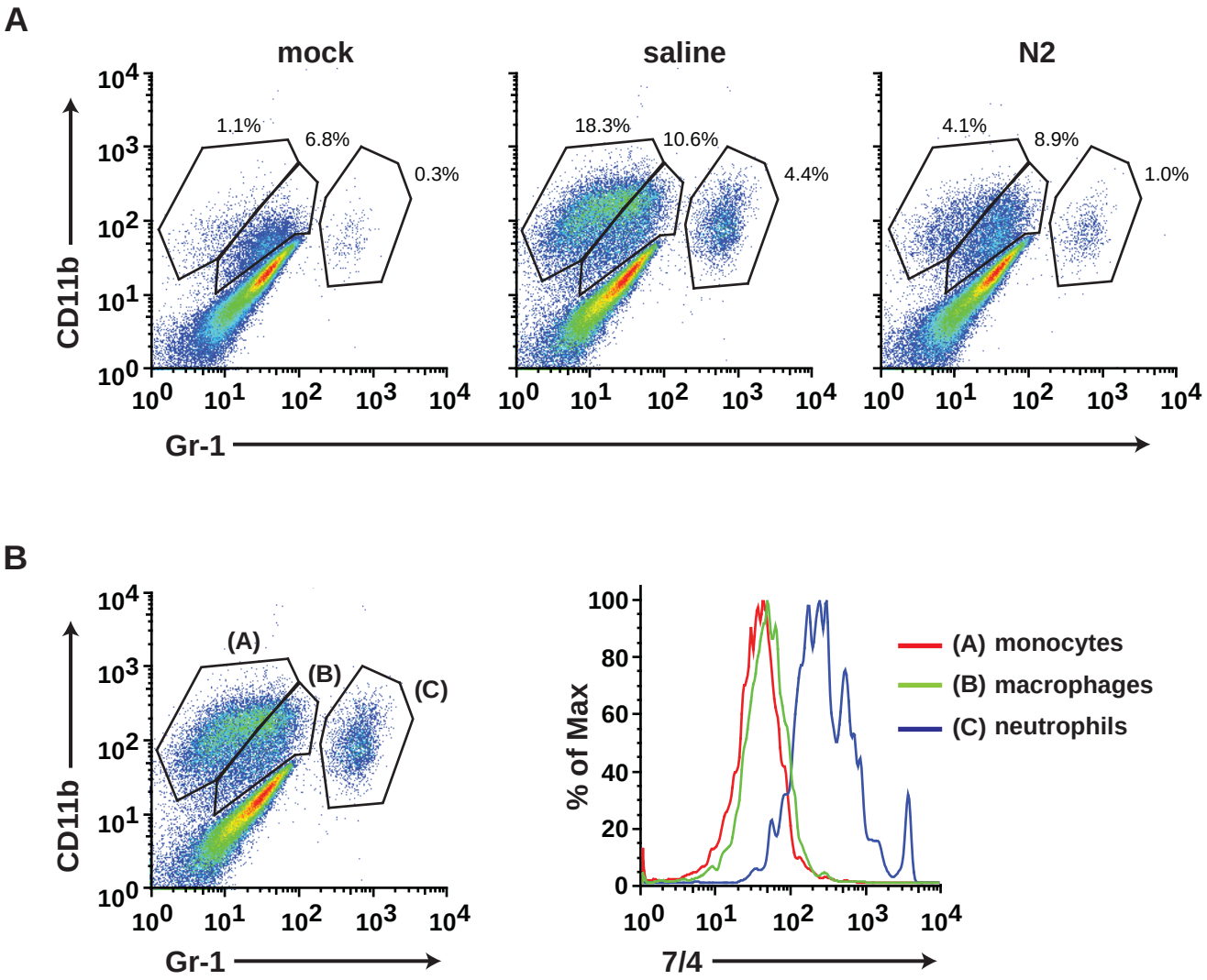
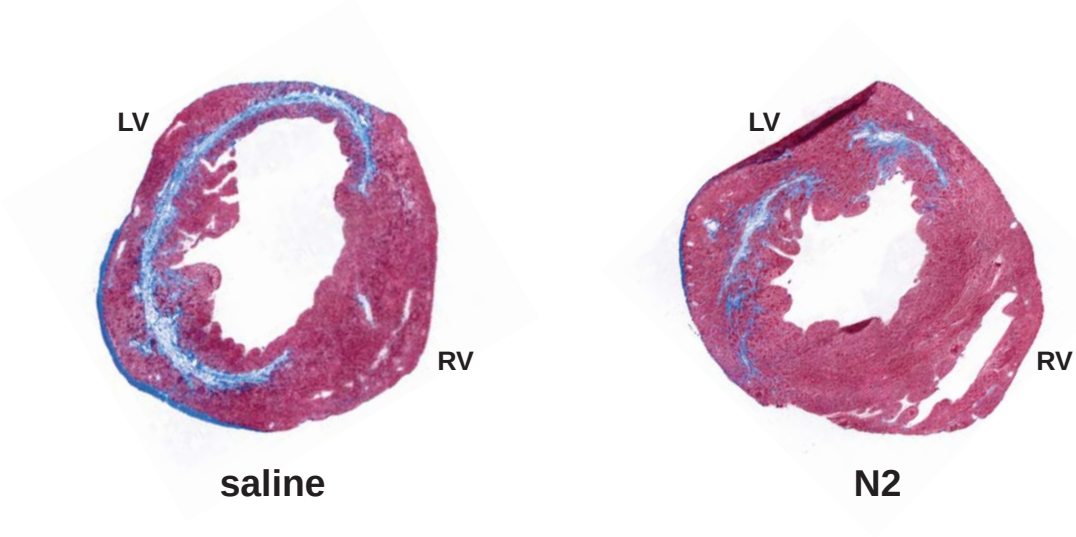
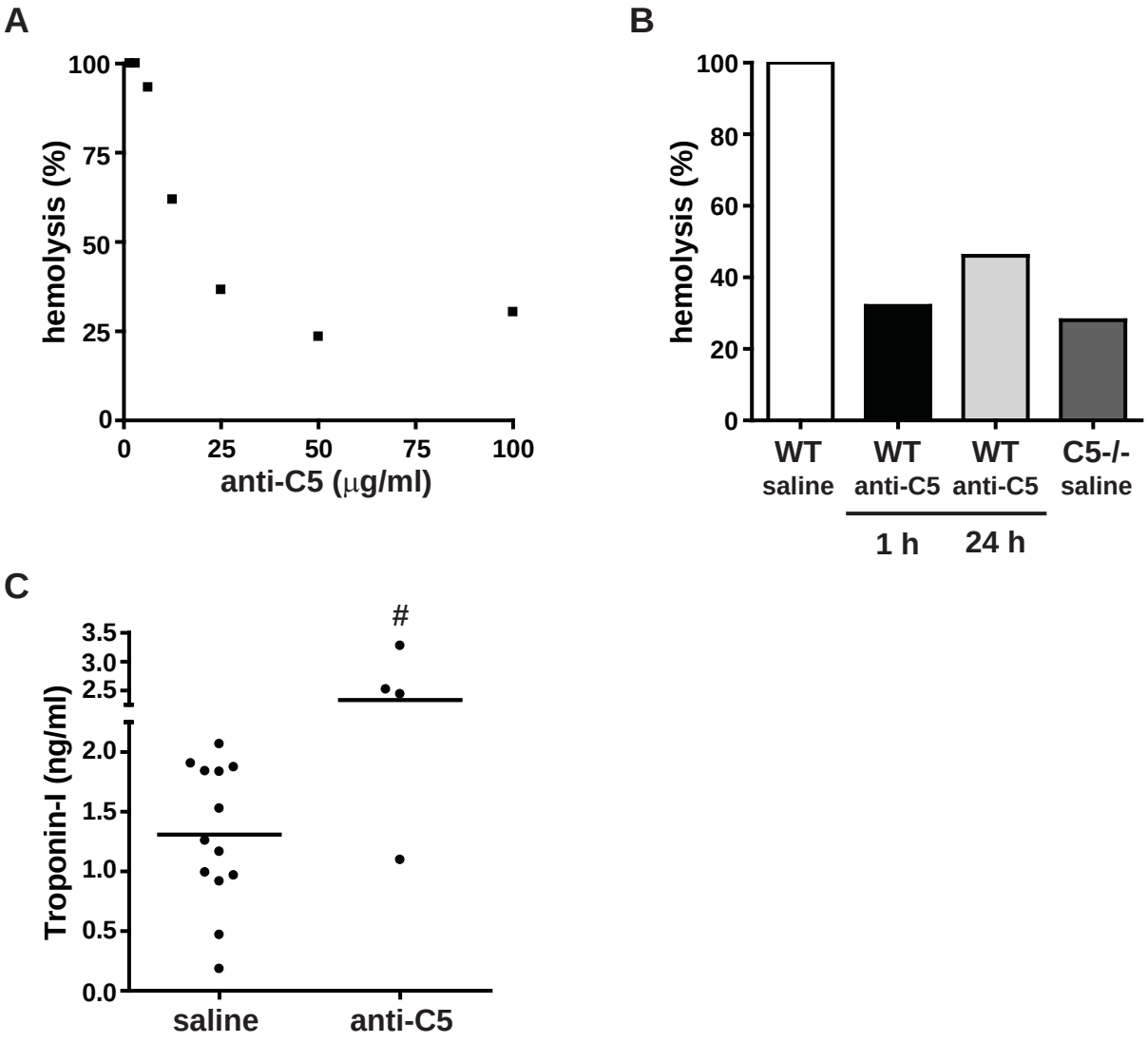

A
mock
saline
N2
1.1%
6.8%
0.3%
18.3%
10.6%
4.4%
4.1%
8.9%
1.0%
CD11b
Gr-1
B
(A)
(B)
(C)
% of Max
7/4
(A) monocytes
(B) macrophages
(C) neutrophils

Figure S3



saline N2

Figure S4


A
hemolysis (%)
100
75
50
25
0
0
25
50
75
100
anti-C5 (μg/ml)
B
hemolysis (%)
100
80
60
40
20
0
WT
saline
WT
anti-C5
WT
anti-C5
C5-/-
saline
1 h
24 h
C
Troponin-I (ng/ml)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
#
saline
anti-C5