**Mechanisms of Acute Hyperglycemic Exacerbation of Myocardial Infarction**

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Presented to the faculty of the School of Engineering and Applied Science at the University of Virginia in partial fulfillment of the degree requirements for Master of Science in Biomedical Engineering

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# Approval Sheet

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is submitted in partial fulfillment of the requirements for the degree of Master of Science

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## Abstract

Coronary artery disease (CAD) and myocardial infarction (MI) remain the most significant causes of morbidity and mortality in the United States. MI is complicated by acute hyperglycemia in up to 30% of patients, and initial blood glucose concentration is strongly associated with 30-day mortality in these patients. Acute hyperglycemia is known to be associated with increased oxidative stress. In the mouse model of reperfused MI, induced acute hyperglycemia leads to larger infarcts compared to euglycemic controls. However, the mechanisms responsible for acute hyperglycemic exacerbation of MI have not been characterized. Furthermore, the French lab has previously shown that CD4+ lymphocytes are important mediators of cardiac ischemia-reperfusion injury. Other researchers at UVA recently demonstrated that natural killer T (NKT) cells are the subset of CD4+ lymphocytes that initiate the inflammatory cascade following ischemia-reperfusion injury to the liver and kidney. Our hypothesis for these studies was that hyperglycemia increases oxidative stress, which is at least partially mediated by the action of CD4+ lymphocytes or NKT cells through the elaboration of pro-inflammatory cytokines or chemokines. An additional possibility is that hyperglycemia generates a pro-inflammatory signal and oxidative stress through activation of the receptor for advanced glycolation endproducts (RAGE). The increased oxidative stress could lead to larger infarct size either directly through increased myocardial damage or through increased endothelial dysfunction and microvascular occlusion.

The specific aims of this research were: 1) Characterize the early mRNA expression changes during myocardial infarction complicated by acute hyperglycemia, and then use network analysis of the transcriptome to evaluate the role of T cells and to look for novel pathways which may be dysregulated in this disease process, and 2) Test the hypothesis that stress hyperglycemic exacerbation of myocardial infarction is mediated by the receptor for advanced glycolation endproducts (RAGE) and/or mediated through natural killer T cell (NKT cell) activity by use of RAGE or NKT cell knock out mice.

The major findings of this research include: 1) Circulating leukocytes demonstrated differential gene expression due to hyperglycemia only in the setting of MI, but not due to MI or hyperglycemia alone; effect of MI is to amplify differences in circulating leukocyte gene expression that result from acute hyperglycemia; network analysis of leukocyte gene differential regulation indicates changes to T cell activation and T cell differentiation; 2) Acute hyperglycemia causes an increase in infarct size regardless of presence of RAGE or NKT cells, which implies that neither RAGE nor NKT cells mediate the pathological effects of acute hyperglycemia; this study is the first to demonstrate that NKT cell deficient mice have smaller infarct size in the reperfused MI model.

## Introduction

**Acute hyperglycemic exacerbation of myocardial infarction is a significant, common, but poorly understood clinical problem.**

Coronary artery disease (CAD) and myocardial infarction (MI) remain the most significant cause of morbidity and mortality in the United States. CAD accounted for 425,425 deaths in the United States in 2006 and 1,255,000 coronary attacks per year according to the National Heart, Lung, and Blood Institute's Atherosclerotic Risk in Communities [ARIC] Study and Cardiovascular Health Study (CHS). [1-2]

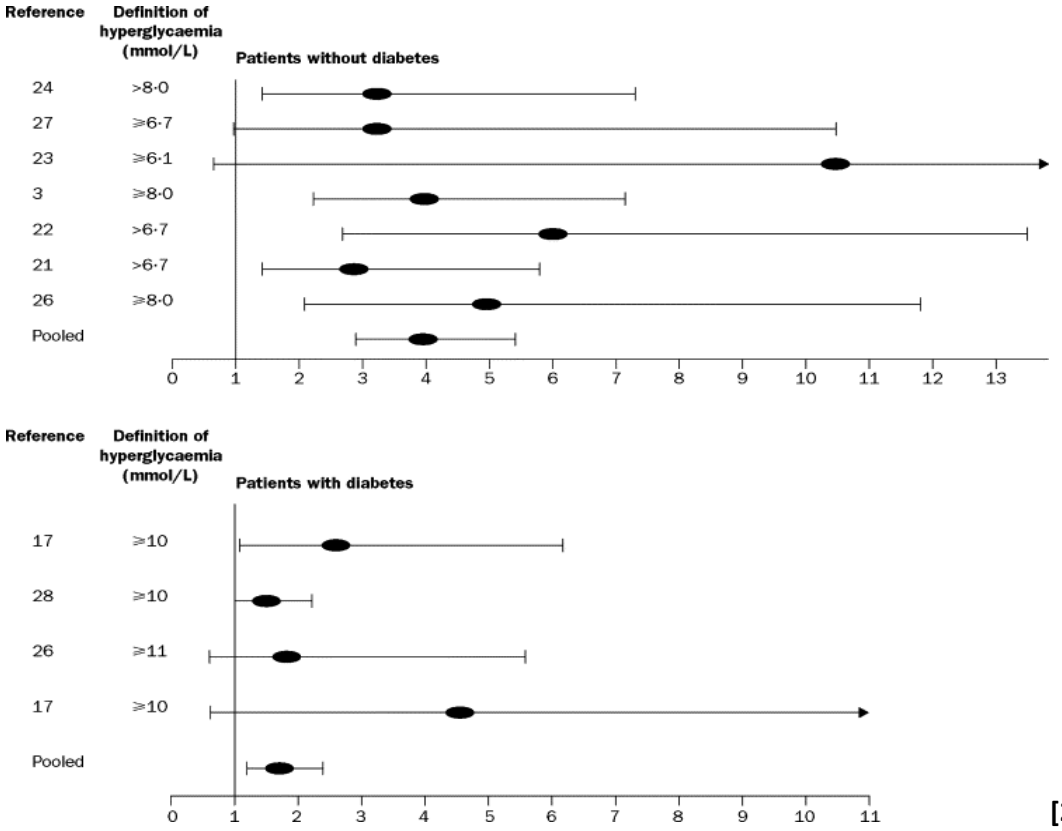
Myocardial infarction is often complicated by acute hyperglycemia. Acute hyperglycemia is a pathophysiological increase in blood glucose concentration in response to acute injury and occurs in both diabetic and non-diabetic patients. In one recent study of 1664 patients with acute MI, over 25% had acute hyperglycemia upon admission but over 30% of those patients had no previous history of diabetes mellitus.[3] Hyperglycemia is a well-established risk factor for poor outcomes in hospitalized patients, especially those requiring intensive care unit stays. Furthermore, insulin therapy to achieve glycemic control improves patient outcomes and is a widely accepted component of critical care medicine. While controversy remains over the precise definition of acute hyperglycemia, blood glucose concentrations greater than 110 mg/dL are generally considered to be elevated. Current recommendations for hospitalized patients include goal glucose concentrations of less than 180 mg/dL.

Patients admitted for myocardial infarction often are found to have acutely elevated blood glucose levels, and initial blood glucose concentration is strongly associated with 30-day mortality and larger infarct size[4-11]. In a retrospective study of 141,680 elderly myocardial infarction patients, 30-day mortality was increased 77% with a rise in initial glucose level from less than 110mg/dL to greater than 240mg/dL.[10] Multiple clinical trials have sought to reverse the deleterious effects of acute hyperglycemia in myocardial infarction with early initiation of insulin therapy with correction of blood glucose levels.[12-14] However, these efforts have failed to demonstrate significant improvements in outcomes. Therefore, at the current time there is no generally accepted treatment for acute hyperglycemia during myocardial infarction.

The AHA recently issued a scientific statement calling for more research into the mechanisms of acute hyperglycemic exacerbation of myocardial infarction, citing both the need to “better understand

the physiological mechanisms responsible for poor outcomes associated with hyperglycemia" and "critical gaps in our understanding of the relationship between hyperglycemia and poor outcomes."[15]

Acute hyperglycemia is a pathophysiological increase in blood glucose concentration in response to acute injury and occurs in both diabetic and non-diabetic patients. Physiological stress is believed to raise blood glucose levels through release of catecholamines and adrenal corticosteroids[18]. Transient elevations in these stress hormones elicit hepatic gluconeogenesis, mobilize stored fatty acids and increase blood lactate levels. This response is an early event in myocardial infarction and probably wanes within approximately five days but is positively correlated with infarct size during this time period [19-20]. Also, initial blood glucose concentration is strongly associated with 30-day mortality[10]. A meta-analysis of clinical outcomes studies confirms the strong correlation between acute hyperglycemia and in-hospital mortality (Fig 1)[21]. The ubiquity of myocardial infarction in Western populations, combined with the common occurrence of associated hyperglycemia, has led several cardiology consulting bodies including the European Society of Cardiology[22], American Heart Association [15] and the Scottish Intercollegiate Guidelines network (2007) to recommend pursuit of euglycemia in hyperglycemic CAS patients.




**[21]**

**Fig 1. Odds ratio of in-hospital mortality for MI in patients with initial hyperglycemia. Acute hyperglycemia is correlated with higher mortality in MI for both diabetic and non-diabetic patients.**

**Early initiation of insulin therapy has failed to improve outcomes from hyperglycemic exacerbation of MI.**

An extensive observational and experimental literature supports a positive association between hyperglycemia and increased morbidity and mortality after myocardial infarction [21, 23]. The consistency and strength of this association has led to a series of investigations on the potential efficacy of insulin in ameliorating this increased morbidity and mortality. Four major clinical trials have studied correction of blood glucose early during MI. The HI-5[24], DIGAMI-2 [13] and the CREATE-ECLA [14] trials did not show clinical benefit of insulin infusion. In contrast, the first DIGAMI trial achieved a significant glucose reduction of 2.1 mmol/liter at 24 hours compared with the control group and a concomitant decrease in mortality. On balance, there is a paucity of high quality clinical data supporting the hypothesis that insulin infusion reduces the adverse impact of hyperglycemia on MI. Therefore, research is needed to discover alternative therapies for treatment for hyperglycemia during MI.

**Acute hyperglycemia has many potentially deleterious effects during ischemia reperfusion injury, especially increased oxidative stress, elaboration of pro-inflammatory cell signaling molecules, and creation of advanced glycated end-products (AGE).**

Myocardial ischemia followed by reperfusion (IRI) can induce an acute inflammatory reaction with death of cardiomyocytes, neutrophil infiltration, reactive oxygen species (ROS), complement activation, mast cell degranulation, cytokine and chemokine release, elaboration of chemotactic factors and cell adhesion molecules. [25-29] Hyperglycemia produces ROS through a variety of mechanisms[30]: auto-oxidation; non-enzymatic glycation of proteins[31]; and metabolism of glucose via aldose reductase. [32] Data suggest that a variety of cells can be affected by oxidative stress during reperfusion injury and play a pro-inflammatory role. High glucose either by itself or co-delivered with pro-inflammatory cytokines to cultured hepatocytes stimulates Nuclear Factor kB-mediated transcription. This transcription was inhibited by PDTC [33] an antioxidant. Exposure of mouse macrophages to ROS can accelerate production of vascular endothelial growth factor via formation of advanced glycated end products. [34] The receptor for advanced glycation endproducts (RAGE) is a 35kD transmembrane receptor belonging to the immunoglobulin superfamily. [35] When RAGE and its ligands interact, genes responsible for inducing inflammation are activated. [36] RAGE is also a known mediator of ischemia-reperfusion injury and $RAGE^{-/-}$ mice have smaller infarcts in the reperfused MI model. Also, mononuclear leukocytes extracted from normal subjects show significant increases in NADPH oxidase activity and ROS production following glucose challenge. [37] Elevated levels of IL-6[38] and IL-18[39] associated with hyperglycemia are related to both short- and long-term mortality in MI. Therefore, multiple pro-inflammatory pathways are known by which acute hyperglycemia could contribute to cardiac IRI.

**T cells play an important role in mediating myocardial infarction.**

The T cell receptor appears to play an important role in the interplay between reactive oxygen species (ROS) and T cell signaling. During reperfusion, hyperglycemia and oxidative stress might synergize to activate CD4+ T cells. [40-41] This synergistic augmenting of T-cell activation could potentially escalate the innate immune response associated with reperfusion injury. A recent clinical study conducted in MI patients experiencing acute hyperglycemia reported enhanced T cell activation along with an increased percentage of natural killer cells and increased CD4/CD8 ratios. [5] Previous work in the French laboratory has demonstrated that transgenic lymphocyte deficient ($RAG^{-/-}$) mice have

smaller infarcts, and that infarct size can be restored by CD4+ lymphocyte transplantation prior to IRI. [42]

**NKT cells are known mediators of ischemia-reperfusion injury.**

Natural killer T (NKT) cells are a subset of T lymphocytes that express T cell receptors (TCR) and the natural killer (NK) cell surface marker NK1.1. [43] Most NKT cells also express CD4. NKT cells are released into the extracellular space by the tissue damage associated with MI and activated by intracellular glycolipids. The glycolipid antigen is presented by an invariant TCR encoded in the Ja18 gene along with CD1d. [44] Following activation, NKT cells can release biologically important levels of both Th1 and Th2 cytokines including IFN-g, TNF-a, IL-2, IL-4, IL-5 and IL-10. [45] Researchers at UVA recently demonstrated that NKT cells are the subset of CD4+ T cells that initiate the inflammatory cascade following ischemia-reperfusion injury to the liver and kidney. [46-47] Since these cells are activated by early tissue damage and elaborate pro-inflammatory signaling, it is possible that NKT cells have an important role in mediating the hyperglycemic exacerbation of cardiac IRI.

**Mouse model of acute hyperglycemia exacerbation of MI**

Previous studies have demonstrated that acute hyperglycemia exacerbation of myocardial infarction can be simulated in the mouse model of myocardial infarction by intraperitoneal injection of dextrose prior to cardiac ischemia-reperfusion injury. [9] These acutely hyperglycemic mice have significantly larger infarcts than euglycemic controls after two hours of reperfusion. Therefore the critical pathophysiological changes induced by hyperglycemia appear in the early period after injury and may explain why interventions aimed at lowering blood glucose have proven ineffective in clinical trials.

**Rationale for Experiments**

**Specific Aim 1: Characterize the early mRNA expression changes during myocardial infarction complicated by acute hyperglycemia and use network analysis of the transcriptome to test the hypothesis that T cells play a role in this process.**

These studies seek to characterize the early mRNA expression changes during myocardial infarction complicated by acute hyperglycemia. Our previous work has shown that T cell deficient mice are protected from cardiac ischemia-reperfusion injury, and we hypothesize that T cells may play a role

in the hyperglycemic exacerbation of myocardial infarction as well. Also, the cellular signaling events initiated by hyperglycemia during cardiac ischemia-reperfusion injury have not previously been characterized. Therefore mRNA expression changes in both circulating leukocytes as well as in the ischemic heart tissue are important to evaluate. This will be achieved using BeadChip microarray genome-wide expression analysis to compare either post-myocardial infarct or sham operated mice under euglycemic or hyperglycemic conditions. By analyzing transcriptome changes, we hypothesize that undiscovered signaling pathways and cell populations activated during hyperglycemic cardiac ischemia-reperfusion injury will be identified. We will then use network analysis of the transcriptome to evaluate the role of T cells and look for novel pathways which may be dysregulated in this disease process.

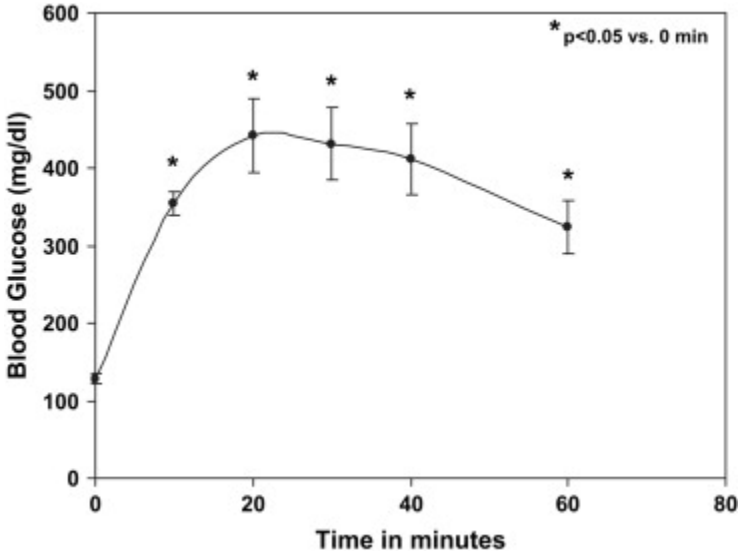
**Specific Aim 2: Test the hypothesis that stress hyperglycemic exacerbation of myocardial infarction is mediated by the receptor for advanced glycolation endproducts (RAGE) and/or mediated through natural killer T cell (NKT cell) activity.**

We previously have shown that simulated acute hyperglycemia induces larger infarct size in wild type mice in our model of reperfused myocardial infarction. RAGE is known to be activated by chronic hyperglycemia while NKT cells are known to be early mediators of ischemia-reperfusion injury in non-cardiac tissues. Myocardial infarction under hyperglycemic and euglycemic control conditions will be repeated in $RAGE^{-/-}$, $CD1d^{-/-}$, and $Ja18^{-/-}$ mice. We hypothesize that acute hyperglycemic infarct exacerbation will be blunted in these transgenic mice if the infarct exacerbation is mediated by RAGE or NKT cells, respectively. Cytokine production profiles of post-MI wild type and $CD1d^{-/-}$ mice under control and acute hyperglycemic conditions will be measured to determine the levels of known NKT cell signaling.

NKT cells are known early mediators of ischemia-reperfusion injury in non-cardiac tissues, and signal through the elaboration of multiple pro-inflammatory cytokines when activated. We hypothesize that NKT cells activate pro-inflammatory cytokine expression through upregulation of NFκB activation, and NKT cell deficient mice will have less pro-inflammatory signaling at the transcriptome level. Other unexpected mechanisms of NKT cell effects during hyperglycemic MI may also be found.

## Preliminary Data

**Acute hyperglycemia can be simulated in mice by intraperitoneal injection of dextrose 20 minutes before cardiac ischemia.** An injection of 20% dextrose (10 μl/g) achieved transient blood glucose levels of 400 mg/dl (Fig 2). The time course of blood glucose levels was then evaluated at this dose in a group of 5 WT mice. After injection, blood glucose increased rapidly to a peak at 20 min post-injection of 442±44 mg/dl, then gradually declined while retaining mean levels of over 400 mg/dl for 30 min. Blood glucose returned to baseline 2 hrs after dextrose injection. For all hyperglycemic MI experiments described in this proposal, hyperglycemia was induced by an injection of 20% dextrose (10ul/g) 20 min prior to LAD occlusion.




[16]

**Fig 2. Time course of blood glucose levels in wild type mice after intraperitoneal injection of dextrose.**

**Infarct size is increased under hyperglycemic conditions compared to euglycemic controls in the mouse model of reperfused myocardial infarction.** WT mice, either hyperglycemic or euglycemic control, were subjected to MI consisting of 30 min coronary occlusion followed by 2 h reperfusion as described below. Hyperglycemic mice have an increase in infarct size as percent area at risk (AAR) (62 vs. 41%, p=0.004) (Fig 3). AAR/LV was similar for both groups. Thus, hyperglycemic exacerbation of myocardial infarction can be readily simulated in our mouse model.

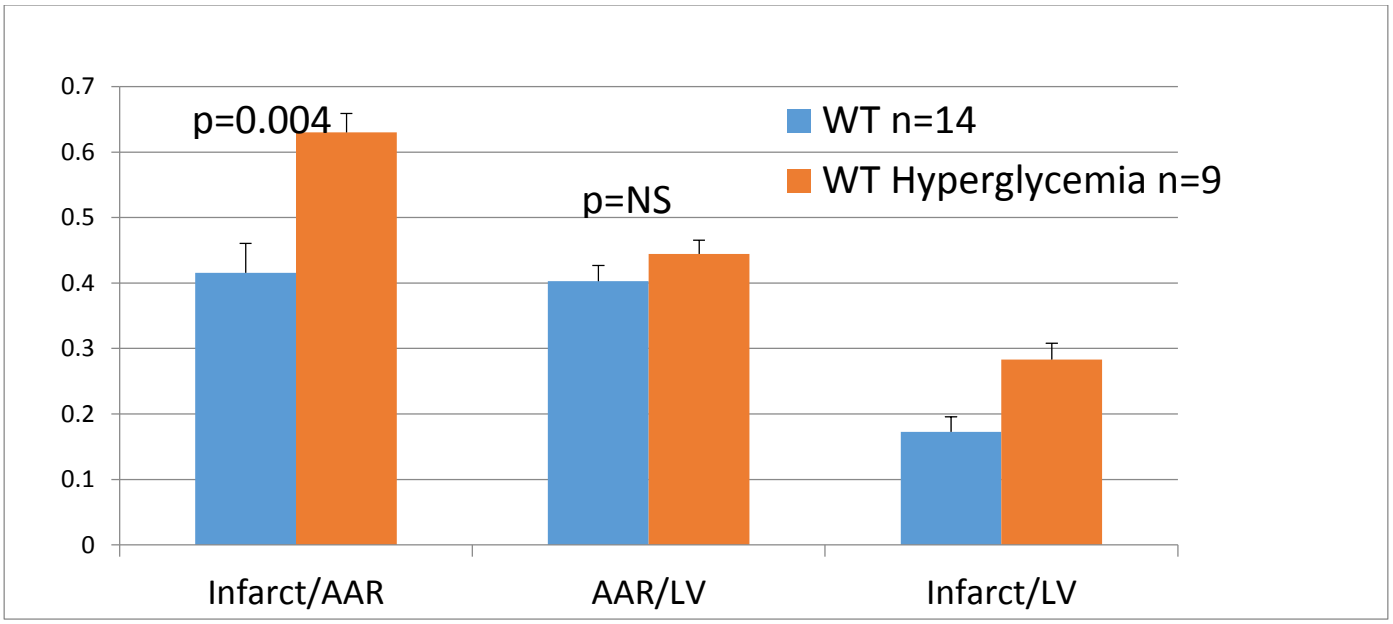
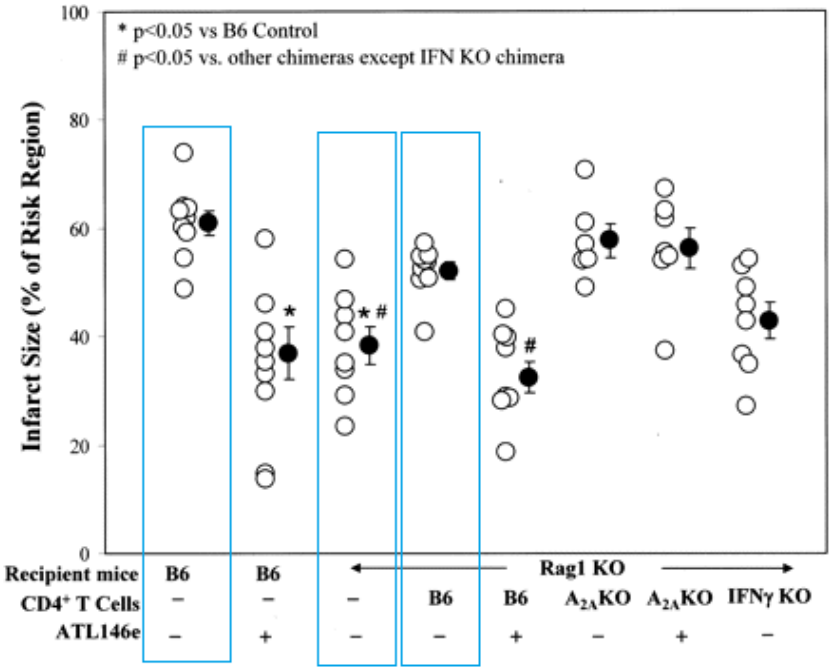



**Fig 3. Infarct size as percent area at risk in mice is significantly increased by hyperglycemia compared to euglycemic controls after 30 min ischemia and 2 h reperfusion.**

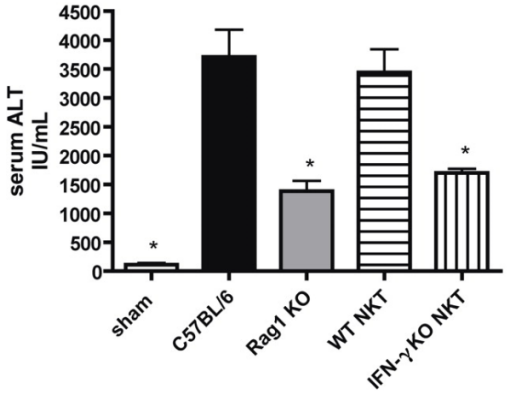
**$CD4^+$ T cells are an important mediator of cardiac ischemia-reperfusion injury in mice as demonstrated by smaller infarcts in $RAG^{-/-}$ mice which is restored by transplantation of wild type $CD4^+$ T cells (Fig 4).** The French lab has previously shown that lymphocyte deficient ($RAG^{-/-}$) mice have smaller infarcts compared to WT controls (Left vs. Middle highlighted columns below). [42] However, $RAG^{-/-}$ mice reconstituted with CD4+ lymphocytes isolated from WT mice have infarct size similar to WT controls (Left vs. Right highlighted columns below). Therefore, CD4+ cells play an important role in mediating cardiac IRI. The subset of CD4+ cells responsible for this effect has not been determined. Of note, reconstitution with CD4+ cells from IFN-$\gamma^{-/-}$ mice does not restore the infarction as well as CD4+ cells from WT mice (Far Right column). IFN-$\gamma$ signaling may be therefore be necessary for CD4+ cells to contribute to myocardial damage during IRI.




[42]

**Fig 4. Lymphocyte deficient ($RAG^{-/-}$) mice have smaller infarcts compared to WT controls after MI. Infarct size can be restored by reconstitution with wild type CD4+ cells, but not by reconstitution with IFN-γ CD4+ cells.**

**NKT cells play a role in hepatic ischemia-reperfusion injury dependent on IFN-γ.** Adoptive transfer of NKT cells restores hepatic injury to $RAG^{-/-}$ mice (fig 5). [55] WT or $RAG^{-/-}$ mice were subjected to 72 min of hepatic ischemia-reperfusion injury or sham surgeries. Purified $CD4^{+}NK1.1^{+}$ T (NKT) cells from WT or IFN-$\gamma^{-/-}$ mice were adoptively transferred into select $RAG^{-/-}$ mice 4 d before hepatic IRI. Reconstitution with WT NKT cells restored hepatic injury, while reconstitution with IFN-$\gamma^{-/-}$ NKT cells did not. Therefore, NKT cells are the subpopulation of CD4+ cells responsible for mediating hepatic ischemia-reperfusion injury in mice, and their effect is likely mediated through IFN-γ signaling.




[55]

**Fig 5. Hepatic IRI is diminished in lymphocyte deficient ($RAG^{-/-}$) mice. Injury can be restored by reconstitution with wild type NKT cells, but not by reconstitution with IFN.**

## Methods

**Specific Aim 1:**

Reperfused myocardial infarction (MI) in mice was surgically induced by the following procedure as previously described. [9-10] All of the mouse surgery performed in this study was conducted under protocols approved by the University of Virginia Institutional Animal Care and Use Committee. Briefly, mice are anesthetized with sodium pentobarbital at a dose of 80 mg/kg, IP. The animals are weighed, placed in a supine position on a heating pad, and orally intubated. Artificial respiration is maintained with a rodent ventilator with a $FiO_2$ of 1.0. Mice were injected with dextrose (10 mL/kg, 20%) or PBS (0.1 mL) intraperitoneally 20 min before ischemia to induce hyperglycemia or eugylcemia as previously described. [9] Blood glucose was measured from tail blood sampling after intubation. Left thoracotomy was performed and the heart was exposed. A 7-0 silk suture was passed beneath the LAD at the level of the left atrial appendage and was tied down over a piece of PE-50 tubing to occlude the LAD for 30 min. Color changes in the risk region were used to confirm successful occlusion. Reperfusion was achieved by untying the ligature and removing the tubing. Sham operation was performed in the same manner except that the 7-0 silk suture was not tied down. Mice were euthanized after two hours of reperfusion. Core body temperature was monitored throughout the operation with a rectal thermocouple probe and was maintained between 36.5-37.5°C with a heating lamp. A schematic time-line of the procedure is shown in fig 6.

Reperfused MI was performed on 16 C57BL6 mice divided into four groups: Eugylcemic sham operation, hyperglycemic sham operation, eugylcemic MI, and hyperglycemic MI. Characteristics of these four groups of mice are shown in table 1. Just before euthanasia of mice, blood samples were collected from right ventricle (~600uL) and allowed to clot at room temperature for 45 min. Blood samples were centrifuged (3000g x 20min) and serum removed. Erythrocytes were lysed by serial washing in lysis buffer (EL buffer, Qiagen), and the remaining leukocyte samples snap frozen at -80C. After euthanasia of mice, hearts were harvested. Infarct zone or corresponding sham region of the heart was trimmed free from the remainder of the uninfarcted heart and snap frozen at -80C.

Total mRNA was isolated from the myocardium and leukocyte samples by standard methods (EasyRNA kit, Qiagen). Samples were converted into biotin-labeled cRNA according to the BeadChip (llumina) manufacture instructions. mRNA quality was verified by northern blot prior to conversion into cRNA. cRNA from each sample was hybridized with a BeadChip murine whole genome expression microarray chip and mRNA expression analysis performed with the Illumina iScan BeadChip system.

Relative mRNA expression (fold change) was calculated between the following groups: eugylcemic sham vs. eugylcemic MI, hyperglycemic sham vs. hyperglycemic MI, eugylcemic sham vs. hyperglycemic sham, and eugylcemic MI vs. hyperglycemic MI. The level of differential expression (fold change) required to consider a gene differentially expressed between two samples depends of the acceptable percent probability of a false positive determined as the false discovery rate (FDR). The number of mRNAs which are differentially expressed between these four groups were quantified for FDRs of $< 0.05$, $< 0.10$, $< 0.20$, and $< 0.40$.

Real time RT-PCR was used to verify the fold change results of the BeadChip system for genes of particular interest. Eight differentially expressed genes in leukocytes in the eugylcemic MI vs. hyperglycemic MI comparison with FDR $< 0.05$ were chosen. These genes (CD2, CD3e, CD27, CD80, CCR5, IL27RA, LCK, and TCF7) are involved in T cell regulation or function. Real time RT-PCR was performed by standard methods with the Qiagen SYBR green kit reagents using the same mRNA samples collected for the BeadChip microarray analysis (four samples from both groups). Average fold change results from RT-PCR was compared to fold change measured by BeadChip analysis and linear regression coefficient ($r^2$) calculated.

Differentially expressed genes in blood leukocytes in the eugylcemic MI vs. hyperglycemic MI comparison were selected for further trend analysis. Differentially expressed genes with greater than

30% fold-change (77 genes) were used to exclude genes with only a modest differential expression. The fold changes for these genes were plotted as a heat map for each group (euglycemic sham and MI, hyperglycemic sham and MI) with red indicating overexpression and blue indicating underexpression to visualize trends in gene expression between the groups (fig 8A). The mean gene expression by microarray analysis for the eight genes chosen for RT-PCR verification were also plotted for each group to visualize trends (fig 8B).

Network analysis was also performed for these 77 differentially expressed genes. Network analysis using HEFalMp (Human experimental functional mapper) software was used to identify known functional associations between the identified genes on the level of the transcriptome. HEFalMp is an online research tool integrating hundreds of publicly available genomic datasets which examines functional associations predicted from an inputted list of genes in the context of a specific biological process, http://hefalmp.princeton.edu/. HEFalMp outputs the biological processes most associated with a given set of genes.

**Specific Aim 2:**

**RAGE$^{-/-}$:** Reperfused MI was performed on wild-type mice (n=18) and RAGE$^{-/-}$ mice (n=16) treated with either dextrose (20% 10ml/kg IP) or saline (10ml/kg IP) 20 min before coronary occlusion. MI consisted of 30 min coronary occlusion followed by 2 h reperfusion as described above. Infarct size as percent area at risk (IF%) was determined by planimetry after TTC and Phthalo blue staining.

**NKT Deficient:** Reperfused MI was performed on wild-type mice (n=8), CD1d$^{-/-}$ mice (n=8), wild-type hyperglycemic mice (n=7), hyperglycemic CD1d$^{-/-}$ mice (n=7) and Ja18$^{-/-}$ mice (n=8). CD1d$^{-/-}$ and Ja18$^{-/-}$ mice lack NKT cells as noted above. MI consisted of 30 min coronary occlusion followed by 2 h reperfusion as described above. Infarct size as percent area at risk (IF%) was determined by planimetry after TTC and Phthalo blue staining.

**Determination of infarct size by TTC staining**: After 120 min of reperfusion, mice are euthanized under anesthesia. The hearts are then excised and cannulated through the ascending aorta with a 23-gauge needle and perfused with 3 ml 37°C 1% TTC in PBS (pH=7.4). The LAD is then reoccluded by retying the suture-ends left in the heart during surgery. The hearts are then perfused with 1 ml 10% Phthalo blue

dye (Heubach Ltd, Fairless Hills, PA) to delineate the non-ischemic tissue. The area not dyed blue is the perfusion bed of the LAD and designated the area at risk (AAR). The hearts are then frozen and trimmed free of atria. The heart ventricle semi-frozen and cut into 5-7 transverse slices, which are then fixed in 10% neutral buffered formalin solution. Each slice is photographed, and the sizes of AAR and infarct area are calculated as percentages of LV by planimetry as previously described.[56-59]

**Cytokine Quantification:** Reperfused MI was performed on wild-type mice (n=5), CD1d$^{-/-}$ mice (n=5), wild-type euglycemic mice (n=5), hyperglycemic CD1d$^{-/-}$ mice (n=5). Sham operation was performed on wild-type mice (n=5) and hyperglycemic wild-type mice (n=5). Serum samples were collected after 30 min coronary occlusion followed by 2 h reperfusion as described above. The following serum cytokine concentrations were measured using a cytokine multiplex analysis system (BioRad): IL-1b, IFN-$\gamma$, TNF-a, IL-2, IL-4, IL-6, IL-12, and IL-17.

## Results

**Specific Aim 1:**

Comparisons of relative mRNA expression in heart tissue and blood leukocytes between euglycemic, hyperglycemic, sham, and MI mice was performed by BeadChip mouse whole genome expression microarray analysis. Characteristics of these four groups of mice are shown in table 1. There was no significant difference in age or weight of mice between groups (ANOVA, p=NS). Blood glucose levels rose significantly after dextrose administration (Euglycemic sham/MI 10.9 +/- 0.8 mM/L and 11.4 +/- 1.0 mM/L, Hyperglycemic sham/MI 27.6 +/-2.3 mM/L and 23.0 +/-3.1 mM/L; ANOVA, $p<0.001$).

The number of genes differentially expressed in these four group comparisons in leukocytes (Table 2A) and infarcted or sham heart tissue (Table 2B) is shown below for FDR < 0.40 to FDR < 0.05.

No differentially expressed genes were found in blood leukocytes between eugylcemic sham vs. eugylcemic MI, except seven genes at FDR < 0.40, nor between eugylcemic sham vs. hyperglycemic sham mice. Very few differences in gene expression were seen between hyperglycemic sham vs. hyperglycemic MI (2 at FDR < 0.1). However, between eugylcemic MI vs. hyperglycemic MI mice, a significant number of genes were differentially regulated (241 at FDR < 0.05). Thus, leukocytes demonstrated differential gene expression due to hyperglycemia only in the setting of MI, but not due to MI or hyperglycemia alone.

Heart tissue demonstrated high levels of differential gene expression due to MI (eugylcemic sham vs. eugylcemic MI, 1790 at FDR <0.05; hyperglycemic sham vs. hyperglycemia MI, 219 at FDR < 0.05). However, hyperglycemia had only a small effect (eugylcemic sham vs. hyperglycemic sham, 8 at FDR < 0.05; eugylcemic MI vs. hyperglycemic MI, 5 at FDR < 0.05).

Fold changes measured by BeadChip microarray for eight genes involved in T cell regulation where were differentially regulated in leukocytes in the eugylcemic MI vs. hyperglycemic MI comparison were verified by RT-PCR. Fold change found by microarray and PCR are given in table 3. The eight genes that were selected for analysis by real-time RT-PCR verification were found to have a linear correlation coefficient of $R^2$=0.94 (fig 7) when their fold changes were compared to those calculated from the BeadChip data.

The heat map (fig 8A) depicts the relative expression levels of the 241 individual genes that are differentially expressed in leukocytes between mice with euglycemic MI and mice with hyperglycemic MI. Euglycemic MI and hyperglycemic MI demonstrate nearly opposite differential expression profiles, i.e. genes overexpressed in eugylcemic MI are underexpressed in hyperglycemic MI and vice-versa. Note that the expression profiles in mice with euglycemic sham surgery and hyperglycemic sham surgery show trends towards those of euglycemic MI and hyperglycemic MI, respectively. Thus the effect of MI is to amplify differences in gene expression that result from acute hyperglycemia. The mean gene expression for the eight genes involved in T cell regulation chosen for RT-PCR verification also exhibit this same pattern of polarization and amplification (fig 8B). The sham operated hyperglycemic and euglycemic mice trend toward the MI hyperglycemic and euglycemic mice, respectively.

Network analysis with HEFalMp was performed to determine which biological systems processes were represented by the genes differentially regulated in leukocytes in the eugylcemic MI to hyperglycemic MI comparison. The 77 genes with greater than 30% fold change were identified as significantly overrepresented in immune system functions, including: regulation of T cell activation ($p < 0.005$), T cell differentiation ($p < 0.005$), and lymphocyte differentiation ($p < 0.05$) (see Table 4).

| Metric | EG Sham | HG Sham | EG MI | HG MI | P-value |
|---|---|---|---|---|---|
| **Age (weeks)** | 11.9±1.4 | 11.3±1.3 | 11.4±1.3 | 12.1±1.2 | NS (0.97) |
| **Weight (grams)** | 27.1±1.0 | 25.3±0.5 | 25.7±0.8 | 27.2±0.7 | NS (0.29) |
| **Peak Glucose (mM/L)** | 10.9±0.8 | 27.6±2.3 | 11.4±1.0 | 23.0±3.1 | <0.001 |

**Table 1. Mouse Characteristics.** Sixteen C57B6 mice were divided into four groups (eugylcemic sham, hyperglycemic sham, eugylcemic MI, hyperglycemic MI). Age, weight, peak glucose are given (AVG +/- SEM). Age and weight do not differ by ANOVA. Glucose is significantly higher in the hyperglycemic mice.

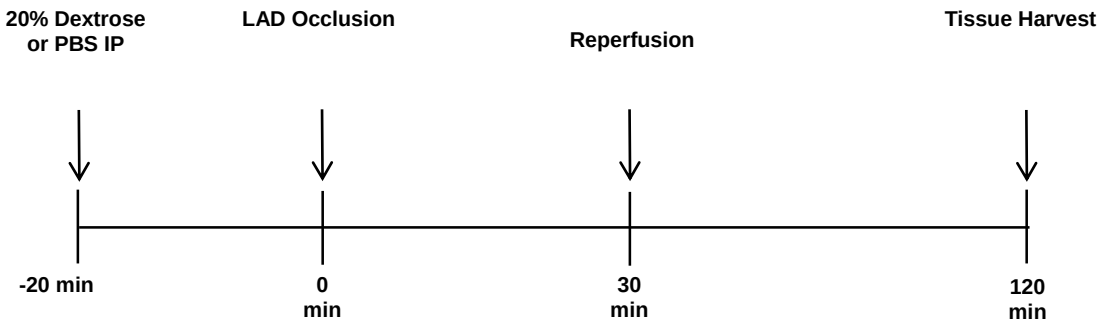



Fig 6. **Time-line of experiment**. Mice injected with dextrose (10mL/kg, 20%) or PBS (10mL/kg). Mice are intubated and left thoracotomy performed. Cardiac ischemia induced 20 min after induction of hyperglycemia. Reperfusion after 30 min followed by tissue harvest at 120 min.

| | False Discovery Rate | | | |
|---|---|---|---|---|
| **Comparison** | **< 0.05** | **< 0.10** | **< 0.20** | **< 0.40** |
| **EG Sham to EG MI** | 0 | 0 | 0 | 7 |
| **EG Sham to HG Sham** | 0 | 0 | 0 | 0 |
| **HG Sham to HG MI** | 0 | 2 | 3 | 52 |
| **EG MI to HG MI** | 241 | 537 | 1320 | 2990 |

**Table 2A: Number of Differentially Expressed Genes in Leukocytes**

| | False Discovery Rate | | | |
|---|---|---|---|---|
| **Comparison** | **< 0.05** | **< 0.10** | **< 0.20** | **< 0.40** |
| **EG Sham to EG MI** | 1790 | 2430 | 3666 | 6086 |
| **EG Sham to HG Sham** | 8 | 12 | 17 | 23 |
| **HG Sham to HG MI** | 219 | 466 | 915 | 2350 |
| **EG MI to HG MI** | 5 | 7 | 147 | 565 |

**Table 2B: Number of Differentially Expressed Genes in Heart Tissue**

Table 2. **A. Number of differentially expressed genes in leukocytes.** mRNA expression by whole genome microarray was measured and relative mRNA expression (fold change) was calculated between the following groups: eugylcemic sham vs. eugylcemic MI, hyperglycemic sham vs. hyperglycemic MI, eugylcemic sham vs. hyperglycemic sham, and eugylcemic MI vs. hyperglycemic MI. The number of mRNAs which are differentially expressed between these four groups were quantified for FDRs of < 0.05, < 0.10, < 0.20, and < 0.40. **B. Number of differentially expressed genes in heart tissue.** Same procedure as above with heart tissue (infarcted region or sham).

| Gene | Microarray FC | PCR FC | Microarray FDR | PCR P-value |
|---|---|---|---|---|
| **CD2** | 0.642 | 0.519 | 0.014 | 0.017 |
| **CD3e** | 0.525 | 0.496 | 0.015 | 0.025 |
| **CD27** | 0.577 | 0.459 | 0.003 | 0.004 |
| **CD80** | 2.072 | 2.734 | 0.005 | 0.024 |
| **CCR5** | 2.58 | 2.599 | 0.012 | 0.008 |
| **IL27RA** | 0.601 | 0.747 | 0.012 | 0.027 |
| **LCK** | 0.55 | 0.568 | 0.015 | 0.084 |
| **TCF7** | 0.576 | 0.831 | 0.041 | 0.403 |

**Table 3: Fold Changes as Measured by Microarray and PCR. Data are from the EG MI to HG MI Leukocyte Comparison.**

Table 3. **Fold changes as Measured by Microarray and RT-PCR**. Data are from the euglycemic MI to hyperglycemic MI leukocytes comparison (n=4 mice per group). Fold changes for eight genes with microarray FDR <0.05 involved in T cell regulation were determined by RT-PCR (avg and p-values). Fold changes by RT-PCR were significant except LCK (p=0.84) and TCF7 (p=0.403).

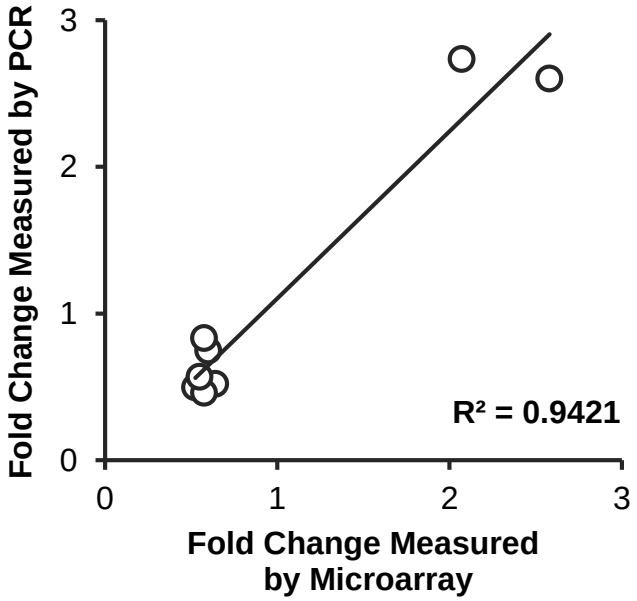



Fig 7. **Plot of Fold Change Measured by RT-PCR vs. Microarray**. Fold change measurements by the two methods have high linear correlation ($R^2$=0.94) indicating a high degree of reliability in the microarray fold change measurements.

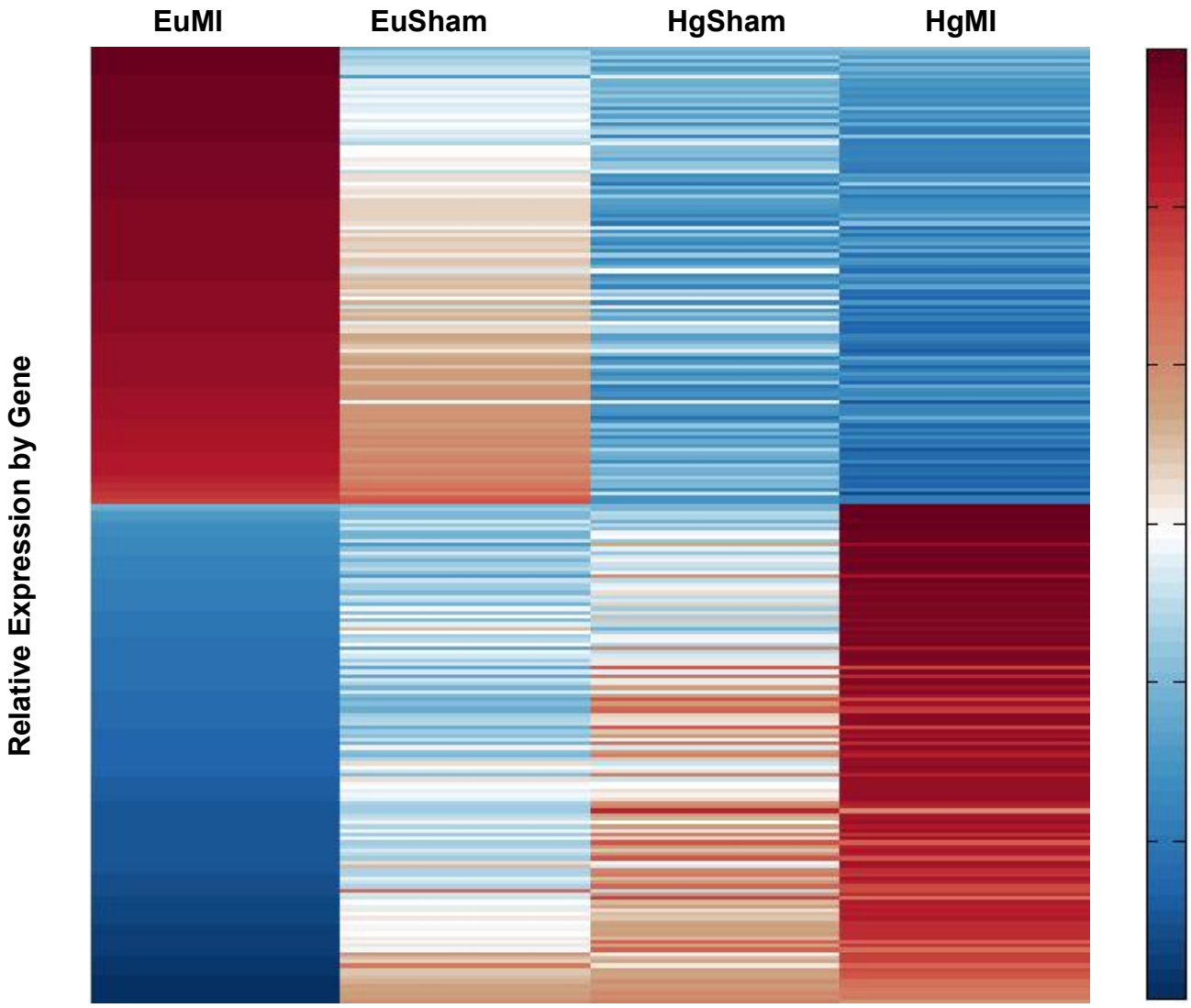



Fig 8A. **Acute Hyperglycemia Polarizes and Myocardial Infarction Amplifies Differential Profiles of Gene Expression in Circulating Leukocytes.** The heat map (A) depicts the relative expression levels of the 241 individual leukocyte genes that are differentially expressed between mice with euglycemic MI and mice with hyperglycemic MI. Note that the expression profiles in mice with euglycemic sham surgery (EuSS) and hyperglycemic sham surgery (HySS) show trends towards those of EuMI and HyMI, respectively. Thus the effect of MI is to amplify differences in gene expression that result from acute hyperglycemia.

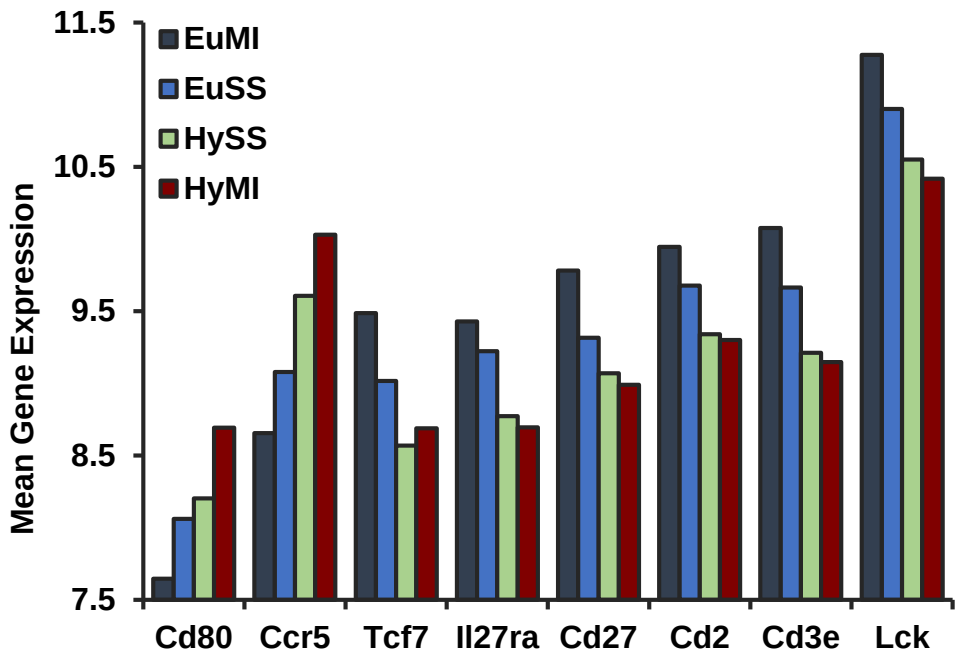



Fig 8B. **Acute Hyperglycemia Polarizes and Myocardial Infarction Amplifies Differential Profiles of Gene Expression in Circulating Leukocytes.** Eight examples from the 241 genes associated with T cell regulation that also exhibit the same pattern of polarization and amplification as the heat map in fig 8A.

| Process | Genes | Network Frequency | P-value |
|---|---|---|---|
| **Regulation of T-Cell Activation** | LAT, LCK, CD27, CD2, THY1, CD3E | 6/77 (7.0%) | < 0.005 |
| **T-Cell Differentiation** | LCK, CD27, CD80, CD2, CD3D | 5/77 (6.0%) | < 0.005 |
| **Lymphocyte Differentiation** | LCK, CD27, CD80, CD2, CD3D | 5/77 (6.0%) | < 0.05 |

**Table 4: Biological Processes and Genes Differentially Regulated After MI in Hyperglycemic Mice.** HEFalMP network analysis tool was used to analyze the 77 most differentially expressed genes in the euglycemic MI vs. hyperglycemic MI comparison in blood leukocytes. Biological processes associated these genes include T cell regulation ($p<0.005$), T cell activation ($p<0.005$), and lymphocyte differentiation ($p<0.05$).

**Specific Aim 2:**

**RAGE$^{-/-}$:** Acute hyperglycemia exacerbates MI similarly in both WT mice (16% increase, 47±5% vs. 63±3%, p=0.01) and RAGE$^{-/-}$ mice (22% increase, 31±5% vs. 53±4%, p<0.01) (Fig 9). Infarct as percent AAR in RAGE$^{-/-}$ mice was reduced by 16% compared to WT mice under euglycemic conditions (31± 5% vs. 47±5%, p= 0.04) and by 10% under hyperglycemic conditions (53±4% vs. 63±3%, p=0.05). The area at risk

for all groups are similar (p=NS). Acute hyperglycemia causes a similar increase in infarct size regardless of presence of RAGE, while RAGE KO causes a similar reduction in infarct size regardless of glycemic status. Thus, the adverse effects of hyperglycemia and RAGE on MI appear to work through independent mechanisms. Furthermore, these results predict that therapies aimed at reducing infarct size through the inhibition of RAGE signaling will be beneficial both in euglycemic and hyperglycemic patients.

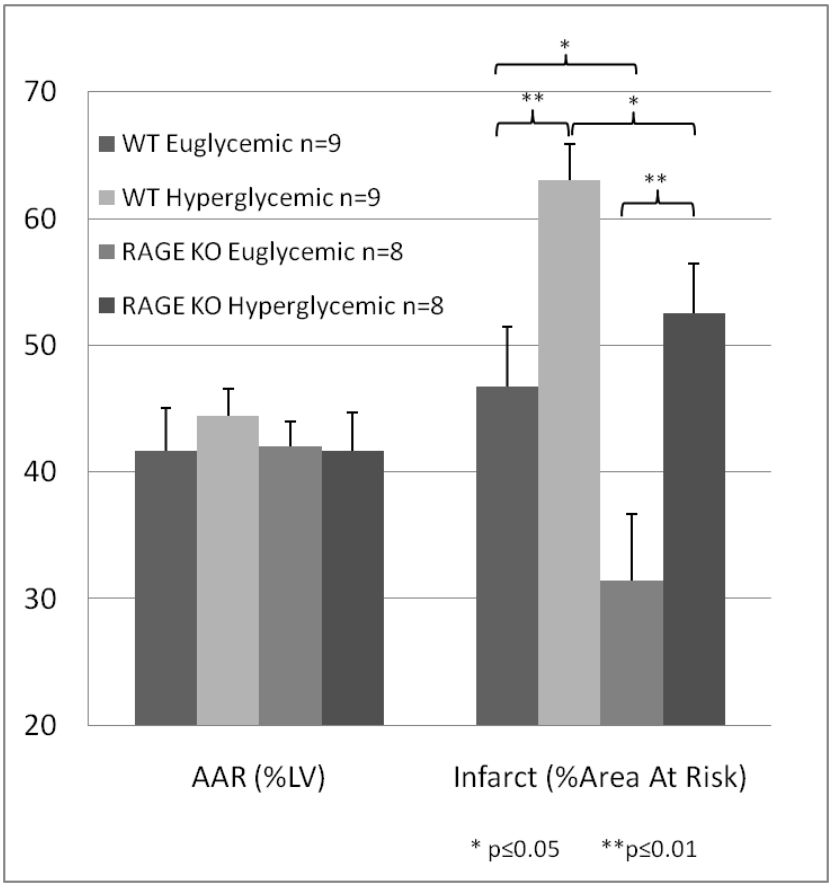



**Fig 9. Hyperglycemia exacerbates cardiac IRI in both WT and RAGE$^{-/-}$ mice.**

**NKT Deficient:** Infarct size was reduced by 16% in CD1d KO mice compared to WT mice (29±5% vs. 45±5%, p=0.04), and was reduced by 19% in Jalpha18 KO mice compared to WT mice (24±4% vs. 45±5%, p=0.009) (Fig 10). There was no significant difference in infarct size between CD1d KO mice and Jalpha18 KO mice (p=NS). The area at risk as percent of the left ventricle (LV) was similar for all groups (p=NS). This is the first direct evidence that NKT cells play an important role in mediating myocardial IRI in mice. Acute hyperglycemia causes a similar increase in infarct size regardless of presence of NKT cells (18% vs. 18%), while lack of NKT cells causes a similar reduction in infarct size regardless of glycemic status (15% vs. 16%) (Fig 11).

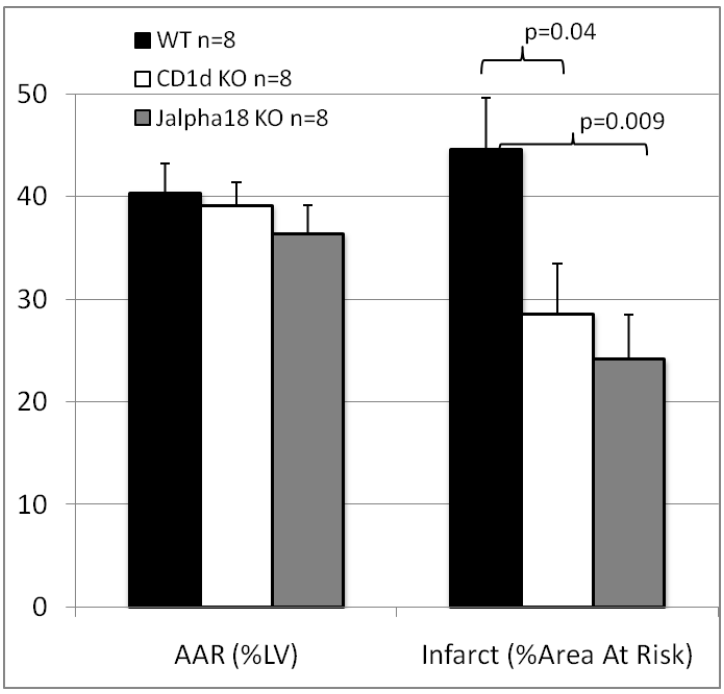



**Fig 10. NKT cells mediate myocardial infarction in mice.**

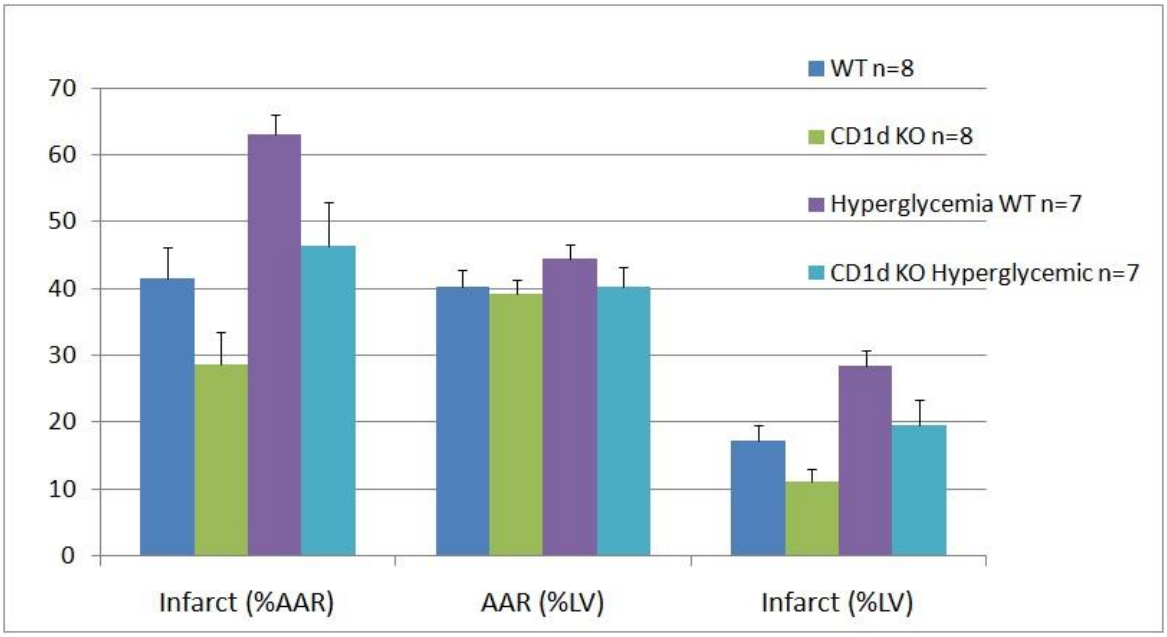



**Fig 11. Hyperglycemia exacerbates cardiac IRI in both wild-type and CD1d$^{-/-}$ mice.**

**Cytokine Quantification:** Serum concentrations of IL-6, IL-1β, IFN-γ, and TNF-α for the 6 groups tested (WT MI, WT Sham, CD1d$^{-/-}$ MI; hyperglycemic and euglycemic) are shown below (fig 12, table 5). IL-6 trends towards an increase in CD1d-/- mice vs. WT (p=0.10). IL-1β, IFN-γ, and TNF-α have decreased levels during hyperglycemia compared to euglycemia (p=0.003, p=0.04, and p=0.19 respectively). These results are unexpected as they imply that systemic pro-inflammatory cytokine release is decreased by acute hyperglycemia.

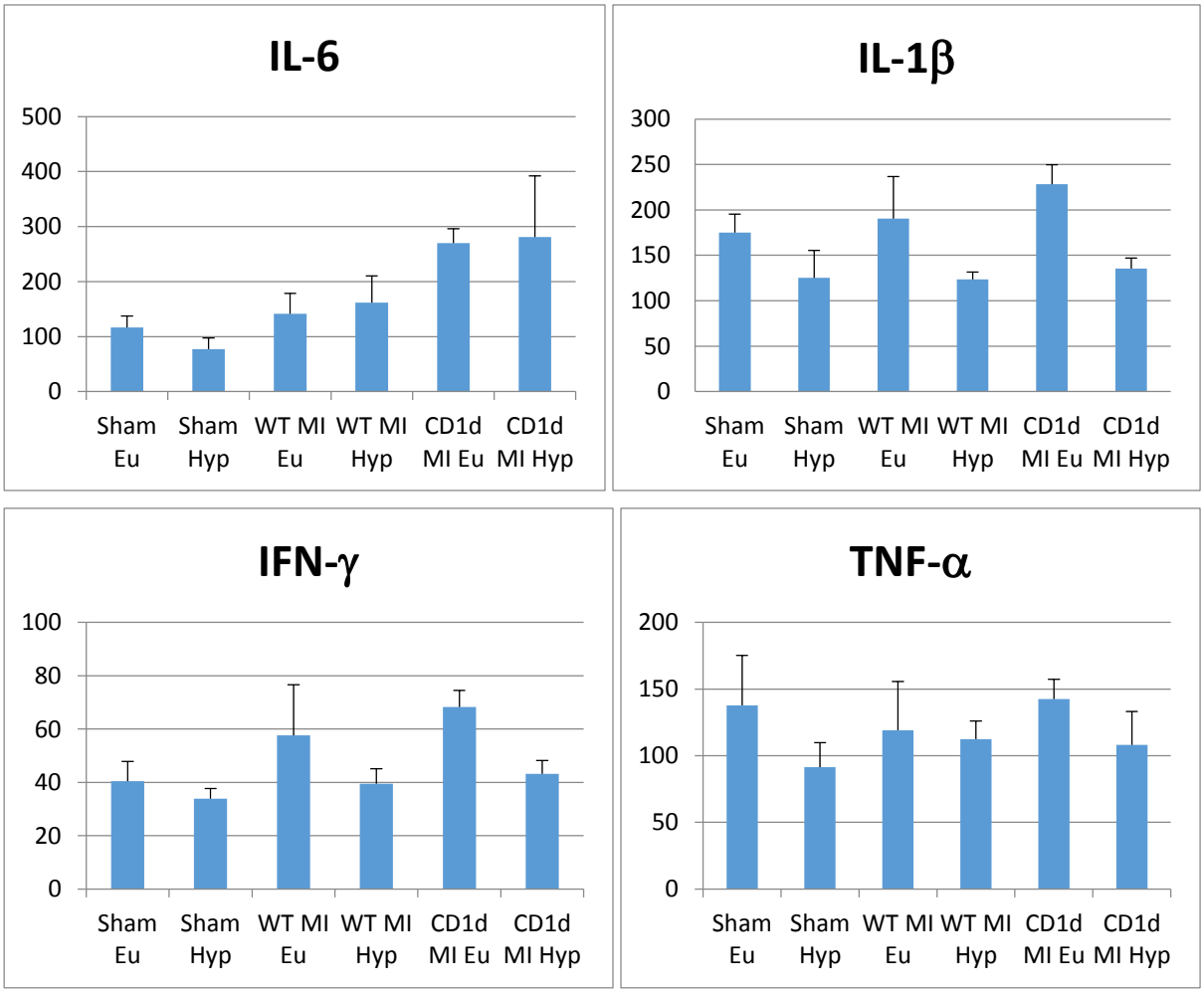



**Figure 12. Serum cytokine levels after reperfused MI in mice.**

| Hyperglycemia vs Euglycemia | |
|---|---|
| Cytokine | p-values |
| *IL-1β* | 0.003 |
| *IFN-γ* | 0.04 |
| *TNF-α* | 0.19 |
| *WT vs. CD1D*$^{-/-}$ | |
| *IL-6* | 0.10 |

**Table 5. p-values for serum cytokine comparisons.**

## Discussion:

These studies performed for specific aim 1 present the first description of mRNA expression changes in mice during the early stages of MI under hyperglycemic conditions. Both mRNA of blood leukocytes and heart tissue was studied. Blood leukocytes demonstrate significant differential mRNA expression between euglycemic MI and hyperglycemic MI mice. Furthermore, heat map analysis of these differentially expressed genes indicate that hyperglycemia appears to impart unique patterns of gene expression that are subsequently amplified by MI to become even more unique. Differentially expressed genes between the euglycemic MI and hyperglycemic MI groups show the same but weaker pattern in the euglycemic sham and hyperglycemic sham groups. This effect holds true for the eight T cell regulatory genes specifically examined. Hyperglycemia may potentiate the circulating blood leukocytes by inducing gene expression changes which may affect their response to concurrent ischemia-reperfusion injury. This altered immune response may underlie the larger infarct size seen in hyperglycemic mice and may contribute to the higher mortality seen in human patients.

The network analysis of these genes support the hypothesis that T cell regulation appears to be involved in the response to hyperglycemia. HEFalMp indicates that T cell activation and differentiation is overrepresented in genes altered in this experiment due to the differential expression of LCK, CD27, CD80, CD2, CD3D, LAT, and THY1. No other subset of leukocytes was implicated in this network analysis.

This experiment provides a snapshot of the leukocyte and heart tissue transcriptome two hours after cardiac ischemia-reperfusion, which is a critical and clinically relevant time period for treatment. However, it is clear that large numbers of genes are already differentially expressed at this time. The window for intervention to prevent or reverse hyperglycemic exacerbation of MI has not been established, but therapies directed at the downstream effects of the hyperglycemia may be needed once the pathways of injury are more fully elucidated.

Myocardial mRNA changes were seen in infarcted tissue, but few due to hyperglycemia alone. Infarction would logically be expected to cause dramatic changes in gene regulation, and perhaps these changes overwhelmed the ability to detect more subtle changes associated with hyperglycemia in heart tissue. The significance of the few mRNA changes seen due to hyperglycemia is unclear in the exacerbation of ischemia-reperfusion injury, and this experiment does not rule out local transcriptome alterations playing an important role in the disease process.

The studies performed for specific aim 2 test the hypothesis that acute hyperglycemic exacerbation of myocardial infarction is mediated by the receptor for advanced glycolation endproducts (RAGE) and/or mediated through natural killer T cell (NKT cell) activity. The RAGE hypothesis was formulated due to its known activation by chronic hyperglycemia and its previously demonstrated role in mediating cardiac ischemia-reperfusion injury. The NKT cell hypothesis was based on experiments performed at UVA demonstrating that NKT cells are the subset of CD4+ T cells that initiate the inflammatory cascade following ischemia-reperfusion injury to the liver and kidney.

Acute hyperglycemia caused a similar increase in infarct size regardless of the presence of RAGE, while RAGE KO caused a similar reduction in infarct size regardless of glycemic status. Thus, the adverse effects of hyperglycemia and RAGE on MI appear to work through independent mechanisms. Furthermore, these results predict that therapies aimed at reducing infarct size through the inhibition of RAGE signaling will be beneficial both in euglycemic and hyperglycemic patients.

Likewise, acute hyperglycemia caused a similar increase in infarct size regardless of presence of NKT cells, while lack of NKT cells caused a similar reduction in infarct size regardless of glycemic status. Thus these experiments provide evidence that acute hyperglycemia does not exacerbate MI by the action of NKT cells. However, this was still an important result as this is first evidence that mice deficient in NKT cells have attenuated injury in the reperfused MI model, and thus it indicates that manipulation of NKT cell function could be a potential treatment strategy for this important clinical problem.

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