The Role of GM-CSF in Myocardial Infarction

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THE ROLE OF GM-CSF IN MYOCARDIAL INFARCTION

by

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I have reviewed this thesis. It represents work done by the author under my guidance/supervision.

Thesis Advisor

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Abstract

Myocardial infarction (MI) is characterized by an obstruction of blood flow to the myocardium, leading to deprivation of oxygen and eventually, irreversible tissue death. After tissue death, intense inflammation occurs, which is characterized by the recruitment of leukocytes to the infarcted heart tissue by chemokines, cytokines, and the complement system. In addition to the immune system’s essential role in healing the infarcted tissue, it also contributes to a deleterious process called ‘ventricular remodeling,’ characterized by negative alterations in cardiac geometry, function, and structure, which can lead to heart failure in the long term. One poorly understood component of the inflammatory response in MI is granulocyte-macrophage colony-stimulating factor (GM-CSF), a growth factor implicated in emergency hematopoiesis in the setting of injury or infection. We hypothesized that GM-CSF plays a critical role in amplifying inflammation after MI, leading to worse outcomes.

In a preliminary survival study using a mouse model of permanent left anterior descending artery (LAD) ligation, the Swirski lab found that GM-CSF knockout (Csf2 KO) mice had significantly higher survival rates than wild type mice as well as decreased leukocyte recruitment into the infarct and improved cardiac function at 21 days post MI in spite of similar infarct size on day 1. Here, we go on to show that GM-CSF expression was shortly and transiently upregulated in infarcted heart tissue after MI, as evidenced by increased GM-CSF mRNA and protein levels. The infarcted tissue was the main source of GM-CSF and within the infarcted tissue, GM-CSF was primarily produced by fibroblasts, but not leukocytes, endothelial cells, or other stromal cells. The primary trigger for GM-CSF expression was found to be DNA, an important danger-associated molecular pattern (DAMP) released after MI that binds to TLR9 and signals through Myd88. Lastly, we found that Ly6C$^{\text{high}}$ monocytes and macrophages in the infarcted tissue responded to GM-CSF by producing CCL2, an important chemokine for monocyte and macrophage chemotaxis. These results support the hypothesis that GM-CSF is a critical cytokine in the inflammatory response after MI and potential target for therapeutic intervention.
Introduction

Myocardial infarction (MI) is characterized by an obstruction of blood flow to the myocardium, leading to deprivation of oxygen and eventually, irreversible tissue death. After tissue death, intense inflammation occurs, a process that for the past 70 years has been observed to occur but is only recently being understood. This inflammation is characterized by the recruitment of leukocytes to the infarcted heart tissue by chemokines, cytokines, and the complement system. The inflammatory response functions to clear the dead cells and matrix debris, as well as facilitate the healing process, resulting in scar formation. In addition to the immune system’s essential role in healing, it also contributes to a deleterious process called ‘ventricular remodeling,’ which is characterized by alterations in cardiac geometry, function, and structure. Because of ventricular remodeling and the heart tissue’s lack of regenerative ability, normal cardiac function is unlikely to be fully recovered and, in the long term, results in congestive heart failure and pump dysfunction. In fact, although the overall incidence of cardiovascular disease and deaths from acute MI have decreased, recent studies suggest that the incidence of heart failure after MI is actually increasing. In order to reduce the incidence of heart failure after MI, a more complete understanding of the cellular and molecular events involved in heart tissue healing and ventricular remodeling are needed in order to develop new therapeutic strategies for acute MI patients.

Although immensely vast and complicated, many key components of the inflammatory response post-infarction are beginning to be understood. The inflammatory response is typically broken up into three key nondiscrete phases: the inflammatory phase, proliferative phase, and maturation phase. The initiation of inflammation in the inflammatory phase occurs with cardiomyocyte death via predominantly coagulative necrosis, which results in the release of intracellular contents. Intracellular contents contain many danger-associated molecular patterns such as alarmins, heat shock proteins, reactive oxygen species, and ATP, all of which can lead to the activation of the complement system and initiate proinflammatory signaling. Along with cardiomyocyte death, extracellular matrix can become damaged and activate toll-like receptors, thereby exacerbating proinflammatory signaling. Proinflammatory signaling activates nuclear factor (NF)-kB, which results in the production of proinflammatory cytokines such as tumor necrosis factor (TNF)-a, interleukin (IL)-1B, and IL-6. Cardiac resident mast cells have also been shown to degranulate and release these proinflammatory cytokines early on post-infarction. Amongst their abundant roles in the inflammatory response, not all of which are understood, proinflammatory cytokines play a key role in inducing the production of chemokines and adhesion molecules, primarily by endothelial cells, which are required for the recruitment of different leukocyte subpopulations to the infarced area.

The first of the newly recruited leukocytes to enter the infarcted area are neutrophils, followed shortly after by inflammatory Ly-6C<sup>high</sup> monocytes, which work in conjunction with pre-existing immune cells that are found in the cardiac tissue to begin the process of clearing the damaged myocardium. These early infiltrators
into the damaged myocardium phagocytose toxic molecules and cell debris, produce proteolytic enzymes, and secrete more inflammatory cytokines. Due to the short lifespans of neutrophils and inflammatory monocytes, their numbers peak around 3 days and begin to drop off. At this time, inflammation is attenuated in a variety of methods. Dying neutrophils release molecules that inhibit continued neutrophil recruitment but simultaneously attract phagocytic cells, mainly macrophages, to enter the scene and begin phagocytosing apoptotic neutrophils. Upon phagocytosing neutrophils, macrophages release anti-inflammatory mediators such as IL-10 and transforming growth factor (TGF)-B. In addition, a subset of anti-inflammatory Ly-6C<sup>low</sup> monocytes appears via reprogramming of inflammatory monocytes or recruitment and contribute to angiogenesis and the synthesis of extracellular matrix by producing vascular endothelial growth factor (VEGF) and TGF-B. Macrophage-colony stimulating factor produced in the proinflammatory environment of the infarct induces monocytes to differentiate into macrophages, which can have the anti-inflammatory actions mentioned previously or attain a proinflammatory phenotype and further the inflammatory response. The signals and microenvironment that dictate what phenotype monocytes and macrophages acquire is not fully understood.

Another key player in the inflammatory response are lymphocytes, which are actively recruited to the inflammatory environment of the infarct. Although myocardial infarction is a sterile wound and intuitively shouldn’t involve the adaptive immune system, lymphocytes and dendritic cells are found in the injured myocardium. Here, effector T cells exacerbate inflammation by secreting proinflammatory mediators while regulatory T cells do the opposite by influencing macrophage phenotype, secreting the anti-inflammatory mediators IL-10 and TGF-B, and interacting with cardiac fibroblasts to reduce protease synthesis. Dendritic cells, although their role in MI is just beginning to be investigated, appear to be involved as immunoprotective regulators during the healing process through their ability to activate regulatory T cells and thus, influence monocyte/macrophage homeostasis.

Non-immune cells such as fibroblasts, endothelial cells, and vascular smooth muscle cells also have important roles in infarct inflammation and healing. Because they are far more resistant to ischemic injury than cardiomyocytes, cardiac fibroblasts play a sentinel role and can sense myocardial injury. When myocardial injury is sensed, they initiate pro-inflammatory mechanisms as characterized by production of cytokines, chemokines, and matrix metalloproteinases (MMPs). Once the peak of inflammation is passed, cardiac fibroblasts undergo a dramatic phenotype change as the proliferative phase after infarction begins. At this point, growth factor signaling in cardiac fibroblasts induces a phenotype change into myofibroblasts, which are characterized by increased proliferation, migratory capacity into the infarct, and expression of contractile, structural, and extracellular matrix proteins. Myofibroblasts play a crucial role in producing structural matrix proteins such as collagens and fibronectin and helping modulate the matrix through expression of MMPs. Once this collagen matrix is laid down, the post-infarction maturation phase commences, in which the matrix is cross-linked and cellular elements, including myofibroblasts, begin to disappear.
The more that is understood about the inflammatory and healing processes that occur after MI, the better it can be appreciated that there are still many unanswered questions and complexity left to comprehend. What can be recognized at this point, however, is the importance of timing and coordination between the different cell types and their actions and influence on others. For example, recent studies have shown that if proinflammatory signaling in the infarct continues for too long, there can be unwarranted damage to existing cardiomyocytes, extension of fibrotic changes into non-infarcted areas, suppressed systolic function of the heart, chamber dilation and tissue breakdown from excessive MMP production, and eventual cardiac rupture or adverse remodeling. These observations have sparked clinical interest in inhibiting excessive inflammation as a potential therapy for MI patients, both to maintain more cardiac function acutely after MI and to prevent heart failure in the long term. In order to design safe and effective therapies for limiting inflammation in the setting of MI, yet still maintain enough inflammation to promote proper wound healing, a more sophisticated understanding of the precise cellular and molecular processes in the inflammatory and reparative stages post-infarction are needed.

When searching for a critical component of the inflammatory response after MI that could be utilized as a therapeutic, a logical place to start would be to look more carefully at the molecular signals that are involved in orchestrating cell recruitment and phenotype. On a macro scale, there is a robust recruitment of myeloid cells from the blood pool immediately after MI, which is replenished either by reserves found in the spleen or bone marrow. In the spleen, several hundred thousand myeloid cells are released within the first day post-infarction and after that, the spleen becomes a site of extramedullary hematopoiesis in order to keep up with demand. The bone marrow also undergoes changes after MI; hematopoietic stem and progenitor cells (HSPCs) ramp up hematopoiesis either by direct stimulation of TLRs by DAMPs released from the infarct or other signals that may travel to the bone marrow. The bone marrow also releases hematopoietic progenitor cells into the circulation, presumably to seed the spleen and begin extramedullary hematopoiesis. The signals that orchestrate the amplification of myelopoiesis after MI will largely dictate the extent of inflammation in the infarct and greatly influence the long-term outcome, but are currently not well understood. One of the signals that increases myelopoiesis in the bone marrow and spleen is the sympathetic nervous system. The sympathetic nervous system triggers release of noradrenaline, which acts on mesenchymal stem cells, leading to mobilization of HSPCs into the circulation for seeding of the spleen. The other major trigger for myelopoiesis from the bone marrow and spleen after MI are molecular signals such as DAMPs/alarmins and cytokines. Hematopoietic cytokines such as granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor, Flt-3 ligand, and erythropoietin, which are known for their ability to induce hematopoietic precursors in the bone marrow to differentiate into leukocytes, proliferate, and mobilize, are currently being studied for their role in the inflammatory response following MI.
Of particular interest to the Swirski lab is GM-CSF, which belongs to a family of cytokines including IL-3 and IL-5 that share a common beta chain in their receptors. GM-CSF is a 23-kD heterodimer that signals through at least three different pathways, JAK-STAT, MAPK, and PI3K.\textsuperscript{12} It regulates the production and activation of granulocytes and monocytes/macrophages, is involved in dendritic cell and T cell function, and has a complexity of other roles that are only now beginning to be appreciated.\textsuperscript{13} Although GM-CSF is not crucial for steady state maintenance of myeloid cell numbers (except in the lung) and steady state serum levels of GM-CSF are highly variable in human adults, it is implicated in emergency hematopoiesis.\textsuperscript{14} In infections, for example, GM-CSF increases production of granulocytes and macrophages in the bone marrow and is involved in their survival and activation within the site of injury. Because of the crucial role of recruited hematopoietic cells in the inflammatory response to and healing of cardiac damage following MI, researchers dove into investigating the potential therapeutic benefits of cytokine administration post MI. Cytokine therapy is an appealing adjunctive treatment to mechanical revascularization because it is noninvasive, but because cytokines can also have many pleiotropic effects that depend on many known and unknown variables, results from animal studies and clinical trials have been highly variable.

Early animal studies investigating the potential use of GM-CSF therapy post-MI were conducted in the early 2000s and focused predominantly on the local effects of GM-CSF in the myocardium and had mostly neutral or negative results.\textsuperscript{15} A study of MI followed by reperfusion in pigs by Terrovitis et al. found that there was no change in infarct size and no improvement in left ventricular function with GM-CSF therapy.\textsuperscript{16} Another group, Maekawa et al., found that administration of a GM-CSF inducing agent into rats post-MI resulted in increased monocytosis and inappropriate collagen synthesis, leading to a marked increase in the infarct region.\textsuperscript{17} A follow up study by this same group found that GM-CSF treatment after MI induces immature dendritic cells (DCs) to differentiate into myeloid DCs in the infarct, which then induce Th1 cell differentiation, thus increasing the Th1/Th2 ratio.\textsuperscript{18} In general, Th1 polarization is associated with inflammation leading to tissue damage, suppression of collagen synthesis, and MMP activation. The authors hypothesize that this could help explain why GM-CSF treatment post-MI was correlated with early LV remodeling in their study. These animal study findings are consistent with data from observational studies in humans. An observational study in humans found that plasma levels of GM-CSF and other adhesion molecules were highest, both acutely and one-month post MI, in patients with MI leading to heart failure manifestations and severe left ventricular dysfunction.\textsuperscript{19} The authors hypothesized that left ventricular dysfunction was caused by GM-CSF’s ability to inhibit leukocyte apoptosis, increased monocyte infiltration into the damaged myocardium, increased levels of adhesion molecules, and propagation of pro-inflammatory cytokine signaling. In stark opposition to these aforementioned studies, however, there was a more recent study published in 2010 by Alturi et al. showing potential beneficial effects of GM-CSF.\textsuperscript{20} After left anterior descending (LAD) artery ligation followed by treatment with GM-CSF
and stromal cell derived factor 1α (SDF), endothelial progenitor cells recruitment was upregulated, allowing for preservation of myocardial function.

In contrast to the focus on GM-CSF’s local effects in the myocardium in early animal studies, there was also strong interest during the early 2000s in utilizing GM-CSF in coronary artery disease (CAD) and MI for its proarteriogenic function, achieved by GM-CSF’s systemic effects on the bone marrow to recruit stem cells and other cell populations. GM-CSF’s proarteriogenic ability had been shown in rabbits and chicks almost a decade earlier and preliminary clinical trials for recombinant human vascular endothelial growth factor and basic fibroblasts growth factor, also proarteriogenic factors, were published that demonstrated their safety and efficacy in improving cardiac perfusion parameters in CAD.\textsuperscript{21–23} GM-CSF was found to be the strongest arteriogenic factor at the time and therefore, a great candidate for therapeutic use.\textsuperscript{24} The first clinical trial for GM-CSF as a proarteriogenic factor in CAD by Seiler et al. found that short-term recombinant human GM-CSF therapy administered both locally (intracoronary) and systemically improved collateral flow index and reduced electrocardiogram signs of myocardial ischemia.\textsuperscript{25} Just 4 years later, a follow-up study by some of the same authors of the first trial investigated using only systemic delivery of GM-CSF in the setting of CAD and also found that collateral flow index was increased in the treatment group.\textsuperscript{26} Surprisingly, however, 2 of 7 patients in the GM-CSF treated group in this study experienced an acute MI during the study and the safety of GM-CSF therapy was called into question. Another clinical trial followed shortly after reporting that GM-CSF was able to effectively mobilize CD34+ bone marrow stem cells over non-treated subjects, which was thought to be beneficial in improving cardiac function and preventing adverse ventricular remodeling.\textsuperscript{27} The authors also noted increased plasma C-reactive protein (CRP) levels, a well-accepted marker of inflammation especially in the context of cardiovascular disease, and warned that further investigation into the local effects of GM-CSF in the myocardium are needed. Although these studies seem to raise red flags in regards to the safety of GM-CSF therapy post-MI, there was a study published in 2011 by Yang et al. that followed a cohort of patients that received GM-CSF therapy after MI without reperfusion.\textsuperscript{28} Follow up at 60 months revealed that subjects that had received GM-CSF had increased left ventricular ejection fraction and reduced left ventricular end diastolic volume, but it is unclear if the size of the infarcts was similar between the two treatment groups or whether their baseline heart function was similar before MI, which makes it difficult to draw definite conclusions from this study.

From these animal studies and human clinical trials, it is easy to appreciate the complexity of GM-CSF’s abundance of local and systemic effects on different cell populations and the reality that we currently understand so little of it. In order to make better informed decisions on the therapeutic potential of GM-CSF, we need to elucidate all aspects of GM-CSF’s role in inflammation and cardiac repair after MI, ranging from the signals that induce production of GM-CSF to its effects on local and distant tissues and finally to its modulation of the phenotypes of different cell types. The Swirski lab, using a *csf2* (GM-CSF gene in mice) KO mouse,
performed a survival study and found that absence of GM-CSF results in significant increases in survival over wild type mice after LAD ligation. Furthermore, functional MRI revealed that \textit{csf2} KO mice have a smaller change in end diastolic volume, end systolic volume, and ejection fraction when evaluated 3 weeks after LAD ligation compared to wild type mice. Lastly, there were notable decreases in the number of leukocytes recruited to the infarcted tissue in the \textit{csf2} KO mice. These preliminary results suggest an overall negative role for GM-CSF in infarct inflammation and repair. Because there are so many conflicting reports regarding GM-CSF’s role in MI, we hope to settle the debate by figuring out the intricacies of GM-CSF biology in MI. Important aspects of GM-CSF’s role to delineate include determining which signals lead to GM-CSF production, which cells produce GM-CSF, what effects it has locally in the infarct as well as systemically on the bone marrow and other organs (and more specifically, on which cells), what effect GM-CSF has on the cytokine/chemokine profile after MI, and what types of phenotypic changes in different cell types are induced by GM-CSF signaling. Understanding these essential aspects of GM-CSF biology will allow for the rationale design of new therapeutic options for patients with acute MI.
Methods

Animal models and in vivo procedures

The mice used in this study were as follows: C57BL/6 (WT), B6.129P2(SJL)-Myd88tm1.1Defr/J, B6.129-Tlr2tm1Kir/J, B6(Cg)-Tlr4tm1.2Karp/J, B6.129S1-Tlr7tm1Flv/J, and C57BL/6J-Tlr9M7Btlr/Mmjax. Csf2−/− and Csf2rb−/− were bred in house on a C57BL/6 background.

The model of myocardial infarction used in this study was permanent ligation of the left anterior descending artery (LAD). Dr. Atsushi Anzai performed all LAD ligations in order to ensure consistency in the MI model used in the mice in all the studies. All mice were males between 8 and 16 weeks old at the time of LAD ligation. In brief, mice were anesthetized with 2% isoflurane using an induction chamber and then the neck and left side of the ribcage were shaved and disinfected. The mice were then intubated with an Inspira Advanced Safety Single Animal Pressure/Volume Controlled Ventilator. A left thoracotomy was performed between the 3rd and 4th rib, the pericardial sac was carefully removed, and the LAD was ligated using a single 8-0 prolene suture. The chest and skin were closed with 7-0 nylon and air was removed from the thorax using a pleural catheter.

Tissue collection and processing for mRNA, ELISA, and single cell suspensions

Single cell suspensions of different tissues isolated from control and MI mice were prepared using a variety of methods. Mice were sacrificed by cervical dislocation following anesthesia. Peripheral blood was collected by retroorbital bleeding into a small amount of EDTA (15 uL of 0.5 M EDTA for ~200 uL peripheral blood) and then stored on ice until processing. For collection of serum, peripheral blood was collected by retroorbital bleeding and allowed to coagulate at room temperature for 2 hours followed by two centrifugations at 2000 x g’s for 20 minutes and 5 minutes (transferring supernatant to clean microtube after each spin) to remove all cells. Vascular perfusion with cold PBS was performed before harvesting internal organs. The heart, spleen, mediastinal lymph node, and right femur and tibia were collected by careful dissection. A small sample of infarct, non-infarct heart, spleen, and lymph node (<100 mg) were frozen at -80C for mRNA isolation and ELISA (heart tissue only), and the rest of the samples were stored in 1X PBS on ice until ready for processing. FACS buffer used in the following protocol was 1X PBS supplemented with sterile 2% FBS and 0.5% BSA.

To prepare leukocytes from blood, 60 uL of the blood solution was cleared of erythrocytes by incubating for 4-5 mins with 2 mLs of 1X RBC Lysis Buffer followed by addition of 1 mL of 1X PBS. The solution was then centrifuged at 1800 rpm (large rotor tabletop centrifuge) for 5 mins and decanted. The lysis was repeated with 1 mL of 1X RBC Lysis Buffer, followed by neutralization of the solution using 2 mLs FACS buffer. The cells were pelleted, decanted, and stored at -80C until further processing to collect total mRNA.

The right femur and tibia were used to collect bone marrow. Bone marrow was harvested by cutting both ends off the bones and then flushing the bone marrow out using an 18 Gauge needle and syringe filled with 1X
PBS. The bone marrow was homogenized by aspirating and flushing out of a 21G needle and syringe multiple times and then passed through a 40-um nylon filtered. Cells were pelleted, decanted, and RBCs were lysed using 1 mL of RBC Lysis Buffer and neutralization with 15 mLs of PBS. After pelleting and decanting again, cells were stored at -80C until further processing to collect total mRNA.

To obtain single cell suspensions of the heart (infarct and non-infarct or whole heart), infarcts were cut out from the remaining healthy heart tissue using a clean blade leaving 1 mm margins (unless whole hearts were used). Hearts were placed in 1 mL of 450 U/mL collagenase I, 125 U/mL collagenase XI, 60 U/mL DNase I, and 60 U/mL hyaluronidase in PBS and then minced into 1mmx1mmx1mm or smaller pieces using clean dissections scissors. The minced solutions were incubated at 37C with shaking for 1 hour to allow for complete digestion. Digested heart solutions were strained through 40-um nylon mesh, pelleted, and decanted. RBCs were lysed by incubating in 1 mL of 1X RBC Lysis Buffer for 30 seconds followed by neutralization of the solution with 10mL FACS buffer. Cells were pelleted, decanted, resuspended in 300 uL of FACS buffer, and placed on ice until ready for staining and cell sorting.

**Heart cell suspension staining and sorting**

Single cell suspensions of the whole heart, infarct, and non-infarcted tissues were stained in FACS buffer using anti-CD45, anti-CD3, anti-CD90.2, anti-CD19, anti-B220, anti-Nk1.1, anti-Ter119, anti-Ly6G, anti-CD11b, anti-F4/80, andri-Ly6C, anti-CD31, anti-MEFSK4, and Zombie Aqua. Viable cells were identified as Zombie Aqua negative. Cardiac cell populations were defined as follows: Ly6C$^{\text{high}}$ monocytes (CD45$^+$Lin$^{-}$CD11b$^+$F4/80$^+$Ly6C$^{\text{high}}$), macrophages (CD45$^+$Lin$^-$CD11b$^+$F4/80$^-$Ly6C$^{\text{low}}$), neutrophils (CD45$^+$CD11b$^+$Lin$^-$F4/80$^+$), CD11b$^-$ (CD45$^+$CD11b$^-$), total leukocytes (CD45$^+$), endothelial cells (CD45$^+$CD31$^+$MEFSK4$^+$), fibroblasts (CD45$^+$CD31$^-$MEFSK4$^-$), and other stromal cells (CD45$^+$CD31$^+$MEFSK4$^+$). Cells were sorted on a BD FACSaria. pSTAT5 staining was performed by stimulating cells in media with or without 20 nig/mL GM-CSF for 30 minutes at 37C followed by fixation in 1 mL of ice cold methanol for 1 minute to permeabilize. Cells were then incubated at 4C for 30 minutes and subsequently washed twice before staining with anti-pSTAT5 antibody for 45 minutes at room temperature.

**mRNA collection and qPCR**

Total mRNA was collected from whole tissue using Trizol Reagent according to the manufacturer’s instructions. For sorted cell populations, 2x10$^4$ total leukocytes, endothelial cells, fibroblasts, and other stromal cells were sorted from infarcted myocardium and total RNA was extracted using the RNeasy Micro Kit according to the manufacturer’s instructions. For whole tissue samples, total mRNA was quantified using a Nanodrop. cDNA was transcribed from 1 ug of total RNA per sample for the whole tissue samples and total mRNA collected for sorted cell samples using the High Capacity cDNA Reverse Transcription Kit. Following
reverse transcription, quantitative real-time PCR was performed using TaqMan primers (Col1a1, Pdgfra, Postn, Csf2, CCl2, and Gapdh). Data were quantified using the $2^{-\Delta \Delta Ct}$ method with Gapdh as the housekeeping gene.

**ELISA for GM-CSF and CCL2 levels in whole tissue and supernatant of stimulated cells**

Heart samples were thawed and homogenized in order to measure GM-CSF protein levels using the Mouse GM-CSF ELISA Kit according to the manufacturer’s instructions.

Sorted Ly6C$^{\text{high}}$ monocytes, macrophages, neutrophils, and fibroblasts were cultured with or with GM-CSF for 48 hours. Supernatant was collected from each culture and then analyzed for GM-CSF protein levels using the Mouse GM-CSF ELISA Kit according to the manufacturer’s instructions. CCL2 protein levels were measured using the CCL2 ELISA Kit according to manufacturer’s instructions.
Results

In previous work performed by Dr. Atsushi Anzai, a survival study of MI in WT and Cs2−/− mice showed that Cs2−/− mice had significantly reduced mortality after permanent LAD ligation (Figure 1a). In this study, mice typically died within 3-7 days after LAD ligation from cardiac rupture. This difference in survival was also accompanied by functional differences in the hearts between WT and Cs2−/− mice at 21 days. Using late gadolinium uptake on MRI to delineate infarct margins, Anzai found that infarct size was similar between WT and Cs2−/− mice on day 1. Despite similar infarct size 1 day after MI, however, there were drastic functional differences on day 21 (Figure 1b). In particular, the end-diastolic and end-systolic volumes were larger in WT mice as compared with Cs2−/− mice, suggesting deleterious changes in cardiac muscle architecture and a loss of contractility. These changes were quantified using fMRI and showed that compared with baseline (day 0, before LAD ligation), the Cs2−/− mice had a significantly smaller change in end-diastolic volume, end-systolic volume, and ejection fraction (Figure 1c). Anzai also showed that there were significant differences in the inflammatory response following MI between WT and Cs2−/− mice (Figure 1d). In particular, there were high total leukocytes on day 3 and day 7 after MI in WT mice. This difference was characterized by higher numbers of neutrophils and Ly6Chigh monocytes at both time points, and higher macrophage numbers on day 7. Total leukocytes on day 1 were mostly neutrophils, on day 3 were primarily comprised of neutrophils, Ly6Chigh monocytes, and macrophages, and finally on day 7 were dominated by macrophages, closely following the well-known pattern of infiltration of leukocytes into the ischemic heart tissue.

In order to further understand GM-CSF’s role in MI, I measured Cs2 mRNA levels in various organs to determine the source of GM-CSF after MI. Cs2 mRNA levels were significantly increased in infarcted heart tissue on days 1, 3, and 7 after MI, but not in non-infarcted heart tissue, spleen, bone marrow, or mediastinal lymph nodes of WT mice (Figure 2a). The increase in Cs2 mRNA peaked on the first day after MI and quickly fell off by days 3 and 7. These results show that GM-CSF expression is shortly and transiently upregulated after MI only in the infarcted heart tissue. Next, I determined GM-CSF protein levels in the hearts of WT, Cs2−/−, and Cs2rb−/− (mice lacking the beta subunit of the GM-CSF receptor) to validate that the change in mRNA levels was consistent with a change in protein levels. On day 1 after MI, GM-CSF protein levels in the infarct of WT mice peaked and then began to fall off, mirroring the mRNA results (Figure 2b). In Cs2−/− mice, virtually no GM-CSF protein could be detected, which was expected since these mice lack the functional gene necessary to produce GM-CSF. On the other hand, Cs2rb−/− mice had levels of GM-CSF protein that were on par with WT mice, another expected result as these mice retain the capacity to produce GM-CSF and the evidence showing that GM-CSF itself is not necessary for production of the growth factor. Cs2rb−/− mice had a trend towards higher levels of GM-CSF than WT, suggesting that the lack of functional GM-CSF receptor may have led to more free GM-CSF in the infarcts.
After determining that the source of GM-CSF was confined to the infarcts, I next determined the cellular source of GM-CSF within the infarcts. Using FACS, major cell populations in the heart were isolated by first gating for CD45+ or CD45- cells, and then taking the CD45- population and delineating three subpopulations by CD31 and MEFSK4 (Figure 3a). Because there is no cardiac fibroblast specific marker, the gene expression profiles of the four isolated cell populations were evaluated by qPCR to validate that the majority of fibroblasts were confined to the fibroblast subpopulation. The subpopulation defined as fibroblasts (CD45-CD31- MEFSK4+) had the highest expression of Postn, Pdgfra, and Colla1, important markers of fibroblasts (Figure 3b). The total leukocyte and endothelial cell subpopulations had no expression of these genes, as expected. In the other stromal cell subpopulation, there was minimal expression of Postn and Colla1, but no expression of Pdgfra. These results suggested that the fibroblast subpopulation truly did contain mostly fibroblasts and that the other subpopulations did not. On day 1 after MI, fibroblasts had a significant increase in mRNA gene levels of Csf2, but there was no increase for total leukocytes, endothelial cells, or other stromal cells (Figure 3c). This result supports that fibroblasts are the main producers of GM-CSF after MI.

In MI, fibroblasts are known to play a crucial role as sentinels for cardiac injury and mediators of the inflammatory and healing responses after MI. As mentioned earlier, ischemic injury and necrosis of cardiomyocytes leads to the release of DAMPs and other intracellular debris. In order to better understand the role of these DAMPs in the upregulation of the GM-CSF gene after MI, I evaluated Csf2 mRNA expression levels in various knockout mice. In this experiment, Myd88−/−, Tlr2−/−, Tlr4−/−, Tlr7−/−, Tlr9−/−, and Il1r−/− mice were subject to MI and sacrificed 1 day later at the peak of GM-CSF expression. Analysis of Csf2 expression in infarcts from these various mice revealed that Myd88−/− and Tlr9−/− mice had significantly decreased expression levels compared to WT mice, meaning that a lack of Myd88 or TLR9 prevented upregulation of GM-CSF expression (Figure 4). Myd88 is an important adaptor protein in the signaling cascade downstream of the TLRs and IL-1 receptor. These results show that TLR9 signaling through Myd88 may be an important pathway for upregulation of GM-CSF expression following MI. TLR9 is stimulated by DNA, suggesting that fibroblasts primarily respond to DNA released by necrotic cardiomyocytes to upregulate GM-CSF and amplify the immune response following cardiac injury.

To better characterize GM-CSF’s effects in the infarcted tissue, I evaluated the expression of CCL2, an important chemokine for attracting neutrophils and other leukocytes. From WT and Csf2rb−/− mice infarcts 2 days after MI, Ly6C<sup>high</sup> monocytes, macrophages, neutrophils, and fibroblasts were obtained via staining and FACS. The different subpopulations were cultured in medium with or without GM-CSF and after 48 hours, the supernatant was evaluated for CCL2 protein concentrations using ELISA. Ly6C<sup>high</sup> monocytes and macrophages from WT mice that were cultured with GM-CSF containing media had significantly increased protein levels of CCL2, whereas those cultured without GM-CSF as well as these subpopulations from Csf2rb−/− mice cultured with GM-CSF had minimal expression of CCL2 (Figure 5a). Neutrophils and fibroblasts from
both WT and Csf2rb\textsuperscript{-/-} mice with or without GM-CSF did not produce significant levels of CCL2. These results show that Ly6\textsubscript{C}\textsuperscript{high} monocytes and macrophages respond to GM-CSF by producing CCL2. As expected, Ly6\textsubscript{C}\textsuperscript{high} monocytes and macrophages that lack the receptor for GM-CSF were not able to respond to GM-CSF and therefore, did not produce CCL2. Of note, fibroblasts from the infarct which are the main producers of GM-CSF, do not respond to GM-CSF and therefore, autocrine activation of fibroblasts does not appear to be a pathway for CCL2 expression. To further validate the response to GM-CSF, Ly6\textsubscript{C}\textsuperscript{high} monocytes, macrophages, neutrophils, CD11b\textsuperscript{-} cells, endothelial cells, fibroblasts, and other stromal cells were sorted from WT mice 2 days after MI were evaluated for phosphorylation of STAT5 (pSTAT5), which is an important step in the signaling cascade downstream of GM-CSF receptor activation. STAT5 is a transcription factor that is activated by phosphorylation, leading to changes in gene expression profiles within a cell. Cell subpopulations were stimulated with or without GM-CSF for 30 minutes and then stained for pSTAT5. pSTAT5 levels were found to be elevated in Ly6\textsubscript{C}\textsuperscript{high} monocytes, macrophages, and neutrophils after simulation with GM-CSF, but not CD11b\textsuperscript{-} cells, endothelial cells, fibroblasts, or other stromal cells (Figure 5b). These results support the previous data showing that Ly6\textsubscript{C}\textsuperscript{high} monocytes and macrophages respond to GM-CSF to produce CCL2.
Discussion

Myocardial infarction is a clinically significant source of morbidity and mortality with up to 25% of MI patients progressing to heart failure, primarily due to systolic dysfunction. Acute MI is primarily treated by reperfusion strategies that aim to restore blood flow to the ischemic myocardium, but this method is most effective only within a very short time interval of 90 minutes and therefore is not always relevant in actual practice and is not without potential negative consequences such as reperfusion injury. Therefore, other strategies to ameliorate the immune response and prevent extensive fibrotic scar formation, adverse ventricular remodeling, and eventual heart failure are imperative at this time. The major goal of MI therapy should be to prevent myocardial damage in the first place, but other strategies include balancing the pro-fibrotic versus pro-regenerative response in the damaged myocardium. Modulation of the immune system is an attractive therapeutic option in that there are so many different components with complex interactions that can be altered with regards to time, location, quality, and quantity.

GM-CSF is an important cytokine whose role in a variety of immunological processes is just beginning to be understood. In the context of MI, there was controversy regarding whether GM-CSF played a detrimental or beneficial role with evidence in mice treated with GM-CSF showing increased infarct size in one study, but another study showing increased endothelial progenitor cell recruitment and myocardial function preservation. Due to the confusion caused by conflicting studies, it was imperative to delineate GM-CSF’s role in the propagation of MI in order to someday explore its therapeutic potential. In preliminary studies performed by the Swirski lab, mice without GM-CSF had significantly increased survival, improved ventricular function, and reduced leukocyte infiltration into infarcts after LAD ligation. We then found that GM-CSF expression was shortly and transiently upregulated in infarcted heart tissue after MI, as evidenced by increased GM-CSF mRNA and protein levels. The infarcted tissue was the main source of GM-CSF and within the infarcted tissue, GM-CSF was primarily produced by fibroblasts, but not leukocytes, endothelial cells, or other stromal cells. The primary trigger for GM-CSF expression was found to be DNA, an important DAMP released after MI that binds to TLR9 and signals through Myd88. Lastly, we found that Ly6C^high monocytes and macrophages in the infarcted tissue responded to GM-CSF by producing CCL2, an important chemokine for monocyte and macrophage chemotaxis.

Here, we have shown that GM-CSF is a critical cytokine in the activation of the immune response immediately following MI that has implications on overall survival and long-term function of the heart. Our results are consistent with previous studies that suggest a detrimental role of GM-CSF in acute MI with GM-CSF administration in rats post-MI leading to increased infarct region and adds to these results by identifying the source and targets of GM-CSF. In stark contrast to results by Atluri et al, we did not find evidence to show increased endothelial progenitor cell recruitment or preservation of myocardial function, however, we did not also administer or even measure stromal cell derived factor-1α, which may have been necessary for GM-CSF to
have this beneficial effect.\textsuperscript{20} The Swirski lab previously discovered a subset of B cells called innate response activator B-cells that produce GM-CSF and are implicated in the immune response in microbial infections such as sepsis and pneumonia.\textsuperscript{32,33} In addition, more recent studies from the Swirski lab have shown that IRA B cells producing GM-CSF alter T cells to a Th1 phenotype that leads to atherosclerosis, a process fundamental to MI.\textsuperscript{34} A recent study has shown that B cells from pericardial adipose tissue are an important source of GM-CSF after MI, leading to recruitment of dendritic cells and induction of IL-17 producing T cells.\textsuperscript{35} We did not look specifically at pericardial adipose tissue nor did we see a significant number of lymphocytes in hearts following MI, therefore, it is difficult to comment on whether this potential source of GM-CSF could have contributed to our results. However, it is worth noting this study’s findings were in a mouse model of exaggerated B cell response and in female mice only and therefore may not accurately reflect the immune response after MI. Given IRA B cells implication in atherosclerosis, it would be interesting to see if these cells play a role in the detrimental outcomes of GM-CSF in MI.

A major limitation of this work is the lack of exploration of the systemic effects of GM-CSF after MI as it is already well known that GM-CSF acts on the bone marrow compartment to stimulate hematopoiesis. In addition, we did not explore the effects of GM-CSF on lymphocyte quantity or quality. Based on our work and other groups work, it seems that there may be two major components to GM-CSF’s role in acute MI. The first being GM-CSF production by cardiac fibroblasts in the immediate period to increase leukocyte accumulation in the heart and the second, a slightly delayed production of GM-CSF by B cells that changes T cell response and phenotype. These separate pathways were delineated using different models, for example we used GM-CSF knockout mice whereas other groups added or induced GM-CSF and therefore, based on this work alone, we cannot be sure how these different mechanisms coexist and interact. Another important limitation of our work is that we do not include human studies. Although previous observational studies have shown that GM-CSF levels were highest in patients with MI leading to heart failure or severe left ventricular dysfunction, it is imperative to show that the results seen here in mice translate over to humans.\textsuperscript{19} Lastly, we used LAD ligation as a model for acute MI, which has known limitations of requiring mice to undergo surgery and having a different pathophysiology than acute MI in humans.

In conclusion, we show here that GM-CSF is a critical cytokine in the inflammatory response after MI and a promising target for clinical intervention.
References


16. Terrovitis, J. *et al.* No effect of stem cell mobilization with GM-CSF on infarct size and left ventricular...


**Figures**

Figure 1a. Survival rates after MI in WT and Csf2−/− mice. **P<0.01.**

Figure 1b. Representative MRI images showing infarct size by late gadolinium uptake on day 1 as well as end-diastolic and end-systolic heart morphology on day 21 in WT and Csf2−/− mice.

Figure 1c. Quantification of change in end-diastolic volume (EDV), end-systolic volume (ESV), and ejection fraction (EF) from day 1 to day 21 after MI in WT and Csf2−/− mice. (n=6-7 per group) *P<0.05.
Figure 1d. Quantification by flow cytometry of cell populations in the hearts of WT and Csf2\(^{-/-}\) mice before (day 0) and 1, 3, or 7 days after MI. (n=3-7 per group) *P<0.05.

Figure 2a. Csf2 mRNA gene expression in infarcted heart tissue (Inf), non-infarcted heart tissue (Non-inf), spleen (Sp), bone marrow (BM), and lymph nodes (LN) of WT mice before (day 0) and 1, 3, or 7 days after MI. (n=3-5 per group) *P<0.05, **P<0.01 vs. control.
Figure 2b. GM-CSF protein level determined by ELISA in the infarcted heart tissue before (day 0) and 1, 3, or 7 days after MI for WT mice and 1 day after MI for $Csf2^{-/-}$ and $Csf2rb^{-/-}$ mice. (n=3-5 per group) *$P<0.05$, **$P<0.01$ vs. control.

Figure 3a. Gating strategy for isolating total leukocytes (Leuks), endothelial cells (ECs), fibroblasts (FBs), and other stromal cells (Other) using FACS.

Figure 3b. $Postn$, $Pdgfra$, and $Col1a1$ mRNA gene expression in whole heart tissue samples and sorted cell populations as shown in Figure 3a from WT mice.
Figure 3c. Csft2 mRNA gene expression in sorted cell populations from the infarcted heart tissue before and 1 day after MI. (n=4-5 mice per group) *P<0.05 vs. day 0

Figure 4. Csft2 mRNA gene expression in infarcted heart tissue 1 day after MI from WT and various knockout mice. (n=4-8 mice per group) *P<0.05 vs. WT heart.
**Figure 5a.** CCL2 protein expression in Ly6\textsuperscript{high} monocytes, macrophages, neutrophils, and fibroblasts from infarcted heart tissue 2 days after MI in WT and Csf2rb\textsuperscript{−/−} mice cultured in the presence or absence of GM-CSF for 48 hours. CCL2 was measured from the supernatant of the cultures using ELISA. Values were normalized to WT with medium only (no GM-CSF). (n=5-6 per group) **P<0.01.**
Figure 5b. Representative flow cytometric histograms showing expression of pSTAT5 in various cell populations sorted from infarcted heart tissue 2 days after MI in WT mice. Cell populations were incubated with or without GM-CSF for 30 mins before staining for pSTAT5.