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Thrombospondin-1-CD47 Blockade Following Ischemia Reperfusion Injury is Tissue Protective

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Abstract

Background—Nitric oxide has pro-survival effects that can limit ischemia-reperfusion (I/R) injuries. However, the matrix glycoprotein thrombospondin-1 is induced following I/R injury and limits nitric oxide signaling by engaging its cell surface receptor CD47. We herein examine whether post-injury blocking of this inhibitory signal can protect from I/R injury in a rat flap model.

Methods—A total of 40 tissue flaps were created in rats based solely on the deep inferior epigastric vessels (DIEV). Microvascular clamps were used to create 45 minutes of ischemia time to the flaps. The flaps were then treated using a monoclonal antibody to CD47 or an isotype-matched control IgG₁ 5 or 30 minutes after clamp removal. Twenty-four or 72 hours post-operatively, the necrotic area of the flap was determined, and serum, DIEV, and flaps were harvested for analysis from 5 rats in each respective group.

Results—Treatment with a CD47 antibody 5 minutes post-reperfusion significantly reduces flap necrosis compared to IgG₁ control (9% vs. 43%; $p < 0.01$). The protective effect is even more dramatic when treatment is delayed until 30 minutes post-reperfusion (10% vs. 88% for control; $p < 0.01$). Markers of neutrophil and endothelial cell activation along with total leukocytes are reduced in CD47 antibody treated flaps, as are tissue malondialdehyde levels. Levels of cGMP are elevated 72 hours post-operatively in the CD47 antibody treated DIEV versus the control flaps.

Conclusions—Therapies targeting the thrombospondin-1 receptor CD47 offer potential for increasing tissue survival in I/R injuries. The ability to protect when given post I/R injury enables a broader clinical applicability.

Introduction

Ischemia-reperfusion (I/R) injury underlies to varying degrees the pathophysiology of many disease states including myocardial infarction, stroke, peripheral vascular disease, traumatic soft tissue wounds, and shock. In the field of reconstructive surgery, I/R injury is a cause of failure of free tissue transfers and microsurgical flaps.¹ The “no-reflow” phenomenon

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commonly observed in such failing flaps is associated with thrombosis, neutrophil accumulation, and reduced perfusion.²

Extensive work has been done to understand the mechanism responsible for these critical elements of I/R injury. The current understanding points towards the reperfusion phase as causing most of the tissue damage and highlights a complex inflammatory response that is ultimately responsible for flow disruption.³ Though an understanding of the importance of the inflammatory response has led to treatments that target these elements, only modest success has been achieved in treating I/R injury. Therapeutic approaches have focused on reducing leukocyte infiltration, the amount of reactive oxygen species generated or other specific pathways within the inflammatory cascade.⁴

Nitric oxide (NO) is a bioactive gas produced by several cell types that has many biological functions.⁵ Based on its ability to promote tissue perfusion and limit inflammation, NO has been studied in the context of I/R injury.² Despite some conflicting data concerning cytoprotective versus cytotoxic roles played by NO, there is an emerging consensus that NO has a pro-survival role in tissue perfusion and flap survival. This is supported by the increased survival of compromised tissue treated with NO donors or NOS substrates, which increase blood flow to ischemic tissues and minimize I/R injury and tissue loss.²

Recently, we discovered that the matrix glycoprotein thrombospondin-1 (TSP1), via engaging the cell surface receptor CD47, blocks the pro-survival signaling of NO.^{6–10} Inhibiting TSP1/CD47 signaling, therefore, maximizes NO signaling and increases tissue blood flow. Notably, expression of TSP1 is strongly induced during reperfusion of ischemic cardiac muscle, kidney, and liver tissues in mouse, rat, and porcine I/R injury models.^{6, 11–13} Kidneys of mice lacking TSP1 were protected from I/R injury, which these authors proposed was mediated by the TSP1 receptor CD36.¹³ However, subsequent studies found that transgenic mice lacking either TSP1 or its receptor CD47 demonstrated similar resistance to liver tissue damage in response to I/R⁶, and pre-treatment of wild type animals with a CD47 monoclonal antibody rendered them resistant to liver I/R injury. This demonstrated CD47 to be the dominant receptor mediating the effects of TSP1 in I/R, potentially via limiting pro-survival NO signaling, and suggested CD47 to be a useful therapeutic target to enhance such signaling following an I/R injury. However, such elective intervention is limited to clinical situations such as transplant surgery and coronary bypass surgery. Most clinical presentation of I/R injury is unpredictable and requires that therapeutic interventions be effective when administered a significant interval after the initial insult.

Our hypothesis was that blocking of TSP1/CD47 signaling can provide a sustainable and pro-survival level of NO that protects from I/R injury even if given post-reperfusion. We examined this theory using a rat island myocutaneous flap model and found that in treated groups, tissue blood flow and survival are maximized as are levels of cGMP, while markers of inflammation are decreased. TSP1-CD47 blockade thus represents a novel therapeutic target for the treatment of tissue flaps in reconstructive surgery and more generally as a potential therapy for I/R injuries resulting from trauma, vascular occlusion, or embolism.

Methods

Animals

F344/Ncr male rats aged 12 to 14 weeks each weighing between 250–300 grams were housed under pathogen free conditions and had ad libitum access to filtered water and standard rat chow. Handling and care of animals under protocol LP-024 was in compliance with the guidelines established by the Animal Care and Use Committees of the National Cancer Institute.

Reagents

A mouse monoclonal antibody to rat CD47 (clone OX101) and isotype-matched control mouse IgG₁ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Island myocutaneous flap model

Anesthesia was induced by and maintained with inhalation 1.5% isoflurane. Body temperature was maintained at 37°C with a heating lamp during the procedure. The ventral surface was clipped of hair and depilated with NairR (Church and Dwight Co., Inc., Princeton, NJ). The skin was then cleansed with surgical soap and alcohol and the animals draped. Flap dimensions were marked on the animal skin surface with the aid of a template to insure consistency. Flaps were situated on the left side of the animal and orientated parallel to the long axis. Using sterile technique, 2 × 6 cm island myocutaneous flaps incorporating the skin, superficial fascia, panniculus carnosus, and subcutaneous tissue were raised according to the method of Petry and Wortham¹⁴, leaving the deep inferior epigastric artery and vein alone as a pedicle. Care was taken to ensure that the epigastric fat pad was raised with all flaps. Complete denervation of these flaps was achieved by dividing the epigastric nerve. I/R injury was initiated by applying 2V non-crushing microvascular clamps (ASSI, Westbury, NY) to the femoral artery and vein both proximal and distal to the deep inferior epigastric vessels (DIEV). Flaps were secured leaving the pedicle edge open with interrupted 5-0 nylon sutures. A minimum number of sutures were employed to limit the effect of suturing on tissue survival. Following a 45 minute interval of warm ischemia, during which proper placement of microvascular clamps was verified with visual inspection of the skin flap, vascular clamps were removed, and flaps were sutured back to their donor bed with 5-0 nylon sutures. After a defined interval of reperfusion of either 5 or 30 minutes, a CD47 antibody or IgG₁ was injected directly into the entire area of the subcutaneous layer of the flap. A total of 40 rats received either IgG₁ or CD47 antibody. Within those two groups, half of the rats received the treatment at 5 minutes where the other half received the antibodies 30 minutes post-reperfusion. Those subgroups were further divided into groups sacrificed 24 hours or 72 hours postoperatively. 10 rats underwent sham operations consisting only of flap elevation and isolation of vessels but not subjected to ischemic injury or antibody treatment and sacrificed at either 24 or 72 hours. Animals were awakened and returned to individual cages and allowed ad libitum access to food and water. The flaps were evaluated prior to excision and sacrifice, and tissues were collected as described below.

Estimation of survival area in flaps

The necrotic area of the flap was determined by color, refill, eschar, and the pin-prick test. The outlines of viable and nonviable areas were traced using transparent film. A ratio comparing flap necrosis to total flap area was obtained by dividing the weight of the flap tracing of the necrotic area by the weight of the original template utilizing the method described by Ueda.¹⁵ Flap necrosis was presented as this ratio multiplied by 100%. Animals were then euthanized and flaps excised, fixed in 10% paraformaldehyde, and processed for histology.

Histopathological analyses

Tissue units were excised, fixed in 10% buffered paraformaldehyde, paraffin embedded, and sectioned at a thickness of 5 μm. Sections were applied to charged glass slides and stained with hematoxylin and eosin (H&E) (American HistoLabs, Inc., Gaithersburg, MD). Review of each slide was performed by an independent observer unaware of the origin of each tissue section.

Biochemical analyses

Samples were collected at either 24 hours or 72 hours postoperatively while the animal was under isoflurane anesthesia as follows. 2 × 0.5 cm tissue sections were taken from the most distal portion of the skin flap (regardless of viability, with or without necrosis). Blood was

taken from the inferior vena cava and immediately centrifuged for serum collection. Care was taken to ensure that no abdominal organs were compromised during this process. The DIEV were then collected, and the flap was excised and fixed in 10% paraformaldehyde. All samples with exception of the skin flap for immunohistochemical analysis were snap frozen in liquid nitrogen and immediately placed in -70°C . Analyses of samples were performed in aggregate at the end of the study.

Serum IFN- γ —Levels of serum IFN- γ were determined using Amersham Interferon Gamma Rat Biotrak ELISA System (GE Healthcare, Buckinghamshire, UK). Serum samples were used undiluted.

Tissue malondialdehyde—Lipid peroxidation in the skin flap sample was determined by measuring levels of malondialdehyde (MDA), an end product of lipid peroxidation. The content of MDA in samples was determined using a calorimetric reaction with thiobarbituric acid as described by Buege and Aust.¹⁶

Vessel cGMP—DIEV were harvested from the takeoff point of the femoral vessels to the attachment of the tissue flap immediately prior to animal sacrifice, 72 hours post-operatively and prepared for analysis by mincing the tissue frozen in liquid nitrogen. These samples were then sonicated and incubated with cGMP lysis buffer. Total cGMP levels were determined using the Amersham cGMP Enzymeimmunoassay Biotrak System (GE Healthcare, Buckinghamshire, UK).

Statistics

Results are presented as the mean $\pm$ SD with significance calculated by the Student's *t* test. One-way ANOVA analysis was used to compare tissue MDA levels between different subgroups. A standard software package (OriginLab Corporation, Northampton, MA) was used, and significance was assigned for a *p* value ≤ 0.05 .

Results

Post-I/R injury CD47 blockade maintains tissue survival

The ability to intervene electively in I/R injury is limited to select surgical situations such as visceral organ transplantation or coronary revascularization. A therapeutic agent that prevents the tissue damage of I/R injury when administered after the insult would have much broader applicability. In a rat model of I/R injury utilizing an island myocutaneous soft tissue flap, CD47 blockade using a rat-specific antibody was performed 5 minutes post-reperfusion. As seen in figure 1A, blockade of CD47 was effective at abrogating tissue ischemia and necrosis and preserving blood flow even in the distal area of the flap as demonstrated by the pinprick test, 72 hours post-operatively. Flaps treated with an isotype matched IgG₁ control antibody experienced a significantly greater degree of tissue necrosis ($43\% \pm 16\%$ versus $9\% \pm 5\%$, Figure 1B, $p < 0.01$). Results were even more dramatic when treatment was delayed to 30 minutes post-reperfusion ($88\% \pm 12\%$ versus $10\% \pm 8\%$, Figure 1C, $p < 0.01$). Sham surgery flaps not subjected to ischemia or antibody treatment showed an average of $12\% \pm 7\%$ tissue necrosis.

Suppression of CD47 increases levels of cGMP postoperatively after I/R injury

In endothelial and vascular smooth muscle cells and platelets, TSP1 signaling through its receptor CD47 prevents NO-mediated activation of soluble guanylate cyclase.^{17–19} In both healthy and ischemic tissues the absence of TSP1 or pharmacological inhibition of this pathway results in elevated tissue cGMP levels.^{18, 20} To test the relevance of this pathway to improved survival in this I/R injury model, we measured cGMP levels after treatment with the CD47

antibody. DIEV harvested 72 hours post-operatively were assayed for cGMP levels in each of the treatment groups (control antibody and CD47 antibody) as well as after sham surgery receiving no treatment. As shown in figure 2, cGMP levels were similar in the non-treatment group (0.65 ± 0.15) and animals subjected to I/R and receiving the IgG₁ control antibody ($0.7 \pm .10$). However, those flaps subjected to I/R followed by a single treatment using the CD47 antibody showed elevated cGMP levels 72 hours post-operatively (1.1 ± 0.12 , $p < 0.01$ compared with the control antibody).

CD47 blockade abrogates elevated circulating IFN- γ levels after I/R injury

The mechanisms underlying I/R injury are complex and include local leukocyte sequestration and activation involving interactions between neutrophils, macrophages, and T cells, which lead to the secretion of pro-inflammatory cytokines/chemokines.^{1, 21–23} IFN- γ is one of the main pro-inflammatory cytokines released during I/R injury that is thought to directly damage through growth arrest and sensitization of epithelial cells to CD95 (Fas/Apo-1)-mediated cell death²⁴ and indirectly through activation of macrophages. We examined serum levels of rat IFN- γ 24 hours after I/R injury $\pm$ treatment given 30 minutes post-reperfusion and compared them to basal levels by immunoassay. Circulating levels of IFN- γ were elevated almost 30% $\pm$ 5% from baseline when treated with the IgG₁ control antibody ($p < 0.01$ compared to baseline). However, after treatment with the CD47 antibody (Figure 3), circulating IFN- γ levels not only decreased in comparison to treatment with the isotype control but were reduced 17% $\pm$ 7% from baseline levels ($p < 0.001$ compared with control antibody).

Tissue lipid peroxidation is reduced after CD47 blocking

Reactive oxygen metabolites play a major role in I/R injury.²⁵ One of the ways in which these metabolites cause damage is through direct reaction with the endothelial membrane, causing lipid peroxidation, disruption of membrane proteins, and ultimately cytoplasmic swelling and cell dysfunction.^{26, 27} Lipid peroxidation in the rat flap tissue specimens was determined by measuring levels of MDA and related reactive lipid metabolites, which are established indicators of lipid peroxidation.^{28, 29} As expected, MDA levels were elevated at 24 hours ($5028.4 \text{ nM/g} \pm 676$) post-operatively and higher at 72 hours ($10,258 \text{ nM/g} \pm 1078$) compared to control tissue subjected to sham surgery ($1976 \text{ nM/g} \pm 201$, Figure 4, $p < 0.01$). Twenty-four hours post-operatively, we saw decreased levels of tissue MDA after treatment with the CD47 antibody ($3998.4 \text{ nM/g} \pm 325$) delivered 30 minutes post-reperfusion when compared to the control treatment. Furthermore, we saw an even greater decrease in MDA levels when tissue samples were taken 72 hours post-operatively ($2378 \text{ nM/g} \pm 345$) when compared to the control IgG₁ antibody treatment (Figure 4, $p < 0.001$).

Decreased inflammation in flap tissues treated with CD47 antibody post-I/R injury

Chemical mediators released following I/R injury are chemotactic to leukocytes mainly through the lipoxygenase and cyclooxygenase pathways by increasing adhesion of leukocytes to vascular endothelium and subsequent diapedesis.²³ Indeed, animal models possessing leukocyte deficiencies exhibit reduced I/R injury.³⁰ In our model, those flaps treated with CD47 antibody showed attenuated leukocyte infiltration when compared to the isotype control antibody as seen on H&E at 72 hours post-operatively (Figure 5A).

At 24 hours post operatively, we examined levels of neutrophil elastase. At this early point in the inflammatory process, activated neutrophils are implicated in tissue damage. Neutrophil elastase, released from such activated neutrophils, is known to mediate tissue damage by degrading components of the extracellular matrix and enhancing intercellular adhesion molecule-1 (ICAM-1) expression in endothelial cells.^{31–34} The latter plays an integral role in I/R injury by facilitating leukocyte adhesion.³⁵ To assess effects of CD47 antibody treatment on this acute inflammatory process, immunohistochemical analysis of elastase was performed

on rat flaps harvested 24 hours post-operatively. Compared to flaps treated with CD47 antibody, flaps treated with the isotype control displayed increased elastase expression (Figure 5B). Flaps harvested 24 hours after sham surgery showed little to no leukocyte infiltration and no elastase expression (results not shown).

Discussion

We have demonstrated previously that pretreatment to interrupt TSP1/CD47 signaling enhances tissue blood flow, survival, and wound healing following various fixed ischemic injuries including skin flaps in both young and aged animals, hindlimb ischemia, and full thickness skin grafts.^{6–8, 10} Furthermore, the same pretreatment was effective in a murine model of subtotal warm liver I/R injury.⁶ However, I/R injury often occurs under non-elective conditions, making pre-injury therapy impossible. Since such conditions are of significant clinical importance, we created a rodent model of defined soft tissue I/R injury whereby treatment to interrupt TSP1/CD47 signaling would occur post insult. We now show that disruption of TSP1/CD47 signaling using a rat CD47-specific antibody administered 5 or 30 min after an I/R injury protects tissue on a gross level, reduces levels of inflammatory cell invasion, lipid peroxidation, and IFN- γ levels, and increases levels of cGMP.

The improved tissue survival is accompanied by decreased neutrophil recruitment and resulting tissue lipid oxidation along with decreased systemic IFN γ levels. These findings indicate a decreased inflammatory response resulting from treatment with the CD47 antibody. This may be due to several targets of CD47 signaling including the NO/cGMP pathway, integrin regulation, and signal regulatory protein (SIRP) signaling.^{36–39}

NO plays major roles in I/R injury to regulate leukocyte mediated inflammation, oxygen free radical formation, and thrombus formation from platelet activation. NO signaling via cGMP inhibits platelet aggregation and neutrophil accumulation, which are often responsible for flap failure.^{40, 41} Low physiologic levels of NO that signal via cGMP also reduce the production of tissue damaging free radicals. The overall effect is that inflammatory mediated tissue injury is reduced, and tissue perfusion is therefore increased. In addition to modulating inflammation, NO/cGMP signaling helps to reduce the vasospasm documented in I/R injury. NO directly relaxes vascular smooth muscle cells, thus decreasing vascular resistance and increasing tissue perfusion and blood flow.⁴²

Due to these pro-survival roles of NO, therapeutics to enhance NO signaling have been aggressively investigated. Even though many of these approaches have been effective at ameliorating I/R injury in experimental models, clinical efficacy has proven elusive and is limited by the potential toxicity of NO as a free radical when present in concentrations that can interact with superoxide to generate reactive nitrogen species that are directly cytotoxic.⁴³ In addition, systemic hypotension is a concern, as widespread vasodilation can actually lower blood pressure enough to compromise tissue perfusion.

Attempts to treat I/R injuries through NO supplementation are thus limited by these concerns in addition to the absence of flow in situations of I/R injury. But perhaps more germane is the finding that TSP1 expression is rapidly induced following I/R injury^{6, 11–13}, which limits the ability of tissues to respond to therapeutic NO- or cGMP-elevated therapeutic agents. This blockade of NO signaling by TSP1/CD47 occurs at several points including sGC and cGK activation.^{17, 19} Thus, TSP1/CD47 signaling limits NO driven changes in blood flow and tissue survival.^{9, 18, 44}

Another reason why CD47 blockade is more effective than NO-based therapeutics is that CD47 may modulate important aspects of I/R injury that are independent of NO/cGMP signaling. CD47 is universally expressed on cells of the immune system and serves as an important

counter-receptor for at least 2 members of the SIRP family. Engaging SIRP α or SIRP γ triggers signaling responses in the relevant cells that profoundly modulate immune cell responses and activation.^{45, 46} Engaging CD47 itself can also regulate immune cell function,^{47, 48} and loss of CD47 can inhibit some immune processes.^{47, 49} In addition, CD47 is an important regulator of integrin activation by its lateral interaction with several integrins in the plasma membrane.^{37, 50} In immune responses, integrins play key roles in diapedesis and tissue recruitment of leukocytes, and CD47 null mice show defects in neutrophil and macrophage recruitment that are consistent with this role. Thus, antibody blocking of CD47 may directly prevent integrin-mediated recruitment of inflammatory cells into tissue after an I/R injury. Finally, CD47 engagement can trigger apoptotic responses in several cells types^{51–57} but only certain CD47 antibodies and ligands have this property,^{58–61} and the antibody used in the present study could potentially block such apoptotic responses in an I/R injury. Therefore, we propose that therapeutic antagonists of CD47 may act at several levels to improve I/R injury responses. Two different species-specific CD47 antibodies have now been validated to protect against ischemic injuries in vivo, suggesting that employing anti-human CD47 antibodies to extend such protection to I/R injuries in patients will be feasible.

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References

1. Khalil AA, Aziz FA, Hall JC. Reperfusion injury. *Plast Reconstr Surg* 2006;117:1024–1033. [PubMed: 16525303]
2. Topp SG, Zhang F, Chatterjee T, Lineaweaver WC. Role of nitric oxide in surgical flap survival. *J Am Coll Surg* 2005;201:628–639. [PubMed: 16183504]
3. Cerra FB, Lajos TZ, Montes M, Siegel JH. Hemorrhagic infarction: A reperfusion injury following prolonged myocardial ischemic anoxia. *Surgery* 1975;78:95–104. [PubMed: 1138403]
4. Mowlavi A, Neumeister MW, Wilhelmi BJ, Song YH, Suchy H, Russell RC. Local hypothermia during early reperfusion protects skeletal muscle from ischemia-reperfusion injury. *Plast Reconstr Surg* 2003;111:242–250. [PubMed: 12496585]
5. Ignarro LJ. Nitric oxide as a unique signaling molecule in the vascular system: a historical overview. *J Physiol Pharmacol* 2002;53:503–514. [PubMed: 12512688]
6. Isenberg JS, Maxhimer JB, Powers P, Tsokos M, Frazier WA, Roberts DD. Treatment of liver ischemia-reperfusion injury by limiting thrombospondin-1/CD47 signaling. *Surgery* 2008;144:752–761. [PubMed: 19081017]
7. Isenberg JS, Romeo MJ, Abu-Asab M, et al. Increasing survival of ischemic tissue by targeting CD47. *Circ Res* 2007;100:712–720. [PubMed: 17293482]
8. Isenberg JS, Romeo MJ, Maxhimer JB, Smedley J, Frazier WA, Roberts DD. Gene silencing of CD47 and antibody ligation of thrombospondin-1 enhance ischemic tissue survival in a porcine model: implications for human disease. *Ann Surg* 2008;247:860–868. [PubMed: 18438125]
9. Isenberg JS, Pappan LK, Romeo MJ, et al. Blockade of thrombospondin-1-CD47 interactions prevents necrosis of full thickness skin grafts. *Ann Surg* 2008;247:180–190. [PubMed: 18156939]
10. Isenberg JS, Hyodo F, Pappan LK, et al. Blocking thrombospondin-1/CD47 signaling alleviates deleterious effects of aging on tissue responses to ischemia. *Arterioscler Thromb Vasc Biol* 2007;27:2582–2588. [PubMed: 17916772]
11. Lario S, Bescos M, Campos B, et al. Thrombospondin-1 mRNA expression in experimental kidney transplantation with heart-beating and non-heart-beating donors. *J Nephrol* 2007;20:588–595. [PubMed: 17918145]

12. Sezaki S, Hirohata S, Iwabu A, et al. Thrombospondin-1 is induced in rat myocardial infarction and its induction is accelerated by ischemia/reperfusion. *Exp Biol Med* (Maywood) 2005;230:621–630. [PubMed: 16179730]
13. Thakar CV, Zahedi K, Revelo MP, et al. Identification of thrombospondin 1 (TSP-1) as a novel mediator of cell injury in kidney ischemia. *J Clin Invest* 2005;115:3451–3459. [PubMed: 16294224]
14. Petry JJ, Wortham KA. The anatomy of the epigastric flap in the experimental rat. *Plast Reconstr Surg* 1984;74:410–413. [PubMed: 6236468]
15. Ueda K, Tajima S, Tanaka Y, Tsujiguchi K, Akamatsu J. Experimental study of delay of venous island flaps. *Ann Plast Surg* 1994;33:519–523. [PubMed: 7857046]discussion 523–514
16. Buege JA, Aust SD. Microsomal lipid peroxidation. *Methods Enzymol* 1978;52:302–310. [PubMed: 672633]
17. Isenberg JS, Ridnour LA, Dimitry J, Frazier WA, Wink DA, Roberts DD. CD47 is necessary for inhibition of nitric oxide-stimulated vascular cell responses by thrombospondin-1. *J Biol Chem* 2006;281:26069–26080. [PubMed: 16835222]
18. Isenberg JS, Wink DA, Roberts DD. Thrombospondin-1 antagonizes nitric oxide-stimulated vascular smooth muscle cell responses. *Cardiovasc Res* 2006;71:785–793. [PubMed: 16820142]
19. Isenberg JS, Ridnour LA, Perruccio EM, Espey MG, Wink DA, Roberts DD. Thrombospondin-1 inhibits endothelial cell responses to nitric oxide in a cGMP-dependent manner. *Proc Natl Acad Sci U S A* 2005;102:13141–13146. [PubMed: 16150726]
20. Isenberg JS, Hyodo F, Matsumoto K, et al. Thrombospondin-1 limits ischemic tissue survival by inhibiting nitric oxide-mediated vascular smooth muscle relaxation. *Blood* 2007;109:1945–1952. [PubMed: 17082319]
21. Gauthier TW, Davenpeck KL, Lefer AM. Nitric oxide attenuates leukocyte-endothelial interaction via P-selectin in splanchnic ischemia-reperfusion. *Am J Physiol* 1994;267:G562–568. [PubMed: 7524346]
22. Lefer AM. Attenuation of myocardial ischemia-reperfusion injury with nitric oxide replacement therapy. *Ann Thorac Surg* 1995;60:847–851. [PubMed: 7677545]
23. Lehr HA, Gohlmann A, Nolte D, Keppler D, Messmer K. Leukotrienes as mediators in ischemia-reperfusion injury in a microcirculation model in the hamster. *J Clin Invest* 1991;87:2036–2041. [PubMed: 1645749]
24. Seidelin JB, Jaattela M, Nielsen OH. Continuous interferon-gamma or tumor necrosis factor-alpha exposure of enterocytes attenuates cell death responses. *Cytokine* 2004;27:113–119. [PubMed: 15271377]
25. Im MJ, Hoopes JE, Yoshimura Y, Manson PN, Bulkley GB. Xanthine:acceptor oxidoreductase activities in ischemic rat skin flaps. *J Surg Res* 1989;46:230–234. [PubMed: 2646473]
26. Sagi A, Ferder M, Levens D, Strauch B. Improved survival of island flaps after prolonged ischemia by perfusion with superoxide dismutase. *Plast Reconstr Surg* 1986;77:639–644. [PubMed: 3952220]
27. Im MJ, Hoopes JE. Improved skin flap survival with nicotinic acid and nicotinamide in rats. *J Surg Res* 1989;47:453–455. [PubMed: 2530401]
28. Del Rio D, Stewart AJ, Pellegrini N. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutr Metab Cardiovasc Dis* 2005;15:316–328. [PubMed: 16054557]
29. Lykkesfeldt J. Malondialdehyde as biomarker of oxidative damage to lipids caused by smoking. *Clin Chim Acta* 2007;380:50–58. [PubMed: 17336279]
30. Breda MA, Drinkwater DC, Laks H, et al. Prevention of reperfusion injury in the neonatal heart with leukocyte-depleted blood. *J Thorac Cardiovasc Surg* 1989;97:654–665. [PubMed: 2709858]
31. Konno T, Ito T, Takai M, Ishihara K. Enzymatic photochemical sensing using luciferase-immobilized polymer nanoparticles covered with artificial cell membrane. *J Biomater Sci Polym Ed* 2006;17:1347–1357. [PubMed: 17260507]
32. Diamond MS, Staunton DE, de Fougères AR, et al. ICAM-1 (CD54): a counter-receptor for Mac-1 (CD11b/CD18). *J Cell Biol* 1990;111:3129–3139. [PubMed: 1980124]
33. Poggetti RS, Moore FA, Moore EE, Bensard DD, Anderson BO, Banerjee A. Liver injury is a reversible neutrophil-mediated event following gut ischemia. *Arch Surg* 1992;127:175–179. [PubMed: 1540095]

34. Jaeschke H, Farhood A, Smith CW. Neutrophils contribute to ischemia/reperfusion injury in rat liver in vivo. *FASEB J* 1990;4:3355–3359. [PubMed: 2253850]
35. Zhang RL, Chopp M, Li Y, et al. Anti-ICAM-1 antibody reduces ischemic cell damage after transient middle cerebral artery occlusion in the rat. *Neurology* 1994;44:1747–1751. [PubMed: 7936308]
36. Matozaki T, Murata Y, Okazawa H, Ohnishi H. Functions and molecular mechanisms of the CD47-SIRPalpha signalling pathway. *Trends Cell Biol* 2009;19:72–80. [PubMed: 19144521]
37. Brown EJ, Frazier WA. Integrin-associated protein (CD47) and its ligands. *Trends Cell Biol* 2001;11:130–135. [PubMed: 11306274]
38. Sarfati M, Fortin G, Raymond M, Susin S. CD47 in the immune response: role of thrombospondin and SIRP-alpha reverse signaling. *Curr Drug Targets* 2008;9:842–850. [PubMed: 18855618]
39. Isenberg JS, Frazier WA, Roberts DD. Thrombospondin-1: a physiological regulator of nitric oxide signaling. *Cell Mol Life Sci* 2008;65:728–742. [PubMed: 18193160]
40. Zimmerman BJ, Granger DN. Mechanisms of reperfusion injury. *Am J Med Sci* 1994;307:284–292. [PubMed: 8160724]
41. Rashid MA, William-Olsson G. Are leukocytosis and lipid peroxidation involved in ischemic or reperfusion injury in cardiac surgery? *Thorac Cardiovasc Surg* 1991;39:193–195. [PubMed: 1948967]
42. Ignarro LJ. Nitric oxide-mediated vasorelaxation. *Thromb Haemost* 1993;70:148–151. [PubMed: 7694386]
43. Thomas DD, Ridnour LA, Isenberg JS, et al. The chemical biology of nitric oxide: implications in cellular signaling. *Free Radic Biol Med* 2008;45:18–31. [PubMed: 18439435]
44. Isenberg JS, Romeo MJ, Yu C, et al. Thrombospondin-1 stimulates platelet aggregation by blocking the antithrombotic activity of nitric oxide/cGMP signaling. *Blood* 2008;111:613–623. [PubMed: 17890448]
45. Bouguermouh S, Van VQ, Martel J, Gautier P, Rubio M, Sarfati M. CD47 expression on T cell is a self-control negative regulator of type 1 immune response. *J Immunol* 2008;180:8073–8082. [PubMed: 18523271]
46. Piccio L, Vermi W, Boles KS, et al. Adhesion of human T cells to antigen-presenting cells through SIRPbeta2-CD47 interaction costimulates T-cell proliferation. *Blood* 2005;105:2421–2427. [PubMed: 15383453]
47. Van VQ, Lesage S, Bouguermouh S, et al. Expression of the self-marker CD47 on dendritic cells governs their trafficking to secondary lymphoid organs. *EMBO J* 2006;25:5560–5568. [PubMed: 17093498]
48. Li Z, Calzada MJ, Sipes JM, et al. Interactions of thrombospondins with alpha4beta1 integrin and CD47 differentially modulate T cell behavior. *J Cell Biol* 2002;157:509–519. [PubMed: 11980922]
49. Lindberg FP, Bullard DC, Caver TE, Gresham HD, Beaudet AL, Brown EJ. Decreased resistance to bacterial infection and granulocyte defects in IAP-deficient mice. *Science* 1996;274:795–798. [PubMed: 8864123]
50. Barazi HO, Li Z, Cashel JA, et al. Regulation of integrin function by CD47 ligands. Differential effects on alpha v beta 3 and alpha 4 beta 1 integrin-mediated adhesion. *J Biol Chem* 2002;277:42859–42866. [PubMed: 12218055]
51. Mateo V, Lagneaux L, Bron D, et al. CD47 ligation induces caspase-independent cell death in chronic lymphocytic leukemia. *Nat Med* 1999;5:1277–1284. [PubMed: 10545994]
52. Mateo V, Brown EJ, Biron G, et al. Mechanisms of CD47-induced caspase-independent cell death in normal and leukemic cells: link between phosphatidylserine exposure and cytoskeleton organization. *Blood* 2002;100:2882–2890. [PubMed: 12351399]
53. Roue G, Bitton N, Yuste VJ, et al. Mitochondrial dysfunction in CD47-mediated caspase-independent cell death: ROS production in the absence of cytochrome c and AIF release. *Biochimie* 2003;85:741–746. [PubMed: 14585540]
54. Saumet A, Slimane MB, Lanotte M, Lawler J, Dubernard V. Type 3 repeat/C-terminal domain of thrombospondin-1 triggers caspase-independent cell death through CD47/alphavbeta3 in promyelocytic leukemia NB4 cells. *Blood* 2005;106:658–667. [PubMed: 15784731]
55. Manna PP, Frazier WA. CD47 mediates killing of breast tumor cells via Gi-dependent inhibition of protein kinase A. *Cancer Res* 2004;64:1026–1036. [PubMed: 14871834]

56. Manna PP, Dimitry J, Oldenborg PA, Frazier WA. CD47 augments Fas/CD95-mediated apoptosis. *J Biol Chem* 2005;280:29637–29644. [PubMed: 15917238]
57. Lamy L, Foussat A, Brown EJ, Bornstein P, Ticchioni M, Bernard A. Interactions between CD47 and thrombospondin reduce inflammation. *J Immunol* 2007;178:5930–5939. [PubMed: 17442977]
58. Pettersen RD, Hestdal K, Olafsen MK, Lie SO, Lindberg FP. CD47 signals T cell death. *J Immunol* 1999;162:7031–7040. [PubMed: 10358145]
59. Kikuchi Y, Uno S, Kinoshita Y, et al. Apoptosis inducing bivalent single-chain antibody fragments against CD47 showed antitumor potency for multiple myeloma. *Leuk Res* 2005;29:445–450. [PubMed: 15725479]
60. Uno S, Kinoshita Y, Azuma Y, et al. Antitumor activity of a monoclonal antibody against CD47 in xenograft models of human leukemia. *Oncol Rep* 2007;17:1189–1194. [PubMed: 17390064]
61. Rath GM, Schneider C, Dedieu S, et al. Thrombospondin-1 C-terminal-derived peptide protects thyroid cells from ceramide-induced apoptosis through the adenylyl cyclase pathway. *Int J Biochem Cell Biol* 2006;38:2219–2228. [PubMed: 16971166]

Figure 1. Tissue protective effects of CD47 blockade treatment given post I/R injury

Myocutaneous rat island flaps measuring 2×6 cm were created on sex- and age-matched F344/NCr rats weighing between 250 and 300g. Deep inferior epigastric vessels (DIEV) were isolated and then clamped for 45 minutes. Five or 30 minutes post-reperfusion, flaps were treated with either an IgG₁ isotype control antibody or rat-specific CD47 antibody ($10 \mu\text{g}$ in 1 ml of sterile PBS) by local subcutaneous injection within the full area of the flap.

Postoperatively 72 hours, rats were re-anesthetized, images were obtained, and the area of flap necrosis was determined. Representative images are presented (A). Percentage of flap necrosis was determined as previously described²⁰ and results represent the mean $\pm$ SD of 5 rats in group of treatment 5 minutes post-reperfusion (B) or treatment 30 minutes post-reperfusion

(C). Sham surgeries were performed as previously described and results represent the mean $\pm$ SD of 5 rats

Figure 2. cGMP and levels are increased in flap vessels treated with CD47 antibody

DIEV were harvested 72 hours post flap creation and treatment with either IgG₁ isotype control antibody or CD47 antibody 30 minutes post reperfusion and compared to DIEV subjected only to flap creation without treatment. Levels of cGMP were analyzed by immunoassay and were increased in those DIEV treated with CD47 antibody (* $p < 0.01$). The results of duplicate samples are presented as the mean $\pm$ SD for 5 rats in each group.

Figure 3. Circulating levels of serum IFN- γ are greatly decreased from baseline following CD47 suppression given post I/R injury

Rat serum was obtained intra-abdominally from the inferior vena cava 24 hours post-reperfusion immediately prior to euthanasia. Serum levels of IFN- γ from rats given either an IgG₁ isotype or CD47 antibody 30 minutes post reperfusion were determined by immunoassay and compared to normal levels of rat IFN- γ . Results represent percentage increase or decrease from baseline IFN- γ levels and are the mean $\pm$ SD of 5 rats in each group

Figure 4. CD47 blockade reduces lipid peroxidation in flap tissue post I/R injury

Levels of malondialdehyde (MDA), which is a stable product of lipid peroxidation, were determined from tissue samples taken from the distal area of each flap. Flaps were subjected to either an IgG₁ isotype control antibody or CD47 antibody 30 minutes post reperfusion, and flap tissue from each group was harvested at 24 or 72 hours after antibody delivery. Tissue samples were also taken from the same area in animals not subjected to I/R injury and used as a normal control. MDA levels were much higher in the IgG₁ isotype control antibody when compared to normal tissue (* $p < 0.01$). However, levels of MDA were greatly decreased after treatment with CD47 antibody 72 hours post I/R (# $p < 0.001$). MDA levels represent the mean $\pm$ SD of 5 rats in each treatment group.

Figure 5. Histopathological evaluation of rat flaps show reduction of inflammatory markers after treatment with CD47 antibody

After 45 minutes of flap ischemia and 30 minutes of reperfusion prior to treatment with either an IgG₁ isotype control antibody or CD47 antibody, age- and sex-matched rats were killed 72 hours post-operatively, and their flap tissue was excised, processed, and stained with hematoxylin and eosin (A; original magnification 4X, inset 20X). Images are representative of tissue flaps from 5 animals of each treatment group. Leukocyte cell infiltrate was determined in 10 high-power fields from each rat tissue section. Flap tissue subjected to the same conditions but harvested 24 hours post-operatively was examined by immunohistochemistry for the presence of elastase, a neutrophil activation marker. Using a rat-specific monoclonal antibody against elastase, staining was performed on both the IgG₁ isotype control antibody or CD47 antibody treated flaps (B; original magnification 10X, inset 20X). Images are representative of tissue flaps from 5 animals of each treatment group.