REDOX MODULATION PROTECTS FROM ANTIGEN-INDEPENDENT AND ANTIGEN-DEPENDENT INJURY IN ISLET TRANSPLANTATION

by

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The preservation of reactive oxygen species (ROS) throughout evolution is evidence that ROS are critical components in immunity. These constituents ignite a multitude of redox-dependent pathways triggering pro-inflammatory cytokine production, dendritic cell maturation, and subsequent T cell activation in response to pathogen, autoimmune, and alloimmune insults. The aim of this thesis is to test the ability of redox modulation to suppress aberrant immune responses in islet transplantation. Presently, islet transplantation is only used in desperate cases of glucose dysregulation due to the high failure rate of the procedure, which forces the majority of recipients to resume exogenous insulin within a year of islet transplantation. Hurdles in islet transplantation include hypoxia during islet isolation and ischemia-reperfusion injury upon transplantation. These insults result in primary non-function of islets, while harsh immunosuppressive agents yield islet toxicity and a multitude of complications for the recipient. We tested a catalytic antioxidant (CA), FBC-007, in islet transplantation based on previous work demonstrating that CA impairs innate-immune ROS and pro-inflammatory cytokine production by inhibiting NFκB-DNA binding, hinders CD4 T cell activation, and prevents the transfer of diabetes into young NOD.*scid* mice.

First, the effects of redox modulation on CD8 T cell effector function in allogeneic and transgenic responses were examined *in vitro*. Human islets were also used *in vitro* and murine experiments were performed *in vivo* to test the ability of CA to protect from streptozotocin-induced islet cell death. Additionally, murine islets were incubated with CA in *in vivo* models of

ischemia-reperfusion injury (antigen-independent) or allogeneic (antigen-dependent) transplantation and separately, CA was used as a systemic therapy for allograft recipients. Further experiments were performed to elucidate *in vivo* protective mechanisms of CA-treatment. An additional approach of interest is the induction of antigen-specific hyporesponsiveness to replace nonspecific immunosuppression. A negative vaccination strategy delivering apoptotic donor alloantigen in a non-inflammatory adjuvant prior to allograft transplantation, was also tested. Collectively, this work demonstrates 1) CA is a non-toxic, islet-sparing, cytoprotective, and immunomodulatory agent capable of promoting islet-function, 2) CA impairs the alloimmune response to induce antigen-specific hyporesponsiveness, and 3) the negative vaccination protocol achieves long-term allograft tolerance.

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First I must thank the people to whom I owe everything, my parents, Barbara and Gene Milton. Their love, support, devotion and belief in me have been amazing and inspiring. I was/am exceedingly fortunate to be raised by such intelligent, caring, witty, wise, loving, fun and generous people. With each passing day I appreciate them more and more as parents and as friends.

My husband, Peter, literally is my "rock" (Πετροσ/Petros) and my best friend. He has been my hero throughout the ups and downs of graduate school and life; always a comfort who rescues my spirits and propels me forward. His love, support, friendship, silliness and caring ways make me feel like the luckiest girl in the world. Thank you for always being there for me, for making me laugh, for letting me dream big, and for cleaning up after I cook dinner and destroy the kitchen.

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collaborator. It is my hope that my thesis work will help move the approach of redox modulation to impair aberrant immune responses from his lab into the clinic someday soon.

I was lucky enough to have a second mentor in the lab, Dr. Hubert Tse. I aspire to achieve his level of science. He was/is an excellent teacher, who is credited for teaching me every technique I know. It helps that he is an all-knowing lab ninja, who is seemingly an expert in every technique that exists and his protocols are superior, always yielding the best results. I thank Hubert for his willingness to help with science questions, revising manuscripts, computer disasters and because he always has an ear to listen to any problem I might be having in or out of the lab. Moreover, Hubert is a friend, who can always put a smile on my face and I wish him much good fortune as he starts his own lab at the University of Alabama.

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Last, but certainly not least, a very special thank you to Dr. Massimo Trucco for his support, guidance, and help throughout my graduate career and beyond, and to the members of his Diabetes Institute at the Rangos Research Center at Children's Hospital, where people are very willing to take time away from their own science to foster another's.

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1.0 CHAPTER 1: INTRODUCTION

1.1 TYPE 1 DIABETES

Type 1 diabetes (T1D) is an autoimmune disease that is mediated by autoreactive T cells working in concert with macrophages to recruit inflammatory cells to islets to target and destroy insulin-producing β-cells of the pancreas in genetically predisposed individuals (Figure 1). According to the World Health Organization approximately 18 million people suffer from T1D worldwide. Though T1D is known as juvenile diabetes, the disease has been diagnosed in patients ranging in ages from babies under 1 year old to middle-aged adults. T1D can be difficult to diagnose as untreated diabetics display symptoms similar to the flu including weight loss and fatigue. In the absence of endogenously produced insulin, type 1 diabetics require administration of exogenous insulin multiple times a day to avoid hyperglycemia and maintain blood glucose levels within a normal range. Hyperglycemia has adverse systemic effects resulting in chronic conditions of the nervous and circulatory systems that can lead to blindness, amputation of limbs, and an increased prevalence of heart attacks. Conversely, hypoglycemia is more acutely dangerous in that low blood sugar can lead to seizures and a coma, which is especially deadly during sleep when hallmark signs of hypoglycemia-like confusion and dizziness go undetected.

Autoreactive antibodies have been characterized in patients with type 1 diabetes, though these markers are not steadfast and thus, not accurate enough to positively identify people who

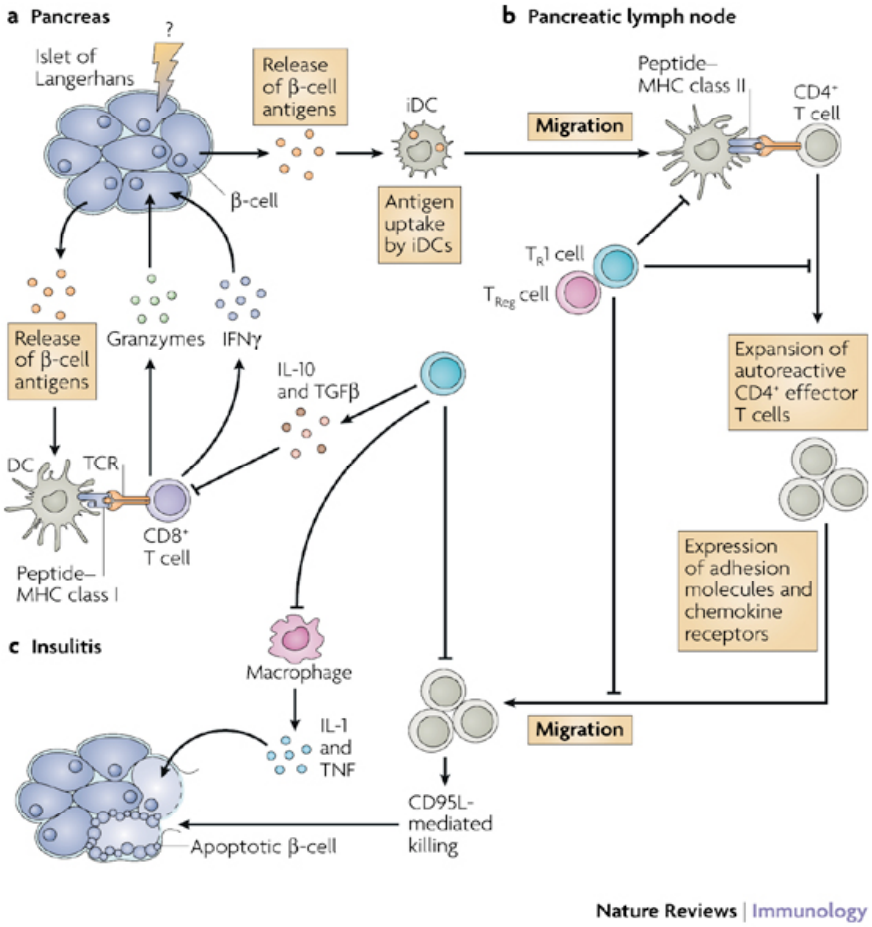

Fig. 1. Pathophysiology of type 1 diabetes. a | First, under still undefined pathogenic conditions, modified islet -cell antigens are released and presented by MHC class I molecules. These previously 'cryptic' antigens are presented by tissue-resident antigen-presenting cells (APCs) and recognized by $CD8^+$ T cells that cause damage to MHC-class-I-expressing cells either through the release of cytotoxic cytokines (such as IFN-γ) or through the perforin–granzyme pathway. **b** | The released islet -cell components are taken up by immature dendritic cells (iDCs) in the pancreatic islets and transported to the draining pancreatic lymph nodes, where the antigens are processed and presented to $CD4^+$ T cells. Lymph-node priming is thought to be the second crucial step leading to expansion of low frequency circulating autoreactive T cells. After clonal expansion, $CD4^+$ effector T cells express adhesion molecules, such as intercellular adhesion molecule 1 (ICAM1) and lymphocyte function-associated antigen 1 (LFA1), and chemokine receptors, such as CC-chemokine receptor 4 (CCR4), CCR5 and CXC-chemokine receptor 3. This allows the effector cells to home to the pancreatic islets, tracing antigen gradients and chemokines induced by the early $CD8^+$ T-cell-mediated inflammatory response. **c** | Once in the pancreas, the activated $CD4^+$ T cells recruit and activate inflammatory cells, causing insulitis. The effector phase of islet -cell destruction is mediated by cytokines (mainly IL-1 and TNF through the induction of pro-apoptotic signalling selectively in islet β-cells and/or by inducing the expression of CD95 by islet -cells, which allows direct killing by CD95 ligand (CD95L)-expressing effector T cells. There is also evidence that the production of free radicals is involved in the pathogenic events leading to islet -cell destruction (not shown). Regulatory T cells can intervene at different stages to control type 1 diabetes. As in graft-versus-host disease, it is likely that regulatory T cells are first activated in the pancreatic lymph nodes. Activated naturally occurring forkhead box P3 $(FOXP3)^+CD4^+CD25^+$ regulatory T (T_{Reg}) cells and T regulatory type 1 (T_R1) cells, through distinct regulatory mechanisms, block the activation and expansion of effector T cells either directly or indirectly through APCs. Expression of adhesion molecules and chemokine receptors by effector T cells is also suppressed by regulatory T cells, with consequent reduced effector T-cell migration to the target organ. The aggressiveness of insulitis is also directly inhibited in the pancreas by regulatory T cells. T_R1 cells, through IL-10 and (TGF-β) production, can inhibit the onset of disease and reduce inflammation.

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will or will not progress to clinical disease. Upon confirmed diagnosis of hyperglycemia, approximately 70% of β-cells have been destroyed (6). Since there is an absence of timely disease predictors or safe treatments for children, when disease onset usually occurs, prophylactic therapy to discourage islet destruction prior to T1D onset is not yet justified. HLA Class II alleles (DRB1*03, DRB1*04, DRB1*03/04) are paramount for T1D development (9), though environmental factors including exposure to certain viruses and bacteria reportedly promote the progression of diabetes onset as evidenced by cases where only one identical twin succumbs to T1D (10). Currently there is no cure for T1D, though innovations like the insulin pump, improved methods of monitoring blood glucose levels, better nutritional knowledge, and the availability of a variety of foods with a low glycemic index have improved survival rates and quality of life for T1D patients. Presently, allogeneic islet transplantation is the only therapy able to achieve any time period of exogenous insulin independence for type 1 diabetics.

1.2 ISLET TRANSPLANTATION

1.2.1 Islet transplantation today

Islet transplantation is a potential cure for T1D patients, however only patients with extreme glucose dysregulation are eligible for the procedure (11). Even though islet transplantation outcomes have improved with the development of the Edmonton protocol, recipients rarely maintain insulin-independence for more than 2 years (12, 13). Poor transplant outcomes can be attributed to the multitude of antigen-independent barriers transplanted islets must overcome prior to the initiation of the antigen-dependent alloimmune response. These

antigen-independent injuries include devascularization, hypoxia, damage during isolation due to enzymatic digestion, donor age and cause of death, and cold storage time. All of these factors contribute to some recipients requiring islets from up to 3 pancreata to achieve any period of normoglycemia (14). Beyond the direct injury to islets in transplantation there is the limited supply of islets available for transplantation. To refine islet transplantation, Ricordi developed a superior method of islet isolation. The number of potential diabetic recipients far exceeds the number of donor pancreata slated for islet transplantation (14-16) as younger, healthier donors are typically reserved for whole organ transplantation, which has a waiting list of its own. The disparity in organ quality between whole pancreas versus islet transplantation contributes to the requirement of multiple islet infusions to normalize a recipient and increased transplant success using the whole organ over islet cell transplants. These factors, coupled with the intrinsically decreased antioxidant defenses of β-cells (17-22), increases islet vulnerability to inflammatory and free radical insults during islet isolation prior to encountering post-transplant complexities (15) of allograft rejection as a result of nonspecific immunosuppressive agents.

Allograft rejection necessitates the use of potent anti-rejection drugs, though some commonly used immunosuppressive drugs are toxic to islets and yield undesirable complications including chronic infection, organ damage, and malignancy (1, 3, 11, 13, 15, 23). Since human islets are delivered into the portal vein en route to the liver, the instant blood-mediated immune response (BMIR) leading to coagulation, complement activation and islet damage must also be overcome. Shapiro, et al at Edmonton outlined criteria for the islet transplant procedure that highlights a corticosteroid-free regimen of treatment for the transplant recipient that is less toxic to β-cells (13). This collective protocol is the basis for clinical islet isolations and infusions worldwide (Figure 2).

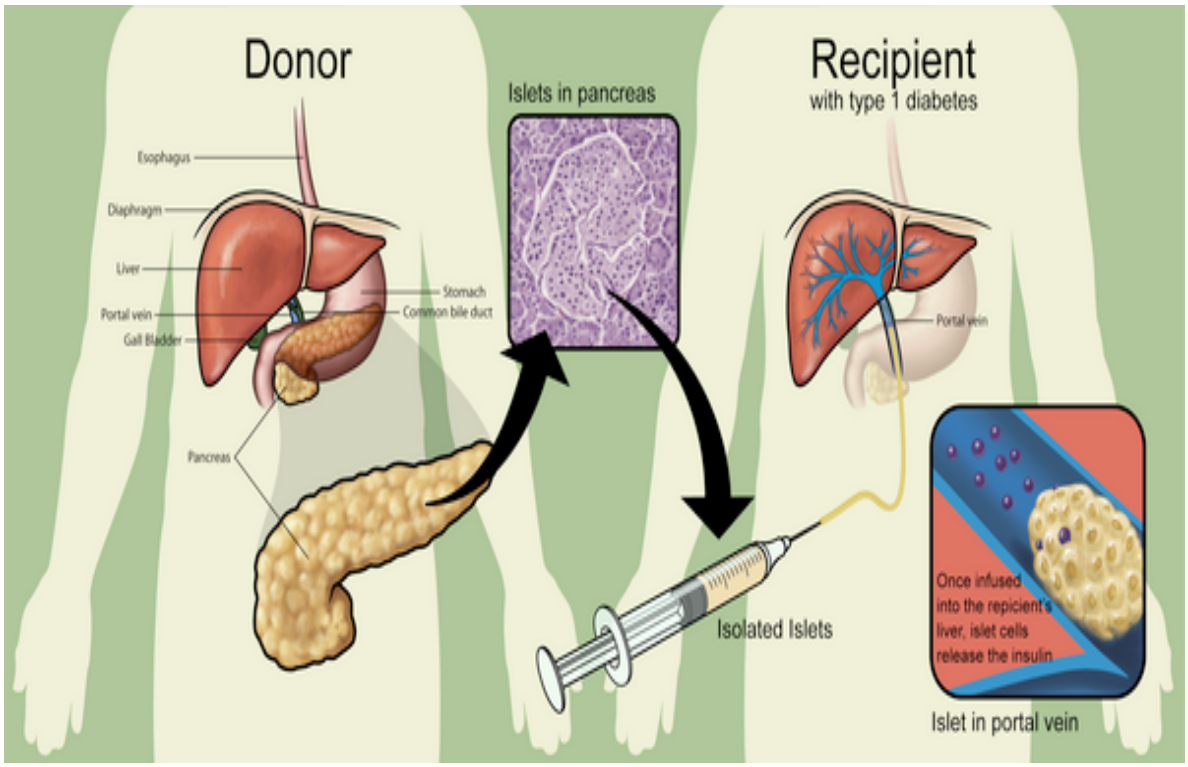

Fig. 2. Central concepts underlying islet transplantation. The main idea of islet transplantation is to process the organ donor's pancreas so as to remove the 95% of the gland responsible for its exocrine functions (secretion of digestive enzymes) and isolate the 5% of the gland responsible for the endocrine hormone secretion— the so-called pancreatic islets. Once isolated, the medical team can infuse the insulin-producing islets through a thin tube, placed in the main vein that transports blood from the intestines to the liver. Once infused, the islets are transported by the bloodstream into the liver, where they lodge, take up residence, and begin making the right amount of insulin to regulate the blood sugar. Illustration: Giovanni Maki
From (3). The Public Library of Science (PLoS) applies the Creative Commons Attribution License (CCAL) to all works we publish. No permission is required from the authors or the publishers.

1.2.2 Clinical islet transplantation: the Edmonton protocol

According to the Edmonton protocol, donor and recipient are not HLA-matched, but are matched for blood type and cross-matched for lymphocytotoxic antibodies. To avoid further complications subtle, but critical, media additives are also used, including the use of human albumin instead of media containing xenoproteins. Initially the pancreas is removed from a brain dead donor while the recipient begins the Edmonton regimen of corticosteroid-free immunosuppression prior to transplant. Next, the pancreas is infused with cold collagenase via the bile duct and then placed into the Ricordi chamber for mechanical dissociation, to separate the islets from the exocrine tissue. Once harvested, the pancreata are maintained in cold University of Wisconsin solution. Islets are purified using Ficoll-diatrizoic acid in an apherisis system and upon purification islets are transplanted without culturing (12). Islets are perfused into the portal vein to facilitate liver engraftment in medium containing 500 units of heparin and 20% human albumin at 4000 islet equivalents (IEQ) per kilogram of the recipient's body weight in the original report from Edmonton (12), though Barker, Naji, Markmann and others believe that more IEQ (≥ 9,000 IEQ/kg) are necessary to achieve increased periods of insulin-independence (11, 24). Any additional success accompanying an increase in IEQ/kg is most likely attributed to increased islet survival after islet loss from hypoxia, isolation-induced stress and post-transplant ischemia-reperfusion injury. Due to perfusion into the portal vein, BMIR is treated with heparin and complement inhibitors, but the antigen-specific T cell-mediated alloimmune response is not easily overcome requiring potent immunosuppressive-agents to inhibit allograft rejection.

1.2.3 Allograft rejection

Damage incurred during the islet isolation process is a prominent reason why whole pancreas transplantation is more successful than islet transplantation, as a whole organ is manipulated less and retains vascularization within the organ (25). However, in a cell or whole organ transplant, removing the pancreas from the donor initiates hypoxia and activation of antigen-independent innate immune ligands through TLR activation. Innate immune activation results in the release of reactive oxygen species and pro-inflammatory cytokines which facilitates an adaptive immune response (2, 5, 7, 26-29). Activation of innate immune ligands induces APC maturation, bridging the innate and adaptive immune systems (2, 5, 7, 26-29). Taken together, antigen-independent inflammatory damage incurred during islet isolation and ischemia-reperfusion injury exacerbates antigen-dependent allograft rejection by triggering inflammatory signals required for adaptive immune function.

Professional antigen presenting cells (APC), such as DCs, phagocytose allopeptide shed from the graft and migrate to the draining lymph nodes to activate naïve allospecific T cells through (1) peptide:MHC:TCR engagement and (2) co-stimulatory molecule interactions between the T cell and the activated DC, which matures as a result of (3) the synthesis of pro-inflammatory innate immune ligands (26-32). The third signal is a culmination of innate immune-derived pro-inflammatory cytokines and ROS and is a precursory requirement with respect to the two other signals for both DC maturation and subsequent T cell activation (Figures 3 and 4).

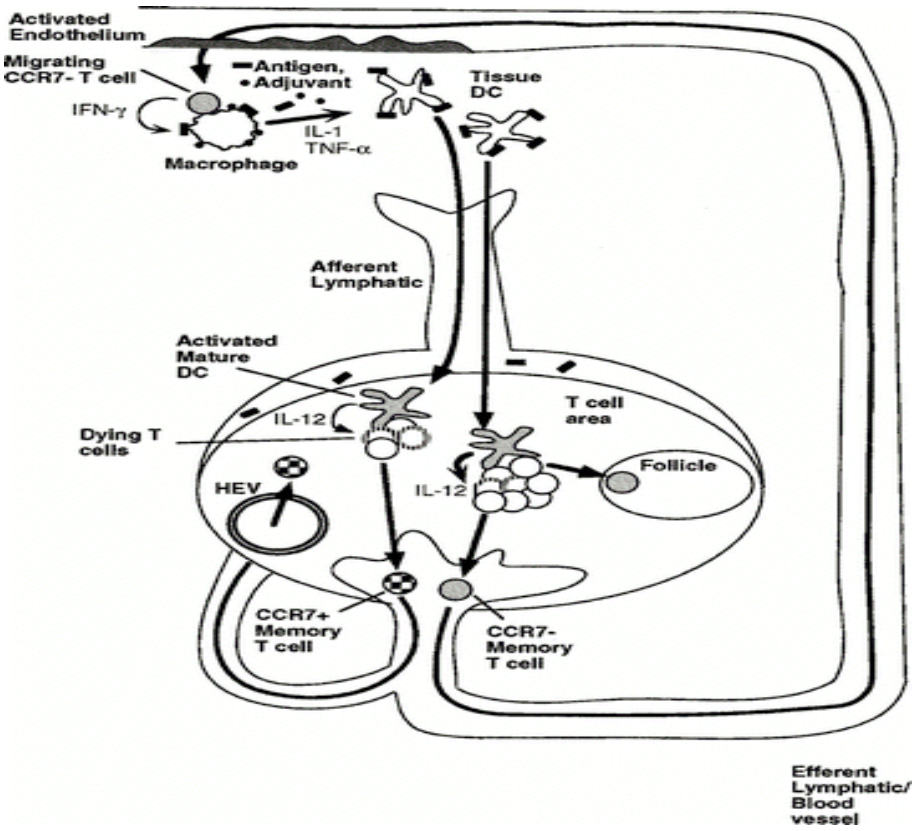

Fig. 3. Diagrammatic representation of a productive primary CD4 T cell response to antigen in the presence of adjuvant-induced inflammation.

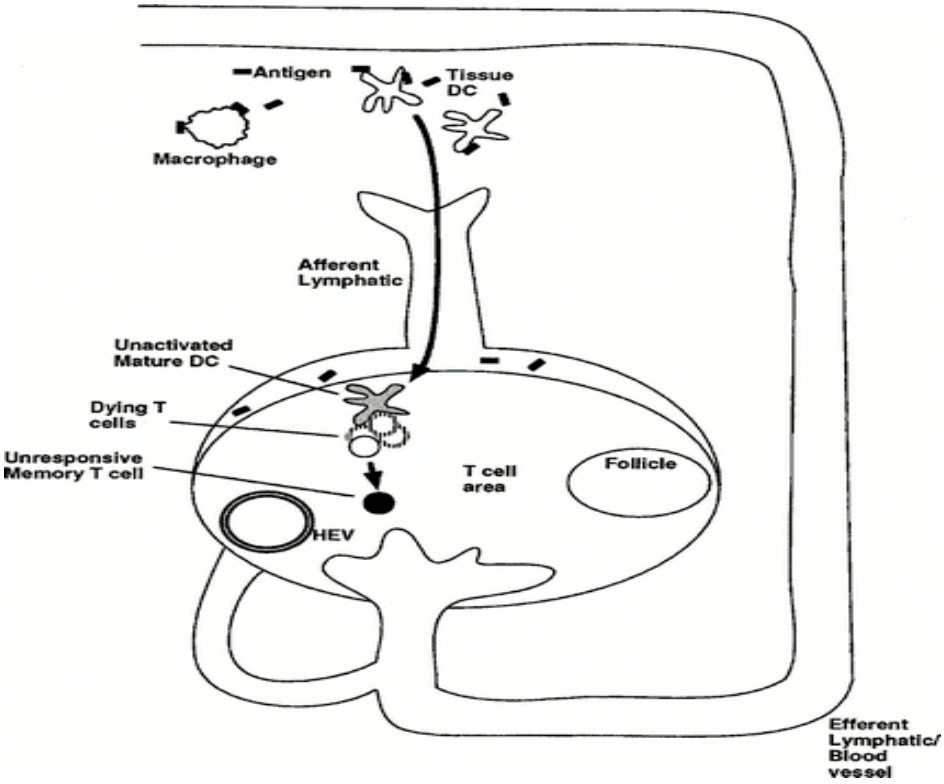

Fig. 4. Diagrammatic representation of an unproductive CD4 T cell response to antigen in the absence of inflammation.

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Allograft rejection occurs through two distinct pathways, the direct and indirect pathways (Figure 5B). In the direct pathway, mature donor DC (passenger leukocytes) migrate to lymph nodes to present their allospecific MHC to allospecific recipient T cells. The indirect pathway contributes to rejection when mature recipient DCs process and present alloantigen to allospecific recipient T cells. Through either pathway, the end result is activated allospecific T cells which engage in acute allograft rejection by acquiring effector function and homing to the site of the allograft to destroy the alloantigen-expressing donor islets (5). The critical difference between immune settings of self-tolerance or allograft rejection correlates to a steady state or inflammatory immune environment, respectively, which dictates the activation/maturation state of DCs (Figure 5A). Even if acute rejection is overcome, the allograft is a constant source of alloantigen that will repeat the activation process to prime allospecific T cells resulting in chronic rejection.

Within these processes, CD8 T cells directly participate in islet death via target cell lysis. CD4 T cells play a role in allograft rejection indirectly by further activating macrophages and B cells and by providing T cell help to CD8 T cells by up-regulating co-stimulatory molecules through APC interactions and the synthesis of T_H1 and T_H17 pro-inflammatory cytokines, like IFN-γ and IL-17. It is also possible that CD4 T cells interact directly with CD8 T cells to enhance the alloimmune response. At the site of the graft, CD4 T cells initiate pro-inflammatory cytokine production by macrophages (reactive oxygen species, TNF-α and IL-1β) to induce direct and indirect islet cytotoxicity, and subsequently, islet dysfunction and death. Most notably, the combination of IFN-γ from CD4 T cells and IL-1β and TNF-α from macrophages prompts β-cell apoptosis, but this combination of cytokines also promotes CD8 cytotoxic lymphocyte (CTL) migration to the allograft (6). Additionally, CD4 T cells activate B cells,

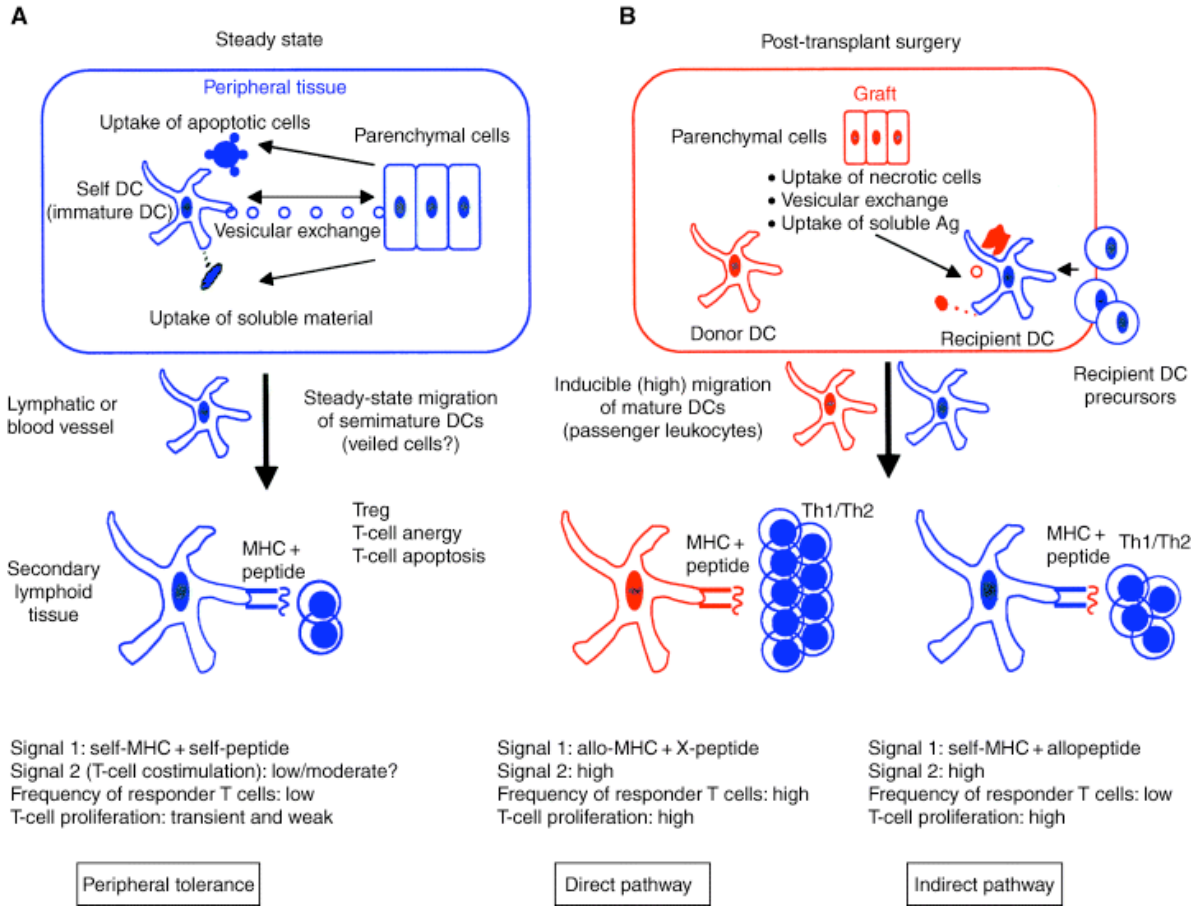

Fig. 5. Role of dendritic cells (DCs) in peripheral tolerance and graft rejection. (A) During steady-state conditions (absence of pro-inflammatory or danger signals), peripheral tissue-resident DCs capture self-antigens from neighboring parenchymal cells via uptake of apoptotic cells, vesicular exchange with living cells, or endocytosis of soluble molecules. DCs mobilize [likely as semi-mature antigen-presenting cells (APCs)] via lymphatic or blood vessels from the periphery to T-cell areas of secondary lymphoid organs. Once there, DCs present self-antigens to autoreactive T cells that escaped thymic selection. Under physiological conditions, DCs in T-cell areas of spleen or lymph nodes express low levels of T-cell co-stimulatory molecules and induce a transient and weak proliferation of autoreactive T cells followed by T-cell deletion, anergy, and, probably, generation of Treg cells. (B) Following transplantation surgery, pro-inflammatory mediators released locally, ischemia–reperfusion injury, and the presence of necrotic cells trigger full maturation of donor DCs (donor tissues and cells are in red) and recipient DCs (recipient cells are in blue) that infiltrate the graft as part of the inflammatory reaction. Both donor- and recipient-derived DCs migrate out of the graft as passenger leukocytes. Once in the T-cell area of the secondary lymphoid organs, mature donor DCs present allo-major histocompatibility complex (MHC) molecules to alloreactive T cells (direct pathway) and mature recipient DCs present allopeptides loaded into self-MHC molecules to recipient T cells recognizing allopeptides in the context of self-MHC molecules (indirect pathway).
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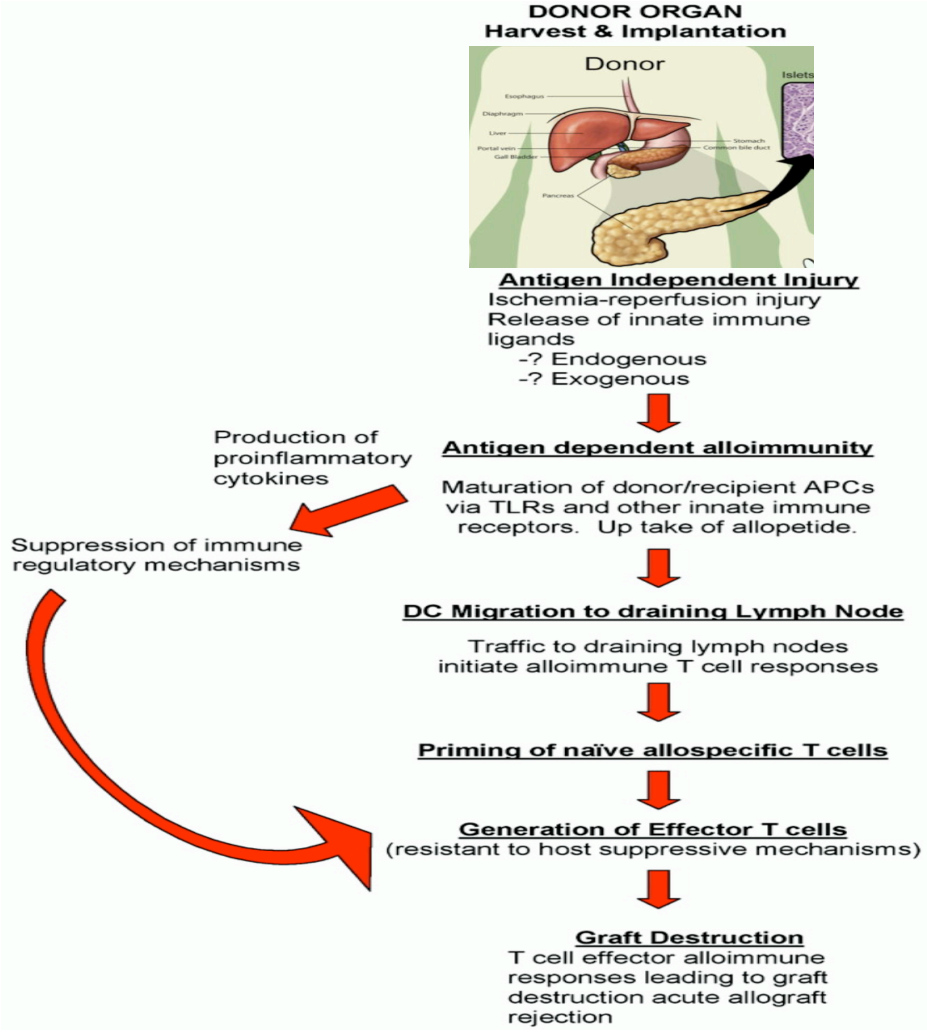

Fig. 6. Model for the role of innate TLR-dependent immunity in acute allograft rejection. Donor organ harvest and implantation results in ischemia-reperfusion injury that may be mediated by TLR signaling and release innate immune ligands. These ligands allow DCs (donor or recipient) to mature and migrate to the draining lymph nodes. The maturation of DCs leads to the production of proinflammatory cytokines that aid in naïve T cell priming and render the generated effector T cells resistant to host suppressive mechanisms. The armed effectors than initiate acute graft rejection.
Modified from (3, 7). Used with permission.

propagating further inflammation and islet death through antibody-mediated responses to the allogeneic islet graft. The concert of these mechanisms leads to destruction of the islet allograft in the absence of immunosuppression (Figure 6), though graft failure often occurs in spite of treatment with anti-rejection drugs.

1.2.4 Immunosuppressive anti-rejection drugs

Due to the alloimmune response, immunosuppression of the recipient is required to protect from graft destruction. The T cell precursor frequency for alloantigen (<1/10,000) is immensely higher compared to the precursory frequency of T cells specific for a nominal antigen (1/200,000) (33-35). Therefore, immunosuppression must be very potent to control an allogeneic response after transplantation. Current anti-rejection drugs used to restrain the alloimmune response, like sirolimus and tacrolimus (Figure 7), are nonspecific and suppress immune function globally. Additionally, the majority of these immunosuppressive agents target the T cell and the adaptive immune response, though it is well-known that the activation state of the APC shapes the adaptive immune response (2, 5, 7, 29, 36). The depressed state of the immune system under immunosuppression often leads to complications including cancer, chronic infection and organ damage. More specifically, commonly used anti-rejection drugs induce β-cell toxicity, adding another hurdle when striving to achieve graft survival.

According to the Edmonton protocol, the recipient is treated with glucocorticoid-free immuosuppression prior to islet transplantation including a loading dose of sirolimus, low-dose tacrolimus (FK506), and daclizumab (anti-CD25). Prophylactic antibiotics (vancomycin and imipenem) are administered prior to transplant and so are the antioxidants, vitamin E, vitamin

B_6, and vitamin A, in attempt to impair free radical production (37). Post-transplant maintenance concentrations of sirolimus and tacrolimus are generally maintained and 4 more doses of daclizumab, inhaled pentamidine once a month to avert pneumocystis, and ganciclovir for 14 weeks to avert cytomegalovirus and lymphoproliferative disease are also administered. Initially, this protocol sustains allograft function as demonstrated by insulin-independence and euglycemia (12). However, in a five-year follow-up study of islet transplant recipients under the Edmonton group, only 5 recipients were normoglycemic after one transplant, 52 patients required two transplants, and 11 patients required three transplants to achieve insulin independence for any length of time. The average duration of insulin independence for all transplant recipients was only 15 months.

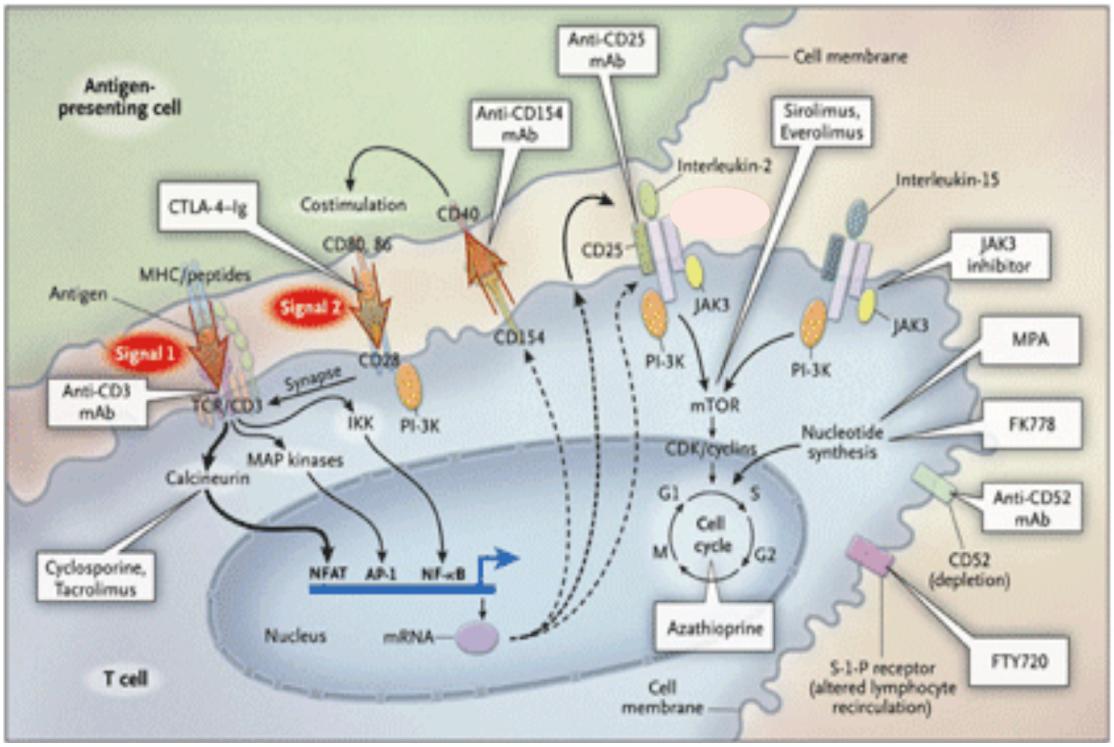

Fig. 7. Individual immunosuppressive drugs and sites of action
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The authors concluded that there is a need for improved islet engraftment and function in addition to the need for novel immunosuppressive drugs uncoupled to toxicity and other harmful side effects like hypertension, fungal pneumonia, and malignancy. These side effects can compromise the transplant and lead to transplant rejection, namely when the side effect requires immunosuppressive regimens to be reduced or eliminated. In the current state of islet transplantation, which is inseparably coupled to complications, an islet transplant may be more of a detriment than of benefit, especially for patients with poorly controlled glucose levels, but preserved kidney function (3). The development of superior immunosuppressives and new protocols to preserve islet viability and function during isolation and engraftment could greatly benefit recipients of islet allografts by promoting long-term insulin-independence and limiting transplant-associated complications.

1.2.5 Difficulties resulting from islet isolation

In transplantation, islets are increasingly exposed to free radical and inflammatory insults compared to whole pancreas transplantation. When a pancreas is excised from donor circulation for a whole organ transplant, vasculature is maintained, however, when a pancreas is excised for islet transplant, the organ endures further manipulation including removal of the organ's vasculature and pancreatic lymph, which promotes allograft tolerance (25). Both whole pancreas and islet transplants incur hypoxic and ischemia-reperfusion injury, though islets must also withstand the process of isolation. Islet isolation is a conundrum as it equates to a less-invasive procedure compared to whole pancreas transplantation, though the islet purification process is harmful to β-cell function and viability. Islet isolation entails additional hypoxic time and

subsequent inflammatory damage fueled by reactive oxygen species and pro-inflammatory cytokines generated by islets, acinar tissue, and resident leukocytes antigen-independent innate-mediated injury. Since antigen-independent, nonspecific inflammatory damage results in primary β-cell non-function and death, cytoprotection of islets is needed during islet isolation and transplantation. The benefits of cytoprotection are repeatedly touted in the literature, but without notable recourse that is practical for use in clinical islet transplantation (2, 15, 38-40).

1.3 REDOX MODULATION BY THE CATALYTIC ANTIOXIDANT, FBC-007

Controlling the innate immune response in the context of allograft transplantation is of increasing interest as more investigators appreciate the role of a mature or immature APC/DC to impart immunity or tolerance, respectively (7, 41-43). There is an overwhelming request for cytoprotective agents for islet allografts, which must endure islet isolation and nonspecific inflammatory cascades prior to post-transplant ischemia-reperfusion injury and the onslaught of the alloimmune response (2, 15, 38-40, 44). These compounding factors necessitate an approach in islet transplantation that is cytoprotective, immunomodulatory, and antigen-specific to achieve long-term, consistent success in islet transplantation.

Redox modulation is capable of addressing several unresolved issues in allograft transplantation since it targets both the innate and adaptive immune systems. The agent used as a molecular probe of redox modulation is the catalytic antioxidant (CA) FBC-007, which is comprised of a metaloporphyrin ring with a manganese ion in the center (Figure 8). CA can scavenge and dissipate a broad range of ROS (4, 45-48) and displays oxido-reductase activity in

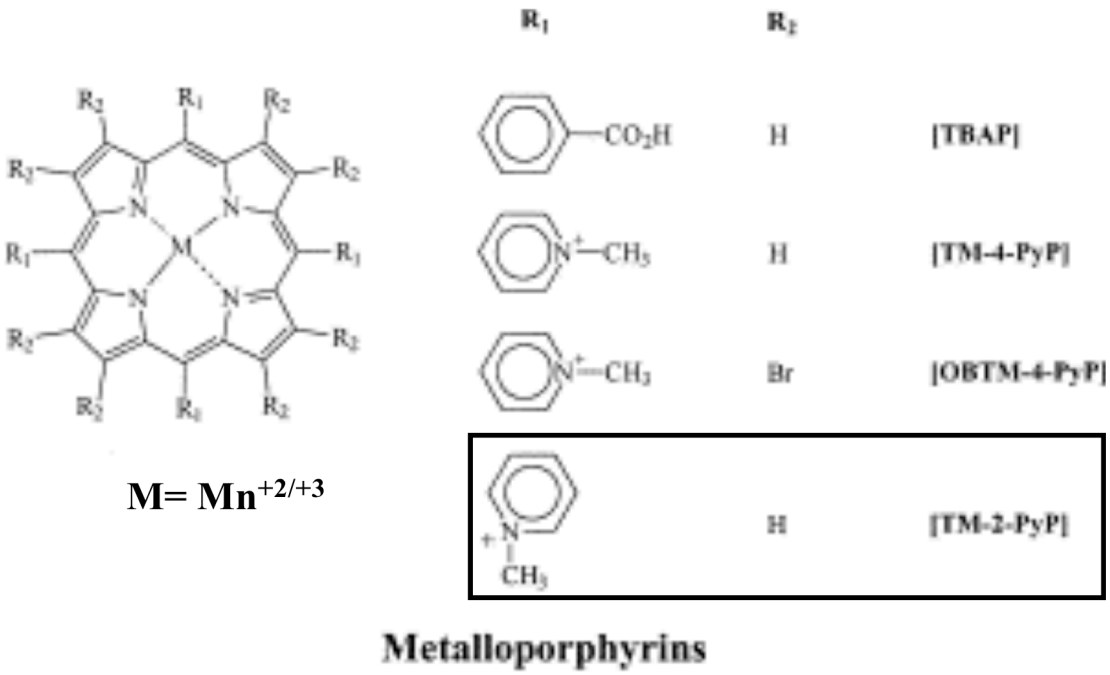


Fig. 8. Structure of FBC-007/CA also known as (manganese(II) tetrakis (*N*-ethylpyridium-2-yl) porphyrin). R groups for FBC-007/CA shown in the black box. *From (4). Used with permission.*

the ability to oxidize and reduce redox-sensitive proteins to exert immunomodulatory effects depending upon the redox environment.

Specifically, CA inhibits the redox-sensitive transcription factor, NF-κB, by oxidizing cysteine 62 of the NF-κB p50 DNA binding site subunit to prevent DNA binding and expression of NFκB-dependent gene transcription in the nucleus (48). Impairment of NF-κB activation is responsible, in part, for downstream effects of CA-treatment including the ability of CA to block the adoptive transfer of type 1 diabetes in young NOD.*scid* mice using a diabetogenic T cell clone (49), to suppress the production of ROS, TNF-α and IL-1β in LPS-stimulated

macrophages and DC (48), and to impair the production of TNF-α thereby disrupting the transition of T cell expansion to effector function (26). CA can be used as islet-directed (cytoprotection) or systemically (immunomodulatory) in small molecule form. Additionally, human islets treated with CA during islet isolation or in culture preserve islet function and viability (38, 39). The benefits of redox modulation using CA warrant use in allograft transplantation when considering the cytoprotective properties of CA and its ability to inhibit innate and adaptive immune effector function without inducing toxicity (Figure 9).

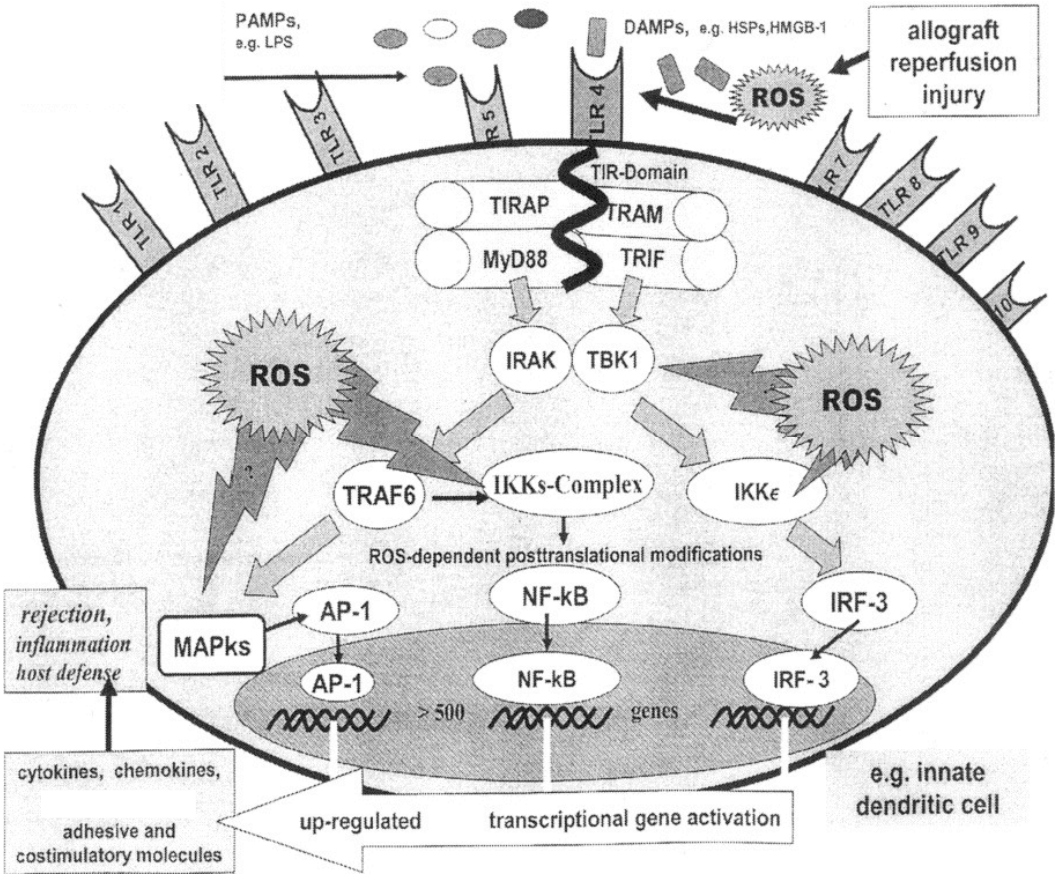


Fig. 9. Simplified schematic illustration of intracellular TLR-triggered/mediated signaling pathways resulting, via activation of transcription factors, in the expression of "innate" genes involved in inflammation, antiviral responses, and maturation of DCs with respect to allograft injury and rejection pathways.
From (2). Used with permission.

2.0 CHAPTER 2: REDOX MODULATION INHIBITS CD8 T CELL EFFECTOR FUNCTION

2.1 ABSTRACT

The evolutionary preservation of reactive oxygen species (ROS) in innate immunity underscores the important roles these constituents play in immune cell activity and as signaling intermediates. In an effort to exploit these pathways to achieve control of aberrant immune activation we demonstrate that modulation of redox status suppresses cell proliferation and production of IL-2, IFN-γ, TNF-α, and IL-17 in two robust CD8 T cell-dependent *in vitro* mouse models: (1) response to alloantigen in an mixed leukocyte reaction (MLR) and (2) CD8 T cell receptor transgenic OT-1 response to cognate peptide (SIINFEKL). To correlate these findings with (cytotoxic lymphocyte) CTL function we performed cytotoxicity assays and found that redox modulation diminishes the ability of alloantigen-specific and antigen-specific OT-1 CTLs to kill their corresponding antigen-expressing target cell. To further examine mechanisms of redox-mediated repression of CTL target cell lysis, we analyzed expression of the effector molecules IFN-γ, perforin, granzyme B, and the degranulation marker, CD107a (LAMP-1). In both models, redox modulation reduced expression of these effector components by at least 5-fold. These results demonstrate that redox modulation quells the CD8 T cell response to

alloantigen and the T cell receptor transgenic CD8 T cell response to its cognate antigen by inhibiting proliferation, pro-inflammatory cytokine synthesis, and CTL effector mechanisms.

2.2 INTRODUCTION

Reactive oxygen species (ROS) are one of the immune system's oldest yet most effective first lines of defense involved in the eradication and control of infectious agents. Throughout evolution, ROS generated during early events in immune system activation have remained an integral part of a complex immune response. In addition to their capacity to directly kill microbes, ROS are critical signaling intermediates linking the innate and adaptive immune systems by triggering the production of pro-inflammatory cytokines (TNF-α, IL-1β), termed third signal molecules, by macrophages and DCs (dendritic cells) of the innate immune system. There is growing interest in molecular pathways and signaling mediators linking the innate and adaptive arms of the immune response (50-52) as the innate immune-derived third signal, required for maturation of the adaptive immune response, is dependent on the redox-sensitive signaling pathways MAPK, AP-1 and NF-κB (27, 28, 48, 50-54).

The events of third signal synthesis leading to APC (antigen presenting cell) activation can occur through the binding of toll-like receptors to microbial pathogen associated molecular patterns (PAMPs) (50-52), the generation of pro-inflammatory cytokines (27, 28, 55, 56), or through natural adjuvants that are endogenous activators of APC (57). NF-κB activation of APCs results in third signal pro-inflammatory cytokine production and contributes to the up-regulation, maturation, and immunostimulatory capability of co-stimulatory molecules (CD40, CD80, CD86) on DC (42, 58-64). Accordingly, this pro-inflammatory third signal is required for

optimizing signal 1 (T cell receptor (TCR), peptide and major histocompatibility complex (MHC) engagement) and signal 2 (co-stimulatory molecule interaction) among APCs and naïve T cells. These events, together with IL-12 production by activated, mature DCs, are required to drive naïve T cells to effector function (29, 54, 65).

We have shown that modulating redox balance using a non-toxic, cell-permeable catalytic antioxidant probe FBC-007 (manganese (II) tetrakis (N-ethylpyridium-2-yl) porphyrin) (CA) led to suboptimal APC priming and inefficient activation of the diabetogenic CD4 T cell clone BDC2.5, thereby preventing the adoptive transfer of type 1 diabetes to young NOD.*scid* mice (49). The CA compound is comprised of a metal center that catalyzes the dismutation of O_2^-, mimicking the active metals in naturally occurring mammalian Cu, Zn, or Mn SODs (66-68), harnessing the ability to scavenge several oxidants including superoxide, hydrogen peroxide, peroxynitrite, and lipid peroxyl radicals (4, 26, 45-48, 66). Manganese metalloporphyrins (CA) display oxidoreductase activity and can oxidize redox-sensitive transcription factors such as NF-κB, AP-1, and HIF-1 without inducing toxicity. Accordingly, CA is able to avert endotoxic shock (69), excitotoxic neuronal cell death (70) and apoptosis (71), lipid peroxidation (4), peroxide-induced mitochondrial DNA damage (72), and significantly prolongs survival in phenotype lethal manganese superoxide dismutase knockout mice (26, 48, 73). Additionally, these compounds have been shown to reduce other inflammatory-mediated diseases by limiting oxidative stress in various *in vivo* rodent models including cancer (74), amyotrophic lateral sclerosis (75), and ischemia and reperfusion injury (76). Our mechanistic analysis of CA-mediated inhibition of APC function revealed that CA acts as an oxidoreductase, affecting the innate immune response of APCs by inhibiting NF-κB-DNA binding and the synthesis of the NF-κB-dependent pro-inflammatory cytokines, TNF-α and IL-1β, which are necessary for

immune cell maturation and for T cells to transition to effector function (27, 28, 48). Most recently, we observed that altering redox balance affects adaptive immune function as demonstrated by decreased antigen-specific CD4 T cell proliferation and type 1 polarizing cytokine synthesis (TNF-α and IFN-γ) as well as the induction of an antigen-specific hypo-responsive T cell population (26).

The focus of this study is to determine if redox modulation, which is known to inhibit APC and CD4 T cell function (26, 48), has a marked effect on CD8 T cell function in the presence or absence of CD4 T cell help. CD4 T cells indirectly augment CD8 T cell function by inducing increased APC immunostimulatory capability through signal 1 engagement. More specifically, increased expression of co-stimulatory molecules on APC, resulting from signal 1 engagement with CD4 T cells, generates a more potent APC which is able to stimulate naïve CD8 T cells to transition to effector function (77). Additional evidence demonstrates that CD4 T cells can directly interact with CD8 T cells through CD40L-CD40 interactions, respectively, to enhance CD8 T cell maturation (78). Our initial studies demonstrating that redox modulation affects CD4 T cell activation and effector function (26), combined with the knowledge that CD8 T cell maturation and effector function is linked to optimal CD4 T cell help, led to the hypothesis that redox modulation would have marked effects on CD8 T cell activation and effector function either directly or indirectly, through impaired CD4-derived T cell help.

To examine if redox modulation can affect CD8 T cell function we employed alloantigen-specific and transgenic *in vitro* mouse models representative of increased T cell precursor frequencies compared to that of a nominal antigen in the presence or absence of CD4 T cell help. For the first model we generated alloantigen-specific CTLs (cytotoxic lymphocytes), inclusive of CD4 T cell help, in a mixed leukocyte reaction (MLR) using MHC-mismatched

C57BL/6J (C57BL/6, H-2b) responders and BALB/cByJ (BALB/c, H-2d) irradiated stimulators. In the second model we determined if redox modulation had a more direct effect on CD8 T cell effector function using the antigen-specific OT-1 T cell receptor transgenic mouse model, dominantly comprised of OVA peptide$_{257-264}$ (SIINFEKL)-specific CD8 T cells (79), in which CD4 T cell help is not necessary, nor available, to augment adaptive immune effector function. Using the two CTL models we demonstrate that redox modulation has a profound effect on T cell proliferation, pro-inflammatory cytokine production, and CTL target cell lysis by reducing the expression of cytolytic effector molecules.

2.3 MATERIALS AND METHODS

2.3.1 Mice and cell lines

OT-1 T cell receptor transgenic mice were bred and housed under specific pathogen-free conditions in the Animal Facility of the Rangos Research Center at the University of Pittsburgh. OT-1 CD8 T cells are transgenic for a TCR specific for a class I-restricted OVA peptide$_{257-264}$ (SIINFEKL-K^b) (79). C57BL/6J (C57BL/6, H-2b) and BALB/cByJ (BALB/c, H-2d) mice were purchased from The Jackson Laboratory. Six to eight week old female mice were used in all experiments. All mouse studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pittsburgh. EL-4 and EG.7 cell lines were purchased from ATCC (Manassas, VA). EL-4 cells were grown in complete media (CM) (DMEM supplemented with 10% heat-inactivated FBS, 10 mM HEPES buffer, 4 mM L-glutamine, 200 μM nonessential amino acids, 1 mM sodium pyruvate, 61.5 μM 2-ME, and 100

μg/ml Gentamicin (Invitrogen Life Technologies, Carlsbad, CA)) and allowed to grow to 10^6 cells/ml in a 75 cm^2 tissue culture flask (Cellstar, PGC Scientific, San Diego, CA) prior to passing. EG.7 cells were grown in CM with 0.4 mg/ml G418 (Sigma-Aldrich, St. Louis, MO) and allowed to grow to 10^6 cells/ml in a 75 cm^2 flask prior to passing.

2.3.2 Reagents

The catalytic antioxidant (CA), FC-007, a kind gift from Dr. James Crapo at National Jewish Research and Medical Center (Denver, CO), was re-suspended in HBSS with Ca^{2+} and Mg^{2+} (Invitrogen Life Technologies) at a stock concentration of 2 mM and filter sterilized (.2μm cutoff) before use. The final concentration of CA used for all experiments was 68 μM as previously published (48). Chicken $OVA_{257-264}$ (SIINFEKL) was purchased from Sigma-Aldrich and used at a final concentration of 1μM. CD4, CD8, IL-2 receptor α-chain (CD25), CD107a (LAMP-1), IFN-γ, granzyme B, perforin, and Foxp3 fluorochrome-conjugated antibodies were purchased from BD Biosciences (San Jose, CA).

2.3.3 Mixed Leukocyte Reactions (MLRs)

Unidirectional MLRs were performed by co-culture of 10^6 C57BL/6 splenocyte stimulators (irradiated with 3000 rads) with 10^6 BALB/c splenocyte responders as described by (80) with minor modification. Cytokine analysis of MLR supernatants was performed by ELISA and intracellular cytokine analysis of cytotoxic lymphocytes (CTLs) was performed in 96-well,

round-bottom plates with or without 68 μM CA in a final volume of 200 μl CM and incubated for 1 to 5 days under normal conditions (37°C in a 5% CO_2 humid air incubator).

2.3.4 OT-1 assays

10^5 OT-1 splenocytes (unless otherwise noted) and 1 μM SIINFEKL peptide were plated with or without 68 μM CA at a final volume of 200 μl CM in a 96-well, round-bottom plate and incubated for 1, 2, or 3 days under normal conditions.

2.3.5 *In vitro* cell proliferation

For proliferation assays, 96-well MLR were prepared as described above and incubated for 5 days. 5 x 10^5 OT-1 splenocytes were plated in 96-well, round-bottom plates and incubated for 2 or 3 days. For MLR and OT-1 assays, [^{3}H]TdR was incorporated on day 5 (MLR) or days 2 and 3 (OT-1) by pulsing wells with 1 μCi of [^{3}H]TdR for 18 h, freezing plates for 18 h, followed by thawing plates before samples were harvested onto glass fiber filters using a sample harvester. The amount of incorporated counts was determined using a beta scintillation counter.

2.3.6 Cytokine measurements by ELISA

MLR assays were performed as described above and supernatants were harvested on days 1 through 5 to measure IL-2, IFN-γ, TNF-α, and IL-17 production by ELISA. OT-1 assays were performed as described above and supernatants were harvested on days 1, 2, and 3 to measure

IL-2 and IFN-γ production by ELISA. In order to detect OT-1-derived TNF-α by ELISA, 5 x 10^5 OT-1 splenocytes were plated and supernatants were harvested on days 1, 2, and 3. IL-2 and IFN-γ, produced in supernatants of 96-well MLR and OT-1 assays, were measured using antibody pairs from BD Pharmingen (San Diego, CA). TNF-α and IL-17 cytokine analysis of both assays was performed using DuoSet ELISA kits (R & D Systems, Minneapolis, MN). ELISA plates were read on a Spectromax M2 microplate reader (Molecular Devices, Sunnyvale, CA) and data were analyzed using Softmax Pro v5.0.1 (Molecular Devices).

2.3.7 Cytotoxicity assay by DELFIA

For MHC-mismatched cytotoxicity assays, a primary MLR was performed as described (80, 81) with minor modification, using 2.5 x 10^6 BALB/c responders and 2.5 x 10^6 C57BL/6 irradiated stimulators in 24-well plates in a final volume of 2 ml CM in the presence or absence of 68 μM CA for 5 days under normal conditions. On day 5, treated and untreated cytotoxic lymphocytes (CTLs) were harvested using cell dissociation buffer (Invitrogen Life Technologies). Simultaneously, EL-4 (H-2b) target cells were labeled with BATDA-reagent and the cytotoxicity assay was performed according to manufacturer's protocol in the DELFIA kit (AD0116: PerkinElmer, Inc, Waltham, MA). 10,000 EL-4 targets were incubated with cytotoxic lymphocytes (CTLs) at 50:1 and 100:1 effector to target ratios for 2 h. Fluorescence of the supernatant was measured in a time-resolved fluorometer. DELFIA data are presented as "percent specific release" based on the formulae in the manufacturer's protocol representing specific lysis of targets cell by effector cytotoxic lymphocytes (CTLs).

For OT-1 antigen-specific CD8 cytotoxicity assays, the DELFIA kit was also used. C57BL/6 splenocytes were irradiated at 3000 rad leaving viable APCs. Five x 10^6 splenocytes were incubated with 1 μg/ml LPS in 24-well flat-bottom plates in a final volume of 2 ml for 3 days under normal conditions to activate the radio-resistant APCs. On day 3, the LPS-activated C57BL/6 APCs were pulsed with 5 μM SIINFEKL for 30 min under normal conditions and then washed twice with CM. OT-1 $CD8^+$ T cells were purified from whole spleen by negative selection using $CD4^+$ and MHC class II magnetic beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's protocol. 2.5 x 10^6 LPS-activated SIINFEKL-pulsed C57BL/6 APCs were incubated with 2.5 x 10^6 OT-1 T cells with or without 68 μM CA in a 24-well flat-bottom plate in a final volume of 2 ml CM under normal conditions for 4 days. On day 4, cytotoxic lymphocytes (CTLs) were harvested and plated with 10,000 target cells at a 50:1 effector to target ratio according to manufacturer's protocol. Two h later supernatants were harvested and fluorescence was measured by a time-resolved fluorometer. Again, data are presented in "percent specific release" as indicated above.

2.3.8 Flow cytometric analysis

Cells were washed in filter-sterilized FACS buffer (1% bovine serum albumin (BSA) in phosphate buffered saline (PBS)) and re-suspended in FACS buffer at the final concentration of 10^7 cells/ml. One million cells were stained with directly fluorochrome-conjugated antibodies at the appropriate dilution (10 μl of each antibody) in FACS buffer for 20 min in the dark at 4°C. Cells were washed in FACS buffer before measuring fluorescence on a FACSCalibur (BD Biosciences).

2.3.9 Annexin V viability experiments

For annexin V viability experiments of whole spleen, 96-well MLRs were prepared as previously described and incubated for 1, 3, or 5 days. At each time point cells were harvested, washed twice in PBS, re-suspended in FACS buffer, and stained with PE-conjugated annexin V antibody (BD Biosciences) before fluorescence was measured as described above. For annexin V staining of the T cell population, assays were performed in the same manner with the addition of an APC-conjugated CD8 antibody (BD Biosciences) stain for all time points.

2.3.10 Intracellular ROS detection by CM-H_2DCFDA

2 x10^6 OT-1 splenocytes were stimulated with 1 μM SIINFEKL, or left un-stimulated, in the presence or absence of 68 μM CA in 24 well plates. After 24 hours, cells were harvested and loaded with 5μM FITC-conjugated CM-H_2DCFDA dye (Molecular Probes) for 30 minutes at 37°C. Some cells were not stained with the dye, and only incubated for 30 minutes at 37°C, as unstained FACS controls. All cells were then washed twice in CM and re-suspended at 10^7 cells/ml in FACS buffer. One million cells were stained with PECy5-conjugated CD8 antibody or left unstained to serve as appropriate controls according to previously mentioned methods. Results are presented as delta (Δ) percent CD8 x CM-H_2DCFDA double positive cells with respect to un-stimulated OT-1 controls.

2.3.11 Trypan blue exclusion viability experiments

For trypan blue viability assays, 96-well MLRs were prepared as previously described and incubated for 1, 3, or 5 days. At each time point cells were harvested, washed twice, and re-suspended in PBS. For each of the groups, a 10 μl cell aliquot was diluted 1:10 in 100 μl and added to 20 μl of trypan blue. Cell samples were vortexed before a 10 μl sample was pipetted into a hemocytometer under a light microscope. Live and dead cells were counted in at least two quadrants for each group at each time point. Results are reported as percent live cells per quadrant equivalent to number of live cells /total number of cells.

2.3.12 Intracellular staining and FACS analysis

Intracellular staining was measured in CA-treated and untreated cells in MLR and OT-1 assays on days 1, 2, 3 with the aid of the Murine BD intracellular cytokine staining kit (BD Biosciences). After stimulation, splenocytes were surface-stained with PECy5 or APC-conjugated CD8 and FITC-conjugated CD107a (LAMP-1) then fixed in BD Cytofix/Cytoperm buffer, washed in BD Perm/Wash buffer, and then stained intracellularly with APC-conjugated rat anti-IFN-γ, APC-conjugated rat-anti-perforin or PE-conjugated rat-anti-granzyme B (BD Biosciences). The appropriate isotype controls were used. Cells were washed twice in BD Perm/Wash buffer and re-suspended in FACS buffer; stained cells were analyzed on a FACSCalibur (BD Biosciences).

2.3.13 Statistical analysis

The difference between mean values for each experimental group was determined by a Student's t test, with $p < 0.05$ for significance, using the JMP statistical discovery software from the SAS Institute. All experiments were performed at least three separate times with data obtained from triplicate wells in each experiment; the only exception being FACS analysis in which data are representative of at least three independent experiments.

2.4 RESULTS

2.4.1 Redox modulation suppresses cell proliferation and the production of IL-2, IFN-γ, TNF-α, and IL-17 in a primary Mixed Leukocyte Reaction (MLR)

Based on our prior studies showing that redox modulation of the innate immune-derived pro-inflammatory third signal diminishes the expansion and effector function of antigen-specific CD4 T cells (26), we initiated studies to determine if redox modulation would also affect the response to alloantigen that is mediated by both, CD4 and CD8 T cells. We performed *in vitro* unidirectional MLRs by co-culturing 10^6 MHC-mismatched BALB/c (H-2d) responders with 10^6 C57BL/6 (H-2b) irradiated stimulators in the presence or absence of a predetermined concentration of CA (68 μM) capable of suppressing production of innate immune-derived pro-inflammatory cytokines (48). Figure 10A demonstrates a significant three-fold decrease in proliferation with redox modulation by day 5 in an MLR assay. Along with reduced proliferation, redox modulation suppresses the production of IL-2 in MLR supernatants by two-

fold at the same time point (Figure 10B). Taken together redox modulation inhibits T cell proliferation and IL-2 production *in vitro*.

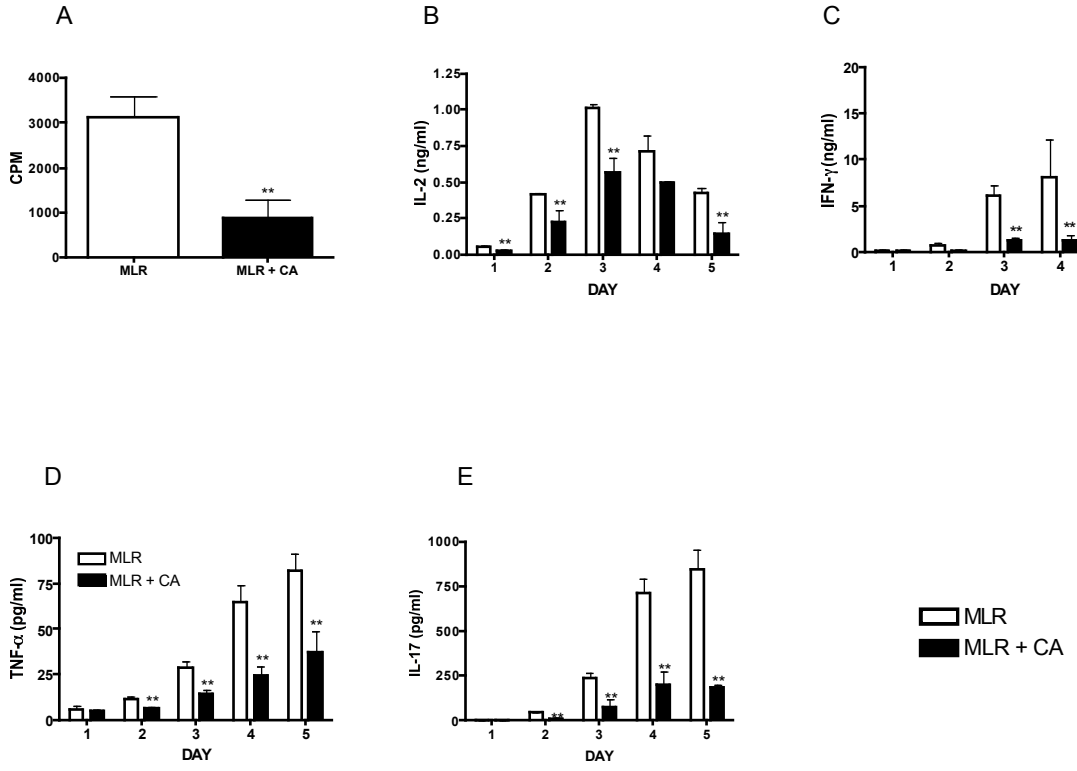


Fig. 10. Redox modulation suppresses cell proliferation and cytokine production in a primary MLR. In a primary MLR, 10^6 BALB/c splenocytes were stimulated with 10^6 irradiated C57BL/6 splenocytes in the presence (■) or absence (□) of 68 μM CA for up to 5 days. On day 5 proliferation was assessed by [^{3}H] TdR incorporation (*A*). On days 1-5 supernatants were harvested and IL-2 (*B*), IFN-γ (*C*), and TNF-α (*D*) were measured by ELISA. Results are presented as the mean (± SEM) of four independent experiments performed in triplicate. **, $p < 0.05$ between the respective treated and untreated (control) group.

To gain further insight into the T cell effector response to alloantigen, IFN-γ production was analyzed in MLR supernatant in the presence or absence of redox modulation. We observed a statistically significant 6-fold decrease in IFNγ levels in CA-treated MLR on Days 3, 4, and 5 compared to untreated MLR (Figure 10C). To determine if TNF-α, an upstream stimulus of IFN-γ production, was also reduced in the presence of redox modulation (26, 82), TNF-α levels were measured by ELISA in untreated and CA-treated MLR supernatants. We observed significant decreases in TNF-α levels from CA-treated samples on days 2-5 (Figure 10D), concomitant with suppressed IFN-γ levels with CA-treatment. In addition, we observed a significant 3-fold repression of the pro-inflammatory cytokine, IL-17, in CA-treated MLRs on Days 2, 3, 4, and 5 (Figure 10E). IL-17 is a cytokine of interest due to its documented role in allograft rejection and indispensable role in autoimmune diseases (83-89). In agreement with several studies (90-93), our data reflects the simultaneous presence of functional T_H1 and T_H17 T cells in response to an alloantigen.

2.4.2 Redox modulation impairs the ability of alloantigen-specific cytotoxic lymphocytes (CTLs) to kill MHC-mismatched target cells

To determine if the redox-dependent decreases in IL-2, IFN-γ, TNF-α, and IL-17 in response to alloantigen equates to an appreciable effect on CTL-induced target cell lysis, CTLs were generated by MLR in the presence or absence of redox modulation. BALB/c CTLs (H-2d) were harvested to determine the capacity of CA-treated and untreated CTLs to kill MHC-mismatched EL-4 (H-2b) target cells. At effector to target ratios of 100:1 and 50:1,

BALB/c CA-treated CTLs exhibited a 3-fold decrease in the ability to kill EL-4 target cells compared to control CTLs (Figure. 11).

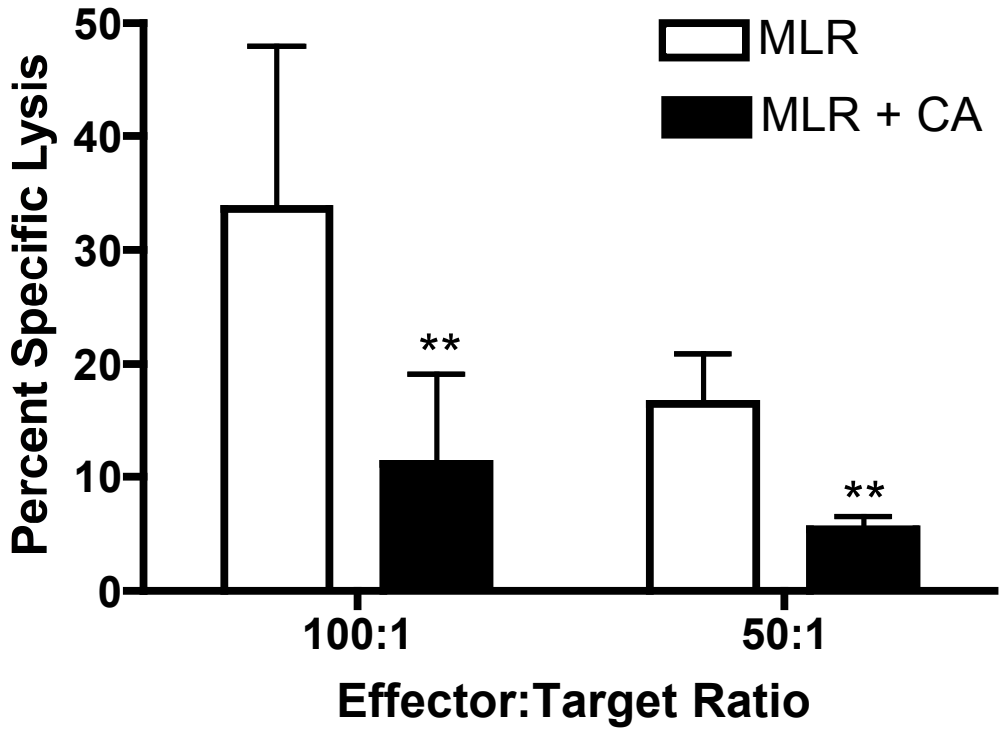


Fig. 11. Redox modulation diminishes the ability of alloantigen-specific CTLs to kill their MHC-mismatched target cells in a cytotoxicity assay. Alloantigen-specific CTLs were derived by stimulating 2.5 x 10^6 BALB/c (H-2d) splenocytes with 2.5 x 10^6 irradiated C57BL/6 (H-2b) splenocytes in the presence (■) or absence (□) of 68 μM CA. On day 5 CTLs were harvested and plated with fluorescently labeled EL-4 (H-2d) target cells at 100:1 and 50:1 effector to target ratios for 2 hours. After 2 hours supernatants were harvested, mixed with Europium reagent, and fluorescence was measured. Results are recorded in percent specific lysis as the mean (± SEM) of three independent experiments performed in triplicate. **, $p < 0.05$ between the representative treated and untreated (control) group.

2.4.3 Redox modulation suppresses OT-1 CD8 T cell proliferation, IL-2, IFN-γ, TNF-α, and IL-17 production in a primary antigen-recall assay

To determine if redox modulation of CD8 T cells could also affect CD8 T cell proliferation and adaptive immune effector function in the absence of CD4 T cell help, we utilized the CD8 T cell receptor transgenic OT-1 mouse model (79). OT-1 splenocytes were incubated with 1 μM SIINFEKL peptide in the presence or absence of CA. Redox modulation of primary OT-1 assays exhibited statistically significant decreases in proliferation on days 2 and 3, though a statistically significant decrease in IL-2 production was only achieved at day 3 (Figure 12A, B). This led us to speculate why redox modulation substantially inhibits proliferation whereas the effect on IL-2 production is not as drastic. For this reason we examined IL-2 receptor α-chain (CD25) expression on the surface of OT-1 CD8 T cells in a primary antigen recall assay. We observed at least a three-fold decrease in CD25 expression on OT-1 T cells harvested from CA-treated OT-1 assays compared to untreated controls on days 2 and 3 (Figure 13), pointing to a possible mechanism by which a several fold decrease in proliferation, though only slight reduction in IL-2 production could occur. This phenomena, however, was not observed in the MLR model as the data demonstrated no significant difference in CD25 expression in the presence or absence of redox modulation (Figure 14A,B).

More importantly, IFN-γ synthesis was suppressed by redox modulation in primary OT-1 assays compared to untreated assays at all time points (Figure 12C). Additionally, redox modulation of OT-1 CTLs was associated with significant inhibition of TNF-α levels on days 1 and 2 (Figure 12D), which may mechanistically contribute to decreased IFN-γ production. Interestingly, we also observed significant decreases in IL-17 in the presence of redox

modulation at all time points (Figure 12E), indicating that antigen-specific transgenic OT-1 CD8 T cells exhibit T_H1 and T_H17 phenotypes having documented the production of both, IFN-γ and IL-17 (Figure 12E). Taken together, these results clearly demonstrate that redox modulation has an inhibitory effect on CD8 T cell proliferation and adaptive immune effector function in the absence of CD4 T cell help.

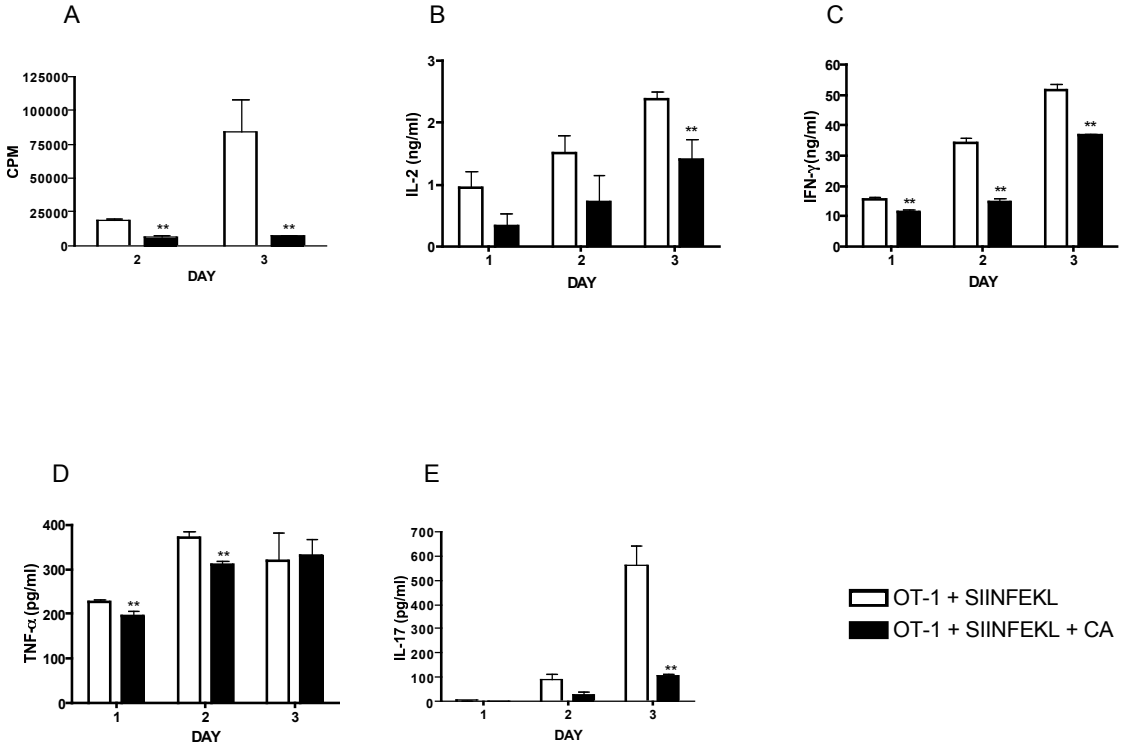


Fig. 12. Redox modulation suppresses proliferation and cytokine production in a primary OT-1 assay. 5 x 10^5 (*A*, *D*) or 10^5 (*B, C*) OT-1 splenocytes were incubated with 1μM SIINFEKL in the presence (■) or absence (□) of 68 μM CA for 1 to 3 days. On days 2 and 3 proliferation was assessed by [^{3}H] TdR incorporation (*A*). On days 1, 2, and 3 supernatants were harvested and IL-2 (*B*), IFN-γ (*C*), and TNF-α (*D*) were measured by ELISA. Results are presented as the mean (± SEM) of four independent experiments performed in triplicate. **, $p < 0.05$ between the respective treated and untreated (control) group.

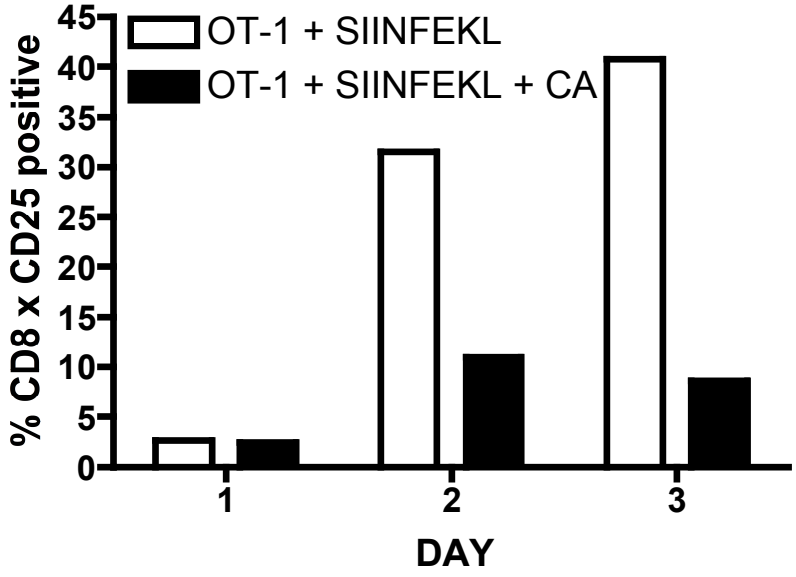


Fig. 13. Redox modulation inhibits IL-2 receptor α-chain (CD25) expression on OT-1 Transgenic CD8 T cells. 10^5 OT-1 splenocytes were incubated with 1μM SIINFEKL in the presence (■) or absence (□) of 68 μM CA for 1 to 3 days. On days 1, 2, and 3 cells were harvested, washed, and results are graphed correlating to surface staining of CD8 x IL-2 receptor α-chain (CD25). Results are representative of three independent experiments shown in the Supplement to Fig. 13.

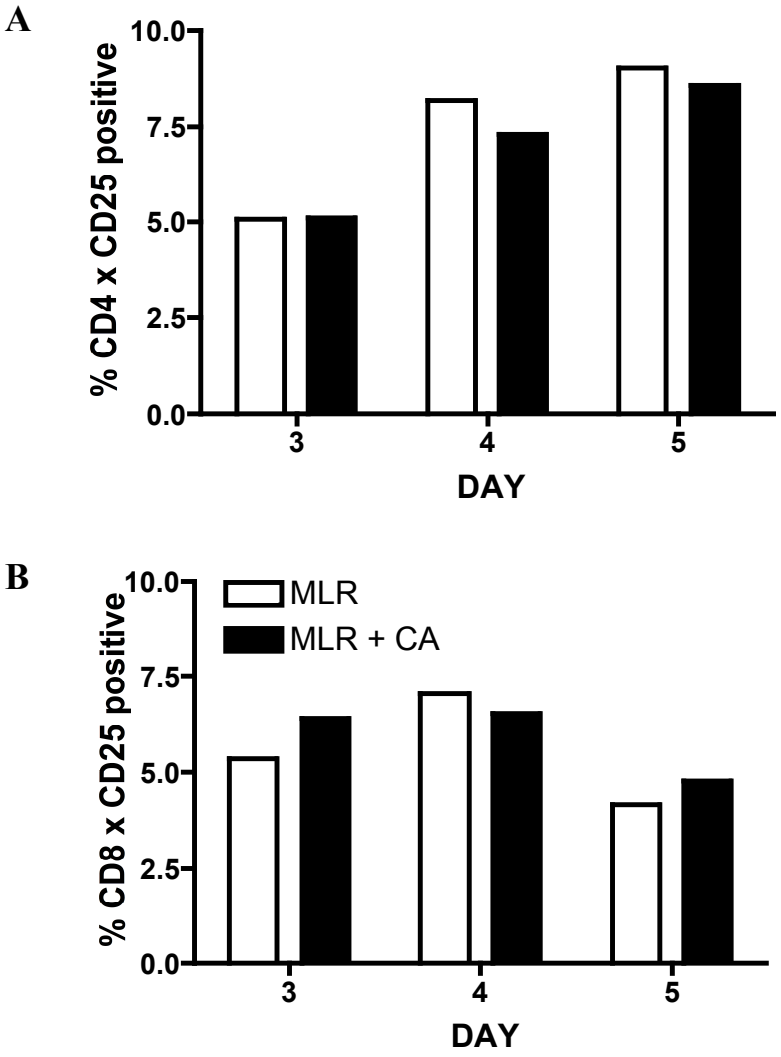


Fig. 14. Redox modulation has no effect on IL-2 receptor α-chain (CD25) expression on BALB/c T cells. 10^6 BALB/c splenocytes were incubated with 10^6 irradiated C57BL/6 splenocytes in the presence (■) or absence (□) of 68 μM CA for 3 to 5 days. On days 3, 4 and 5 cells were harvested, washed, and results are graphed correlating to surface staining of (A) CD4 x IL-2 receptor α-chain (CD25) or (B) CD8 x IL-2 receptor α-chain (CD25). Results are representative of three independent experiments shown in the Supplement to Fig. 14.

2.4.4 Redox modulation impairs the ability of antigen-specific OT-1 cytotoxic lymphocytes (CTLs) to kill their corresponding antigen-expressing target cells

Redox modulation, known to inhibit the innate immune-derived pro-inflammatory third signal, was efficient in suppressing adaptive immune effector cytokine synthesis in OT-1 primary assays, but whether redox modulation also decreased CTL activity remained to be determined. To explore this, OT-1 CTLs were generated in the presence or absence of CA and used as effectors against the OVA-expressing tumor cell line, EG.7 (H-2b), and the non-OVA-expressing parent cell line, EL-4 (H-2b). As expected, both CA-treated and untreated OT-1 CTLs failed to exhibit significant specific lysis of the non-OVA-expressing EL-4 targets at a 50:1 effector to target ratio (Figure 15). At the same effector to target ratio, CA-treated OVA-specific OT-1 CTLs exhibited a 3-fold decrease in specific lysis of the OVA-expressing EG.7 cells compared to untreated OT-1 CTLs (Figure 15). This cytotoxicity assay further demonstrates that redox modulation is having a profound effect on T cell receptor transgenic antigen-specific CD8 T cells by impairing their capacity to be direct effectors of antigen-specific target cell lysis.

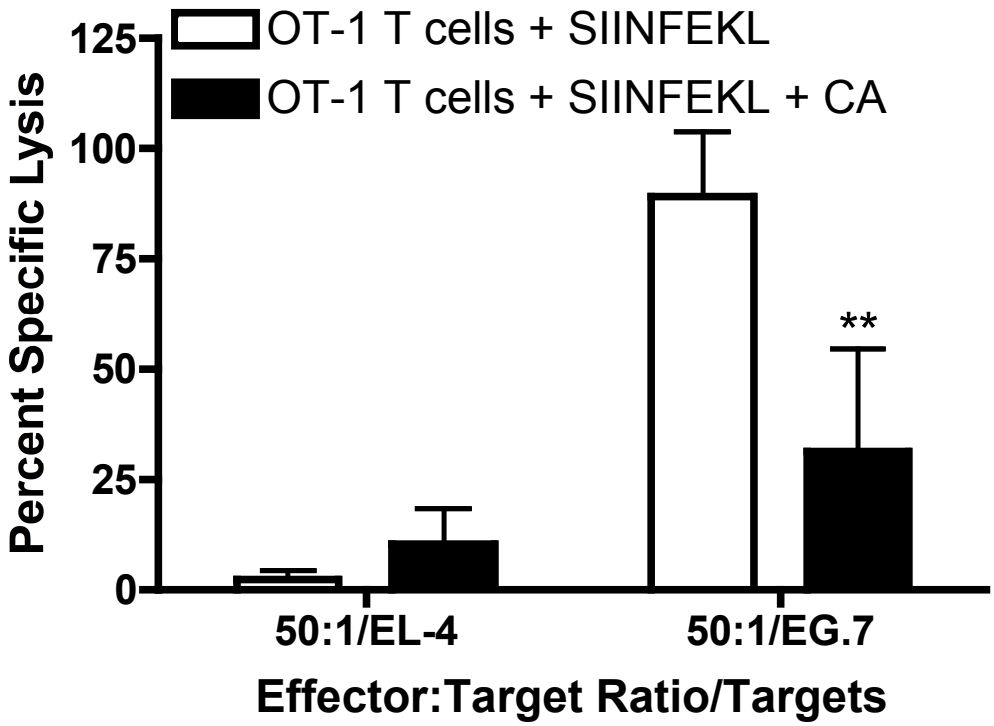


Fig. 15. Redox modulation diminishes the ability of antigen-specific OT-1 CTLs to kill their corresponding antigen-expressing target cells in a cytotoxicity assay. OT-1 CTLs were generated by activating 5 x 10^6 C57BL/6 APCs with 1μg/ml LPS for three days, loading the APCs with 5 μM SIINFEKL for 30 minutes, washing several times, then plating the activated, loaded APCs with 2.5 x 10^6 OT-1 T cells in the presence (■) or absence (□) of 68 μM CA for 4 days. On day 4 treated and untreated CTLs were harvested and plated with fluorescently labeled (non-expressing) EL-4 or (OVA-expressing) EG.7 target cells at 50:1 effector to target ratio for 2 hours. After 2 hours supernatants were harvested, mixed with Europium reagent, and fluorescence was measured. Results are recorded in percent specific lysis and are presented as the mean of three independent experiments performed in triplicate (± SD). **, $p < 0.05$ between the representative treated and untreated (control) group.

2.4.5 Cell toxicity is not a mechanism of immunoregulation by CA

To ensure that CA-treatment does not inhibit effector function by an apoptotic mechanism, we performed assays to compare the viability of untreated and CA-treated samples. Experiments were preformed in the MLR model on days 1, 3, and 5. Viability was assessed by trypan blue exclusion (Figure. 16A, C, E) and annexin V staining (Figure 16B, D, F). We observed a significant increase in cell viability in CA-treated samples by trypan blue exclusion on days 1 and 5 (Fig. 16A and E) and by annexin V staining on days 3 and 5 (Figure 16D, F). There was no significant difference in cell viability at other time points for either assay.

To specifically analyze CD8 T cell viability, we performed annexin V and CD8 double staining by FACS analysis. In these experiments we observed no significant difference in annexin V positive CD8 positive T cells in the presence or absence of CA, though a trend for increased cell viability, as indicated by increased percentages of live cells, was present in CA-treated samples compared to untreated control samples (Table 1). Day 5 was specifically chosen for our alloantigen-specific cytotoxicity experiments since this day is the optimal time point for the generation of CTL from a primary MLR assay. Taken together, the viability data indicate that redox modulation inhibits pro-inflammatory cytokine production and CTL target cell lysis, while promoting cell viability. These results further support our hypothesis that redox modulation imparts regulatory capabilities by preventing the transition from expansion to effector function in the absence of cell death.

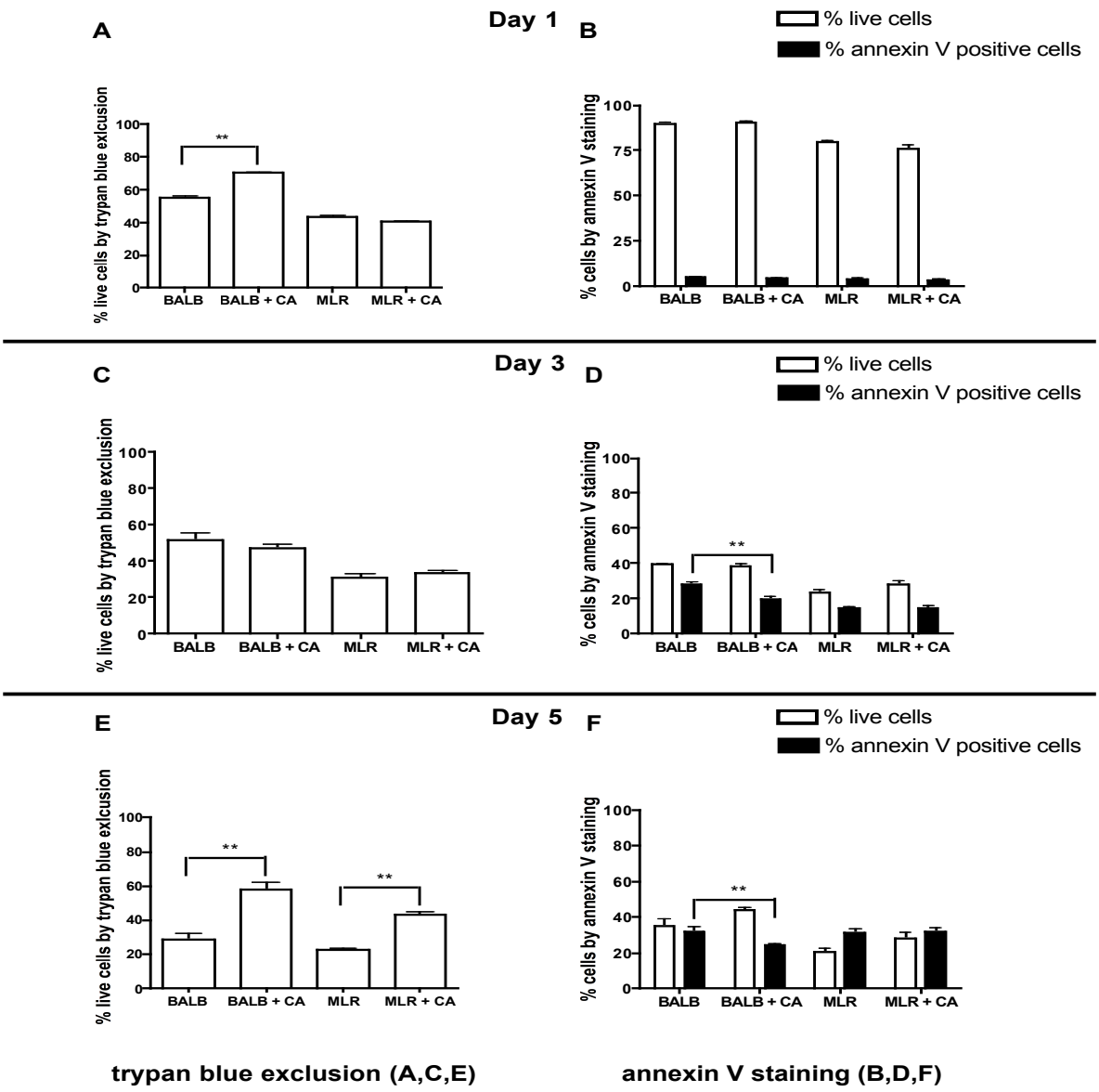


Fig. 16. Redox modulation enhances cell viability. In a primary MLR, 10^6 BALB/c splenocytes were stimulated with 10^6 irradiated C57BL/6 splenocytes in the presence or absence of 68 μM CA for up to 5 days. On days 1, 3 and 5, viability was assessed by trypan blue exclusion (A, C, E) or annexin V staining (B, D, F). Results are presented as the mean (± SEM) of three independent experiments. **, $p < 0.05$ between the respective treated and untreated (control) group.

Table 1. CD8 T cell viability is not significantly affected by redox modulation

% cells	double-negative (live cells)	annexin V single-positive	CD8 single-positive	CD8/annexin V double-positive
BALB	32.35 +/- 9.69	62.25 +/- 10.25	4.70 +/- 0.70	0.75 +/- 0.07
BALB + CA	40.90 +/- 0.28	53.15 +/- 0.21	5.30 +/- 0.28	0.70 +/- 0.14
MLR	25.00 +/- 4.24	71.35 +/- 3.32	2.25 +/- 0.63	1.45 +/- 0.35
MLR + CA	25.90 +/- 4.67	69.15 +/- 5.87	3.30 +/- 0.85	1.60 +/- 0.28

CD8 T cell viability is increased in the presence of redox modulation. In a primary MLR, 10^6 BALB/c splenocytes were stimulated with 10^6 irradiated C57BL/6 splenocytes in the presence or absence of 68 μM CA for 5 days. On day 3 cells were harvested, washed and stained with CD8 and annexin V. Results are presented as a percentage of the cell population as the mean (± SD) of two independent experiments.

2.4.6 Induction of a regulatory population is not a mechanism of immunoregulation by CA

To determine if redox modulation induces a classic regulatory population we assessed intracellular expression of Foxp3 and IL-10 production. To analyze Foxp3 expression in T cell populations primary MLRs and OT-1 assays were performed. Cells were harvested from MLR assays on days 3, 4 and 5 and at 18, 42, and 66 hours for OT-1 assays and T cells were surfaced stained for CD4 or CD8 and CD25 and then stained for intracellular Foxp3. OT-1 T cells appear to show a trend for decreased Foxp3 expression in the presence of redox modulation, while CD4 and CD8 T cells in an MLR assay show a slight, but negligible, increase in Foxp3 on days 3 and 4 (Figure 17). On day 5, CD4 and CD8 T cells show a prominent decline in any Foxp3 population, regardless of CA-treatment. Perhaps this phenomenon is an indication that the regulatory mechanisms have been overcome by the robust response to alloantigen (7).

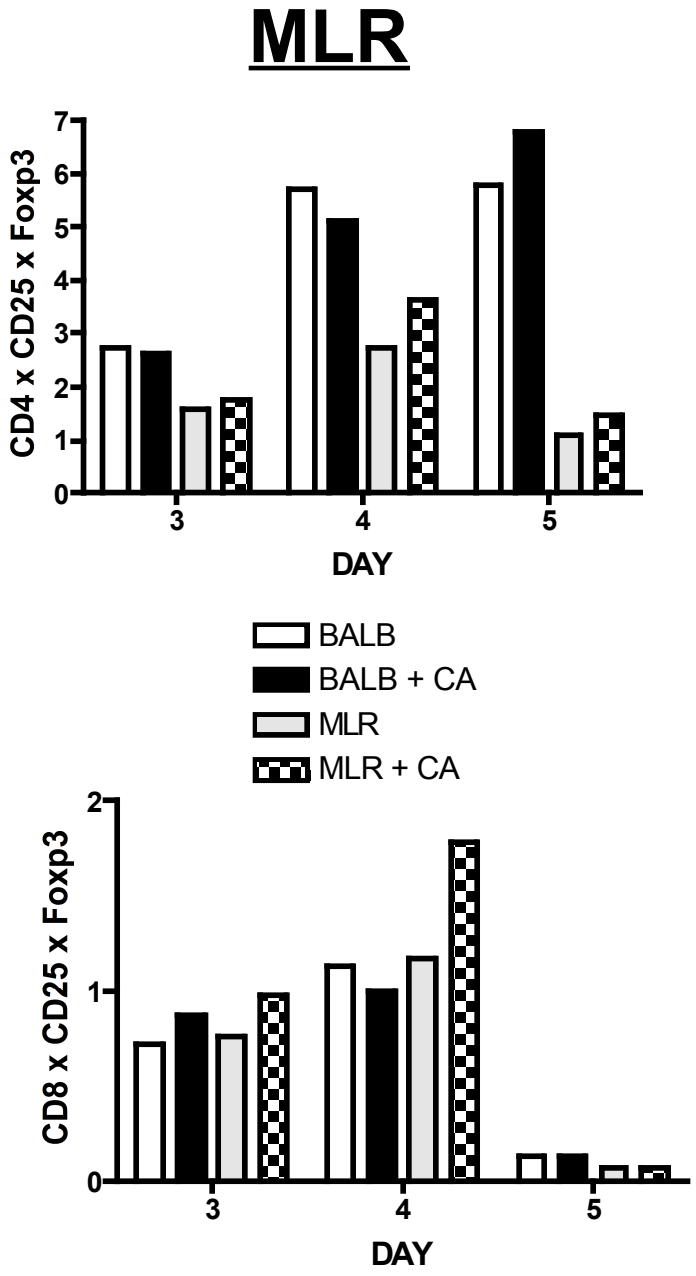


Fig. 17. Redox modulation does not induce Foxp3 expression. In primary MLRs, 10^6 BALB/c splenocytes were stimulated with 10^6 irradiated C57BL/6 splenocytes in the presence or absence of 68 μM CA for up to 5 days. MLR cells were harvested on days 3-5, surface-stained with CD4/CD8 and CD25, and then stained intracellularly with Foxp3. Results are representative of three independent experiments shown in the Supplement to Fig. 17.

To further examine the induction of any regulatory mechanisms in the presence of redox modulation IL-10 production was also analyzed in primary MLR and OT-1 assays. Supernatants were harvested from MLRs on days 1-5 and from OT-1 assays on days 1-3. In both assays, no significant difference was found between untreated and CA-treated wells, though there is a trend of decreasing IL-10 production on days 3-5 of the MLR and on day 3 in the OT-1 assay (Figure 18). IL-10 production could not be detected in wells receiving no stimulation by alloantigen or peptide for MLR and OT-1 assays, respectively (data not shown). These data demonstrate that redox modulation does not induce a regulatory T cell population or increased IL-10 production by any cell type.

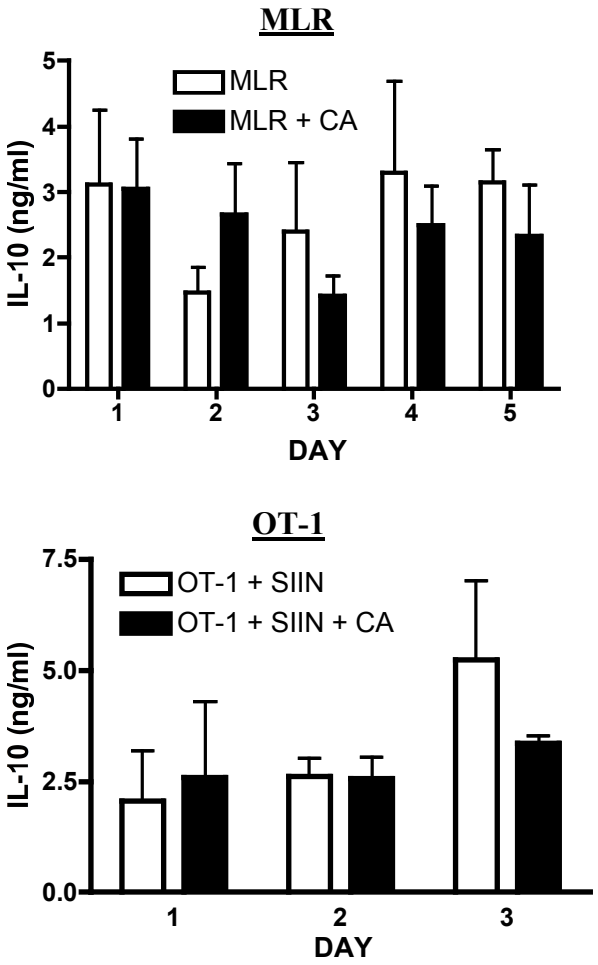


Fig. 18. Redox modulation does not induce increased IL-10 production. In a primary MLR, 10^6 BALB/c splenocytes were stimulated with 10^6 irradiated C57BL/6 splenocytes in the presence (■) or absence (□) of 68 μM CA for up to 5 days. 10^5 OT-1 splenocytes were incubated with 1μM SIINFEKL in the presence (■) or absence (□) of 68 μM CA for up to 3 days. IL-10 production was measured by ELISA. Results are presented as the mean (± SEM) of three independent experiments performed in triplicate. **, $p < 0.05$ between the respective treated and untreated (control) group.

2.4.7 Redox modulation hinders intracellular ROS production in CD8 T cells

We have previously shown that CA can inhibit ROS production in APCs (26), and now want to determine if CA can also hamper ROS production in CD8 T cells. T cell receptor transgenic OT-1 splenocytes were stimulated with cognate peptide in the presence or absence of redox modulation for 24 hours after which the cells were harvested and labeled with CD8 and CM-H_2DCFDA, an intracellular indicator of reactive oxygen species, for FACS analysis. The data demonstrate that CA inhibits ROS production as ROS could not be detected in OT-1 CD8 T cells stimulated with cognate peptide in the presence of redox modulation, while OT-1 CD8 T cells stimulated with cognate peptide in the absence of CA show almost a 7% increase in intracellular ROS production over the background (Figure 19).

Fig. 19. Redox modulation inhibits CD8 T cell ROS production. 2 x 10^6 OT-1 splenocytes were stimulated with 1 μM SIINFEKL or left unstimulated in the presence or absence of 68 μM CA in a primary OT-1 assay. After 24 hours cells were harvested, stained with CM-H_2DCFDA, washed, and then stained with CD8. Results are presented as delta (Δ) percent CD8 x CM-H_2DCFDA double positive cells with respect to un-stimulated OT-1 controls. Results are representative of three independent experiments.

2.4.8 Redox modulation decreases intracellular expression of IFN-γ, perforin, granzyme B and surface expression of CD107a (LAMP-1) on alloantigen-specific CTLs and OVA-specific OT-1 CTLs

CTL effector molecules were measured to determine how redox modulation controls alloantigen-specific and OVA-specific CTLs from killing their respective targets. Specifically, we performed intracellular staining assays for IFN-γ, perforin, and granzyme B expression and assessed degranulation using the surface marker CD107a (LAMP-1), on allospecific and OT-1 T cell receptor transgenic CTLs generated in an MLR or in an OT-1 primary assay, respectively.

Analysis of intracellular IFN-γ levels in MLR and OT-1 primary assays demonstrated at least a 5-fold decrease in expression of IFN-γ-producing $CD8^+$ T cells in CA-treated samples versus untreated controls (Figures 20A and 21A). Perforin, granzyme B and CD107a (LAMP-1) expression was also notably inhibited by at least 5-fold on CA-treated CTLs versus untreated controls at all time points (Figures 20B-D and 21B-D). These results indicate that redox modulation restrains allospecific and T cell receptor transgenic CTL target cell lysis by decreasing CTL effector molecule synthesis including IFN-γ, perforin, granzyme B, and the degranulation marker CD107a (LAMP-1), regardless of CD4 T cell help.

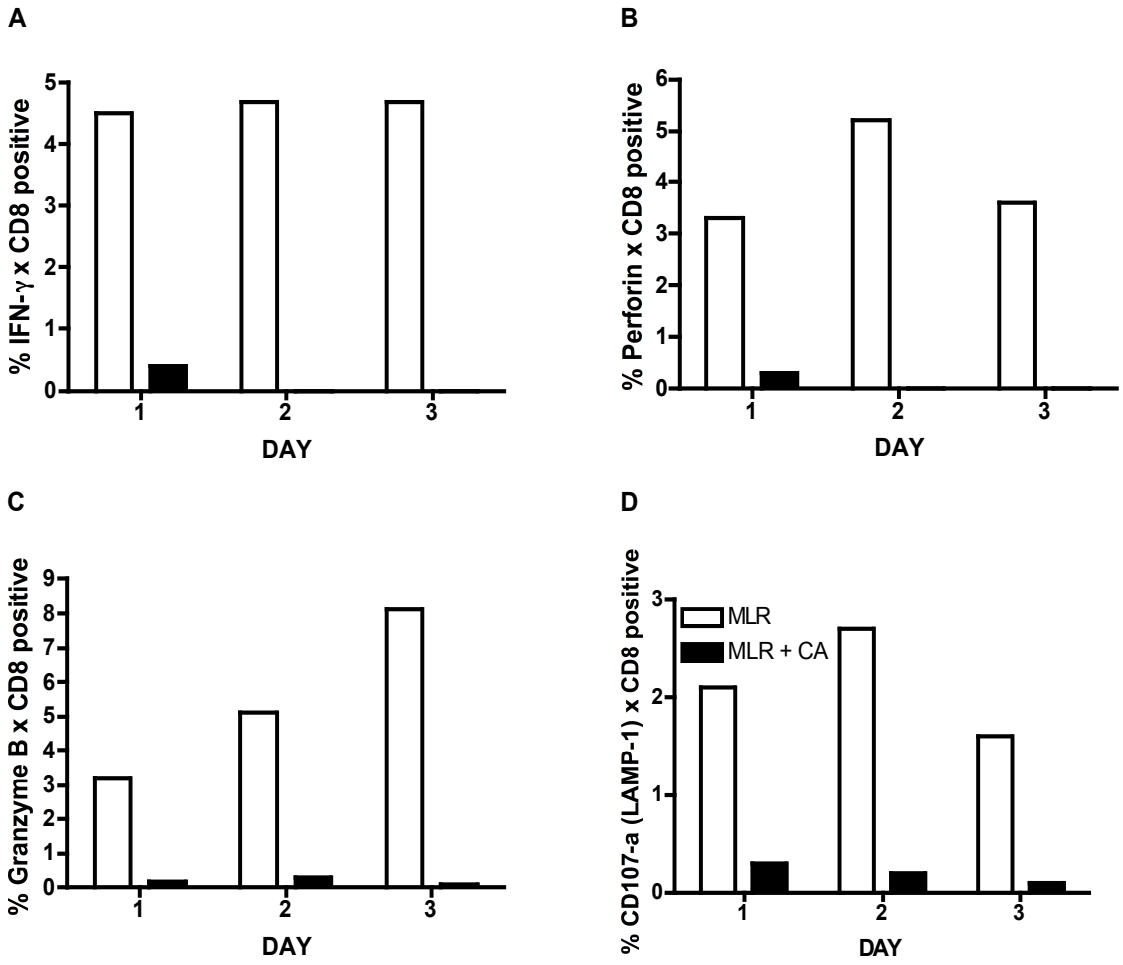


Fig. 20. Redox modulation decreases intracellular expression of IFN-γ, perforin, granzyme B, and surface expression of CD107a (LAMP-1) on alloantigen specific CTLs. Alloantigen-specific CTLs were derived by stimulating 10^6 BALB/c splenocytes with 10^6 irradiated C57BL/6 splenocytes in the presence (■) or absence (□) of 68 μM CA for 1, 2, or 3 days. On days 1, 2, and 3 CTLs were harvested, washed, and results are graphed correlating to staining of surface CD8 x intracellular IFN-γ (*A*), surface CD8 x intracellular perforin (*B*), surface CD8 x intracellular granzyme B *(C*), or surface CD8 x surface CD107a (LAMP-1) (*D*). Results are representative of three independent experiments shown in the Supplement to Fig. 20A-D.

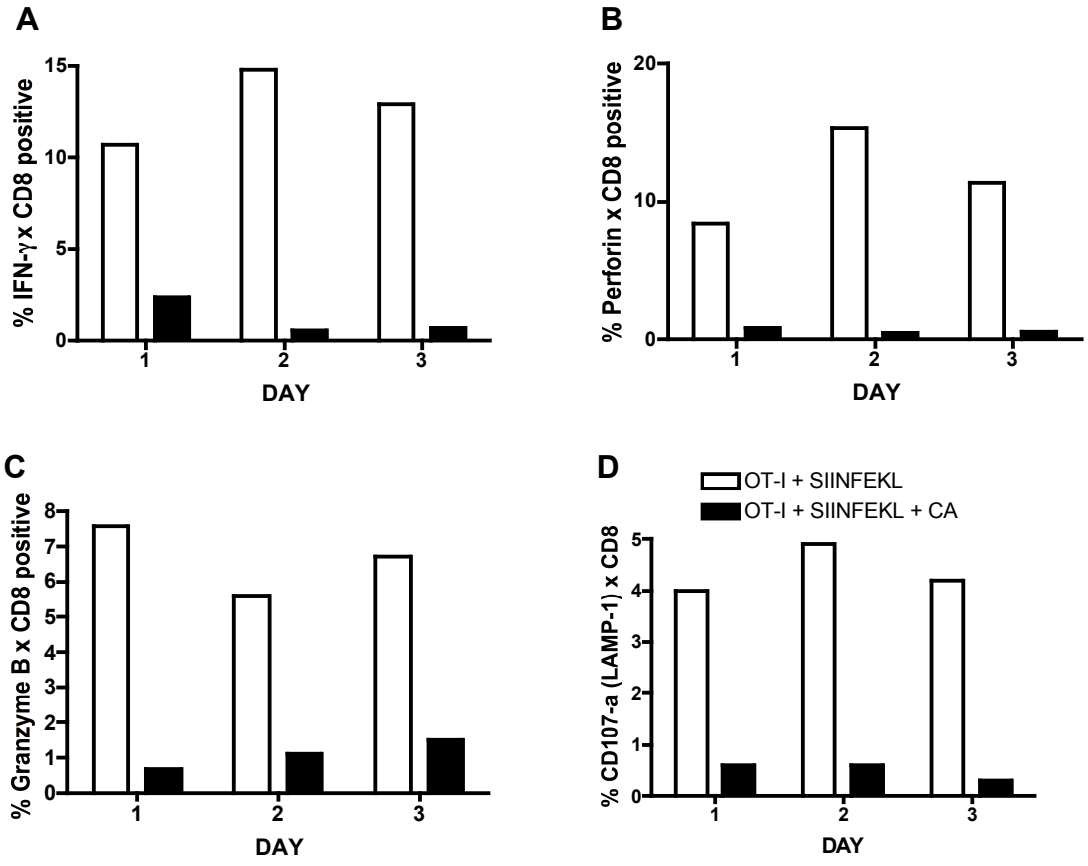


Fig. 21. Redox modulation decreases intracellular expression of IFN-γ, perforin, granzyme B, and surface expression of CD107a (LAMP-1) on OT-1 CTLs. OT-1 CTLs were derived by stimulating 10^5 OT-1 splenocytes with 1 μM SIINFEKL in the presence (■) or absence (□) of 68 μM CA for 1, 2, or 3 days. On day 1, 2, and 3 OT-1 CTLs were harvested, washed, and results are graphed correlating to staining of surface CD8 x intracellular IFN-γ (*A*), surface CD8 x intracellular perforin (*B*), surface CD8 x intracellular granzyme B (*C*), or surface CD8 x surface CD107a (LAMP-1) (*D*). Results are representative of three independent experiments shown in the Supplement to Fig. 21A-D.

2.5 DISCUSSION

A potential consequence of hindering CD4 T cell activation is a downstream effect on CD8 T cell effector function (94-98), therefore we examined if redox modulation could impact CD8 T cell effector function, either in the presence or absence of CD4 T cell help. Our data demonstrate that redox modulation inhibits a response to alloantigen and a CD8 T cell receptor transgenic T cell response as exemplified by reduced proliferation, IL-2, IFN-γ, TNF-α and IL-17 production. More importantly, redox modulation significantly impaired antigen-specific CTL target cell lysis and the synthesis of cytolytic effector molecules, IFN-γ, perforin and granzyme B, as well as the expression of the surface degranulation marker, CD107a (LAMP-1).

Using two robust *in vitro* experimental models, our data further support the pivotal role of redox status for generating a CD8 T cell effector response. Furthermore, the results using the purified CD8 T cells from the OT-1 T cell receptor transgenic mouse demonstrate the likelihood that CD8 T cells, themselves, are directly affected by redox modulation. The T cell precursor frequency involved in peptide recognition in an alloantigen mismatch is significantly elevated compared to that of nominal antigen recognition and the precursor frequency in a T cell receptor transgenic T cell population is elevated to an even higher degree over that of an allomismatch. Our experiments using CA to affect redox status were performed in the presence of increased precursor frequency in order to assess the efficacy of this type of regulation in two robust *in vitro* systems.

Interestingly, CA-treatment of the OT-1 primary recall assay had a profound effect on T cell proliferation, though IL-2 levels were not significantly inhibited at all time points (Figure 12A and 12B). Correlating with our previously published studies using human islets (38), our viability assays demonstrate that CA-treatment does not induce apoptosis, but leads to an increase in viable cells over the duration of the culture period (Figure 16 and Table 1). In spite of the increased number of viable cells in CA-treated groups, pro-inflammatory cytokine production and cytotoxic lymphocyte (CTL) target cell lysis is hindered in the presence of CA (Figures 10, 11, 12, 15), indicating that CA is disrupting effector function in the absence of direct cell toxicity.

Examination of IL-2 receptor α-chain (CD25) expression in CA-treated OT-1 assays exhibited greater than a 2-fold decrease in CD25 expression on days 2 and 3 (Figure 13). The decrease in CD25 expression would impact the IL-2 autocrine loop resulting in a decrease in CD8 T cell proliferation. Evidence indicating that CD25 expression is NF-κB-dependent (99-101) taken together with our previous work in APCs, demonstrating that redox modulation inhibits NF-κB DNA-binding (48), leads us to speculate that this mechanism may be responsible for the decrease in proliferation in CA-treated OT-1 assays. We did not observe a significant difference in CD25 expression in CA-treated mixed leukocyte reaction (MLR) assays (Figure 14), however, a greater decrease in IL-2 production was observed (Figure 10B) compared to the CA-treated OT-1 assays (Figure 12B).

The inhibition of cytokine production (IL-2, IFN-γ, TNF-α, IL-17) indicates that regulation by redox modulation is capable of overcoming a higher precursor frequency as compared to a nominal antigen (Figures 10 and 12). Furthermore, this effect translated to a decrease in type 1 cytotoxic effector function, as demonstrated by a significant decrease in

extracellular and intracellular IFN-γ in MLR and OT-1 primary assays (Figures 10C, 12C, 20A, 21A). Impairing the production of IFN-γ is critical in suppressing undesirable immune responses as IFN-γ induces respiratory burst by macrophages, activates NF-κB, and induces MHC Class I expression (102). In agreement with our previous studies using CD4 T cells (26), redox modulation significantly decreased TNF-α and IFN-γ production in allospecific and T cell receptor transgenic primary recall assay models (Figures 10C, 10D, 12C, 12D) and subsequently inhibited CTL antigen-specific target cell lysis (Figures 11 and 15). In response to alloantigen, it is likely that impaired CD4 T cell function by redox modulation abates the availability of CD4 T cell help that enhances CD8 effector function. Since CD8 T cells are highly dependent on CD4 T cell help for an optimal CTL effector response (77, 103, 104), antigen-specific CTL precursors are tolerized, instead of activated when CD4 T cell function is impaired (104).

In addition to a decrease in the T_H1 cytokine response (Figures 10C, 10D, 12C, 12D) we found that redox modulation has a dramatic effect on the pro-inflammatory T_H17 cytokine, IL-17. IL-17 has been implicated in a variety of autoimmune diseases and in allograft transplantation (83-85, 88, 89, 105, 106). With anti-IL-17 emerging as a potential therapeutic proven to ablate arthritis, experimental autoimmune encephalomyelitis, and allograft rejection in animal models (83, 87, 107-111), there is growing interest in further characterization of T_H17 T cells in autoimmunity and allograft rejection. In this study we report a significant reduction in IL-17 production in response to alloantigen with CA-treatment (Figure 10E). Additionally, we have found that CD8 T cell receptor transgenic OT-1 T cells produce IL-17 in response to cognate peptide and that redox modulation can inhibit this production (Figure 12E).

Since our previous work revealed that redox modulation inhibits TNF-α, IL-1β and ROS production in LPS-stimulated murine macrophages by inhibiting NFκB-DNA binding (48), it is

probable that redox modulation is limiting IL-17 production by impeding a pro-inflammatory feedback loop involving NF-κB, IL-17, and NO, in addition to hindering optimal synergistic T_H17 activation by suppressing ROS, IFN-γ, TNF-α and IL-1β production (26, 48, 84, 112-114). In combination with the previously cited literature, our data suggest that inhibition of NF-κB, and therefore, the innate-derived pro-inflammatory third signal (TNF-α, IL-1β, ROS) by redox modulation can efficiently hinder IL-17 production. Using two robust *in vitro* models, our work reveals that redox modulation of pro-inflammatory third signal synthesis can affect both CD4 and CD8 T cell effector function leading to a reduction in T_H1 and T_H17 pro-inflammatory cytokine synthesis and CTL effector molecules necessary for target cell lysis.

Furthermore, we observed several-fold decreases in target cell lysis and the expression of the cytolytic effectors, perforin and granzyme B, and in the expression of the surface degranulation marker, CD107a (LAMP-1) on CA-treated alloantigen-specific CTLs (Figure 20B-D). To further delineate how redox modulation affects CD8 T cells, we generated antigen-specific OT-1 CTLs. We isolated the effect of redox modulation on CD8 T cells one step further since C57BL/6 APCs were pre-activated with LPS and pulsed with SIINFEKL, separately, prior to incubation with naïve OT-1 CD8 T cells (CTL generation) and CA-treatment. In this experiment, the activated APCs were capable of producing pro-inflammatory third signal cytokines and to up-regulate co-stimulatory molecule expression for 3 days prior to CD8 interaction and CA-treatment. Addition of naïve OT-1 T cells and CA to the activated, SIINFEKL-pulsed APCs demonstrated that redox modulation was able to efficiently decrease CTL target cell lysis in the presence of pre-activated antigen-pulsed APCs (Figure 15). Additionally, redox modulation inhibited the expression of perforin, granzyme B, and CD107a (LAMP-1) in the presence of CA (Fig. 21B-D) quite possibly by inhibiting the NF-κB-dependent

expression of co-stimulatory molecules. By impairing NF-κB activation and subsequent production of pro-inflammatory third signal cytokines, it is likely that redox modulation reduces APC maturation/up-regulation of CD40, CD80 and CD86, resulting in suboptimal signal 2 interaction and ineffective activation of naïve T cells. This hypothesis is currently under investigation in our laboratory, though the possibility that redox modulation has a direct effect on the T cell cannot be overlooked.

The immunomodulatory capabilities of redox modulation on APC function have been described (48), however, a mechanistic analysis of the effect of redox modulation on T cells has not been fully characterized. We have initiated studies to investigate the possible T cell mechanisms altered by redox modulation and hypothesize that CA-treatment can directly affect CD4 and CD8 T cells by inhibiting NF-κB-DNA binding (48), or by impairing T cell receptor signaling and/or STAT pathway activation. Evidence that redox modulation may have an impact on T cell-specific signaling pathways was demonstrated when we observed decreased intracellular expression of IFN-γ in both CA-treated CTL models (Fig. 20A; Fig. 21A). Moreover, it has been demonstrated that IFN-γ production is required for optimal perforin-mediated CTL effector function (115, 116), suggesting that redox modulation inhibits perforin-mediated CTL target cell lysis, in part, by inhibiting IFN-γ production (Figures 10C, 12C, 20A, 21A).

Redox modulation of aberrant immune function is emerging as a viable immunotherapy since (a) oxidative stress contributes to allograft failure by triggering activation of the innate immune system (117), (b) redox modulation inhibits the pro-inflammatory innate immune-derived third signal required for CD4 T cell adaptive immune maturation and effector function (48), (c) induces antigen-specific hypo-responsiveness (26), (d) suppresses CD8 T cell

proliferation (CPM and IL-2), (e) inhibits the synthesis of pro-inflammatory cytokines (IFN-γ, TNF-α, IL-17), and (f) impairs CTL target cell lysis by depressing CD8 CTL effector mechanisms (perforin, granzyme B, CD107a (LAMP-1)), and quite possibly, CTL generation. Agents such as CA, which affect immune function through redox modulation, may be alternative therapeutics to use in conjunction with decreased doses of late stage co-stimulatory blockade agents or calcineurin inhibitors to combat both innate and adaptive immune responses in transplantation.

3.0 CHAPTER 3: REDOX MODULATION PROTECTS ISLETS FROM TRANSPLANT-RELATED INJURY

3.1 ABSTRACT

Due to reduced antioxidant defenses, β-cells are especially vulnerable to free radical and inflammatory damage. Commonly used anti-rejection drugs are excellent at inhibiting the adaptive immune response, however, most are harmful to islets and do not protect well from reactive oxygen species (ROS) and inflammation resulting from islet isolation and ischemia-reperfusion injury. The aim of this study is to determine if redox modulation, using the catalytic antioxidant (CA), FBC-007, can improve *in vivo* islet function post-transplant. The abilities of redox modulation to preserve islet function were analyzed using three models of ischemia-reperfusion injury: 1) streptozotocin (STZ) treatment of human islets, 2) a STZ-induced murine model of diabetes, 3) a murine model of syngeneic islet transplantation; and one murine model of MHC-mismatched allogeneic transplantation. CA-treatment of human islets protects from STZ-induced islet damage and systemic delivery of CA ablates STZ-induced diabetes in mice. Islets incubated with CA prior to syngeneic transplant exhibited superior function in an intraperitoneal glucose tolerance test (IPGTT) compared to untreated controls. Allograft rejection was significantly delayed when CA-treated islets were transplanted into MHC-mismatched murine diabetic recipients and treating recipients systemically with CA further extended the delay in

allograft rejection. Pre-treating donor islets with CA protects from antigen-independent ischemia-reperfusion injury incurred during transplantation. Treating systemically with CA protects islets from antigen-independent ischemia-reperfusion injury and impairs the antigen-dependent alloimmune response. These results suggest that the addition of a redox modulation strategy would be a beneficial clinical approach to preserve islet function in transplantation.

3.2 INTRODUCTION

Hypoxia is the leading cause of β-cell death during islet isolation and transplantation (40) with the highest percentage of islet graft loss and dysfunction occurring just days after transplantation (118, 119). Because islets are a cellular transplant, devoid of intrinsic vasculature (40, 120), they are exceptionally susceptible to ischemia-reperfusion injury. Islets are also increasingly vulnerable since they have inherently decreased antioxidant capacity (17-22), making them prone to oxidative/nitrosative/free radical damage. The antigen-independent complexities of islet transplantation increase the incidence of primary graft non-function and β-cell death thus requiring protection for islets at early stages of the transplant procedure (121).

In addition to antigen-independent innate-mediated inflammatory injury, islet allografts are also plagued by the antigen-dependent T-cell mediated alloimmune response, which necessitates immunosuppressive drugs for allograft survival. Commonly used anti-rejection drugs are excellent at inhibiting the antigen-dependent adaptive immune response, though most are harmful to islets and do not protect well from antigen-independent reactive oxygen species (ROS) and inflammation during islet isolation and ischemia-reperfusion injury (15, 23, 122). In their review, Balamurugan, et. al. concluded that successful islet transplantation in type 1

diabetes (T1D) necessitates islet-sparing immunosuppressive agents that combat recurrent autoimmunity with low islet toxicity (15). Predominantly, the field of islet transplantation is devoid of cytoprotective agents that promote islet survival and function by inhibiting nonspecific innate-mediated inflammation during islet isolation and early inflammatory events in islet transplantation (7, 15, 16, 38, 39, 44, 121).

The first phase of immunity involves innate immune activation and subsequent pro-inflammatory signals required for optimal T cell activation (26-28), yet the majority of immunosuppressive drugs only target adaptive immune function (1, 7), the second phase of immunity. A nontoxic, cell-permeable catalytic antioxidant (CA) redox modulator, FBC-007 (manganese (II) tetrakis (N-ethylpyridium-2-yl) porphyrin) is able to depress free radical and cytokine production by antigen presenting cells (APC) (48) and T cells in transgenic and allospecific mouse models (26, 123). Additionally redox modulation inhibits CTL (cytotoxic lymphocyte) target cell lysis by reducing the production of intracellular cytolytic molecules (perforin and granzyme B) in a mixed leukocyte reaction without toxicity (123), preserves and promotes human islet function *in vitro* (38, 39), prevents the transfer of diabetes into young NOD.*scid* mice (49), and inhibits innate-immune NF-κB activation (48). Thus, islet-sparing agents, which decrease the production of free radicals, and therefore, pro-inflammatory cytokines, may have a positive impact on islet function post-transplant.

Since islet transplantation can benefit from agents that inhibit early inflammatory cascades to preserve islet function (44), we hypothesize that redox modulation holds potential as a therapy in islet transplantation to decrease the incidence of β-cell primary non-function. In order to further test the effects of redox modulation we treated human islets with STZ *in vitro* and treated mice *in vivo* with STZ, both in the presence or absence of CA, to mimic the antigen-

independent free radical and inflammatory damage of post-transplant ischemia-reperfusion injury. To examine the effects of islet-directed CA-treatment on innate-mediated (antigen-independent) primary islet non-function *in vivo*, we performed syngeneic (175 islets/recipient) islet transplants in diabetic mice and assessed islet function by fasting intraperitoneal glucose tolerance tests (IPGTT). Lastly, we performed allogeneic (300 islets/recipient) islet transplants in diabetic recipients to assess islet function in the presence or absence of islet-directed or systemic redox modulation in the presence of both, innate (antigen-independent) and adaptive (antigen-dependent), immune responses. Our results demonstrate that islet-directed and systemically delivered redox modulation are protective of islet function post-transplant.

3.3 MATERIALS AND METHODS

3.3.1 Human islets

Human pancreata were obtained from CORE (Center for Organ Recovery and Education, Pittsburgh, PA) and were harvested using standard multi-organ recovery techniques and islets were isolated as previously described (38).

3.3.2 Human islet experiments

Islet preparations were cultured in flasks at 37°C in an atmosphere of 5% CO_2 in humidified air in human islet medium containing CMRL-1066 (Gibco-BRL) medium supplemented with 10% FCS, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM L-

glutamine (Life Technologies, Grand Island, NY). The islets were cultured for 3 days and hand-picked on the 4th day using a dissecting microscope. Groups of 60 hand-picked islets were randomly assigned to control and experimental groups. Each group was sub-cultured in 60 x 15-mm Falcon dishes at a concentration of 12 islets/ml for 8 hours in the previously described islet media. The CA group was treated with 68 μM CA and the STZ group was treated with 11 mM STZ while the control group was cultured in islet media alone. The group treated with CA and STZ was treated with 68 μM CA 20 minutes prior to the addition of 11 mM STZ.

3.3.3 Human islet viability assays

Islet viability was determined by simultaneous staining of live and dead cells using a two-color fluorescence assay (acridine orange (green=live) and ethidium bromide (red=dead), Sigma, St. Louis, MO). After the 8-hour incubation, islets from each group were transferred into separate microcentrifuge tubes, washed with PBS, and spun at 2000 rpm for 2 minutes. Supernatants were carefully aspirated, leaving ~ 25 μl to allow re-suspension of the cell pellets. Next 1.3 μl of dye mix (100 μg/ml acridine orange + 100 μg/ml ethidium bromide in PBS) was added to each tube to stain all islet cell nuclei. The tube was mixed gently, 25 μl of the cell suspension was transferred to a microscope slide, and a cover slip was placed on top of the suspension. Cells were visualized at 10x magnification using a fluorescence microscope with an excitation of 450-490nm. At least 3 fields from each group were analyzed by ImageJ (NIH, Bethesda, MD) software. The percentage of viable and dead cells was determined by linearly converting ImageJ arbitrary units into percentages.

3.3.4 Mice

Male 6-8 week old C57BL/6 and BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME). C57BL/6-Ins2$^{akita/+}$ breeder pairs (female C57BL/6 + male C57BL/6-Ins2$^{akita/+)}$ were also purchased from Jackson Laboratories, but bred in-house at the Rangos Research Center (Pittsburgh, PA). Male C57BL/6-Ins2akita/+ mice develop spontaneous diabetes by 4 weeks of age and do not require exogenous insulin or fluids/electrolytes to thrive. This strain is well-suited for transplantation studies because they become spontaneously diabetic, ease of care, and ability to be rendered indefinitely euglycemic via syngeneic islet transplants (300 C57BL/6 islets), yet reject islet transplants from MHC-mismatched donors (300 BALB/c islets) (124).

3.3.5 Systemic treatment with CA to inhibit STZ-induced diabetes

C57BL/6 male mice were injected IP with 10 mg/kg CA for 7 days. On day 2 all mice were given an intravenous (IV) injection of 170 mg/kg STZ. Blood glucose was measured every other day. Two consecutive blood glucose readings over 300 mg/dl were indicative of STZ-induced diabetes. Diabetic animals were sacrificed following the second consecutive reading.

3.3.6 Syngeneic transplants

8-12 week old C57BL/6 males were used as donors and recipients. Recipients were injected intraperitoneally (IP) with 240 mg/kg STZ (Sigma) on day 1. Animals were tested for

diabetes using urine-test strips (Bayer) on day 3. All diabetic animals received 1 unit of insulin (Lantus) and 500 μl Ringers solution on days 3-5. On day 5, islets were isolated from naive donor C57BL/6 mice as described in (125). The same day, islets were simultaneously picked, counted, and redistributed into petri dishes containing 175 islets each. Each dish of islets contained islet media (10% heat-inactivated FBS, 2% Hepes [1M], 1% Penicillin/Streptomycin [10,000 mg/ml], 1 % L-glutamine [200mM], and 0.1% 2-β-mercaptoethanol [50mM] in RPMI sterile-filtered) alone or islet media plus 68 μM CA. Treated and control islets were incubated for 24 hours at 37 degrees C and 5% CO_2. Non-fasting blood glucose levels were tested in recipient mice on day 6 by obtaining a small blood sample from the retro-orbital sinus. Only animals with blood glucose 400 mg/dl and above were used as recipients in this study. On day 6, each recipient was transplanted with 175 syngeneic CA-treated or untreated control islets inserted under the kidney capsule as described in (125). Blood glucose was tested every other day. All mice normalized post-transplant.

On post-operative day 9 and 70, transplant recipients were subject to a fasting intraperitoneal glucose tolerance test (IPGTT). Food was removed from the recipients' cages 18 hours prior to IPGTT with water available ad lib. Mice were weighed and a baseline (zero minute) blood glucose was taken immediately before each recipient was injected with a 10 μl/g body weight dose of sterile-filtered 20% glucose solution in dH_20. Blood glucose readings were taken at 30, 60, 90, and 120 minutes post glucose injection. Nephrectomies were performed on Day 77 as described in (126).

3.3.7 Islet-directed CA-treatment to delay allograft rejection

Male C57BL/6 mice were used as transplant recipients and male BALB/c mice were used as islet donors. C57BL/6 mice were rendered diabetic with an intraperitoneal (IP) injection of 240 mg/kg STZ. Recipients were prepared for transplant as previously described and islets were incubated in islet media alone or in islet media with 68 μM CA for 24 hours. Three hundred CA-treated or control-treated BALB/c islets were transplanted under the kidney capsule of diabetic C57BL/6 recipients as previously mentioned. All diabetic recipients became euglycemic post-transplant. Blood glucose was checked every other day. Two consecutive blood glucose readings over 400 mg/dl were indicative of allograft rejection. Diabetic animals were sacrificed following the second consecutive reading.

3.3.8 Systemic delivery of CA in pellet form to delay allograft rejection

Male C57BL/6-Ins2$^{akita/+}$ mice were used as transplant recipients and male BALB/c mice were used as islet donors. This work is based on (124) and the transplants were performed as previously mentioned, transplanting 300 BALB/c islets into each spontaneously diabetic C57BL/6-Ins$^{2akita/+}$ recipient on day 0. Three days before transplant, a placebo or 21-day CA pellet (0.1 mg/day or 5 mg/kg/day) (Innovative Research of America, Sarasota, FL) was inserted into each recipient. An additional pellet was inserted into mice on post-operative day 16, as pellets were administered every 20 days. All diabetic recipients became euglycemic post-transplant. Blood glucose was checked every other day. Two consecutive blood glucose

readings over 400 mg/dl were indicative of allograft rejection. Diabetic animals were sacrificed following the second consecutive reading.

3.3.9 Hematoxylin and eosin (H&E) staining of islet-bearing kidneys

Mouse kidney samples were fixed in 4% paraformaldehyde for 3hrs then transferred to 30% sucrose. After imbedded in frozen section medium (Richard-Allan Scientific, Kalamazoo, MI.), cryosections (10μm) were cut using a cryostat (Microm HM550, Germen) and mounted onto gelatin coated or pre-cleaned slides. Hematoxylin and eosin staining was performed on pre-cleaned slides using a Frozen Section Staining kit (Thermo Electron Corporation, Pittsburgh, PA). Images were captured at 40x magnification using a Nikon confocal microscope (Nikon D-ECLIPSE C1, Japan).

3.3.10 Statistical analysis

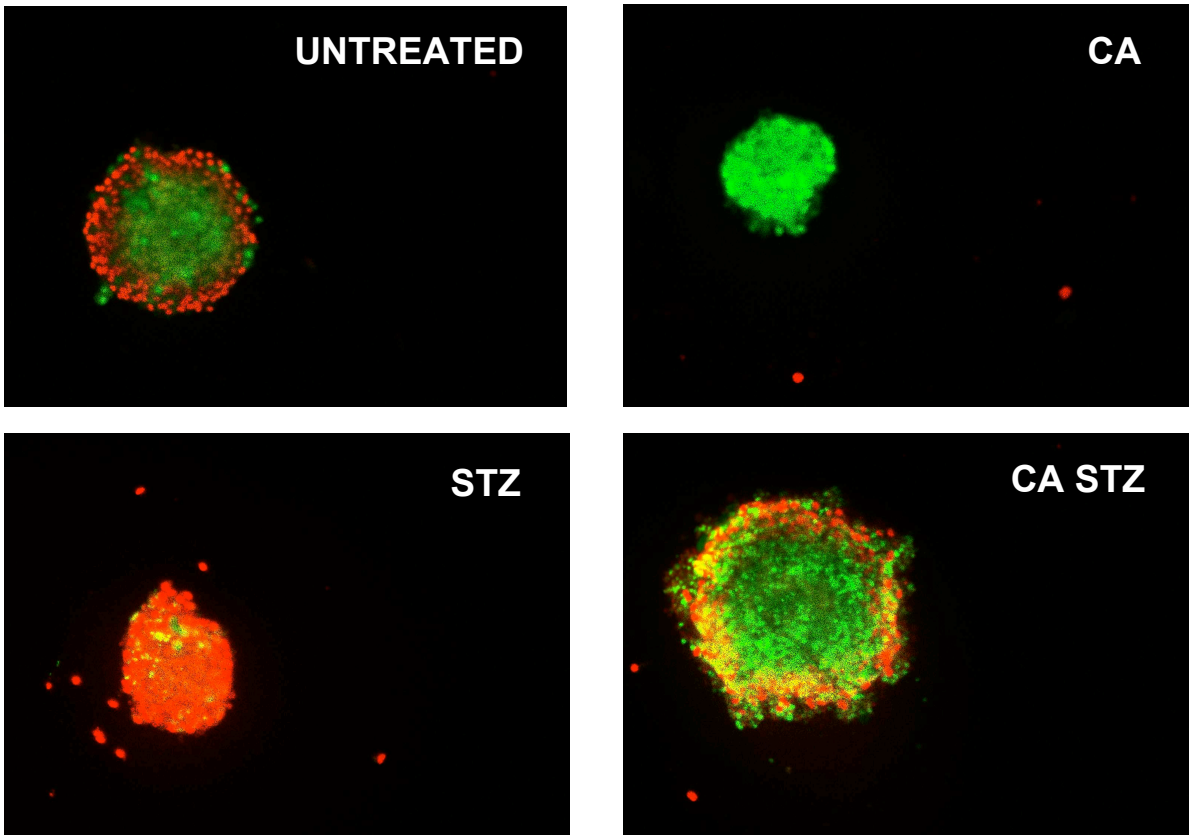
Mean data are expressed as standard error of the mean (SEM). The difference between mean values was determined by a Student t test and the area under the curve was determined using the trapezoidal rule. Kaplan-Meier survival plots were analyzed by the Log-rank test. All statistical analysis was performed with the aid of PRISM (Graphpad, San Diego, CA) and JMP statistical software from the SAS institute using $p<0.05$ to achieve significance.

3.4 RESULTS

3.4.1 CA protects human islets from STZ-induced cell death

Using STZ to mimic free radical damage and inflammation in ischemia-reperfusion injury human islets isolated from donor pancreata were divided into 4 groups and were cultured in 1) media alone (untreated), 2) CA, 3) STZ, or 4) CA+ STZ. After an 8-hour incubation, a double-fluorescence viability assay was performed using acridine orange, which penetrates the plasma membrane of living cells and stains their nuclei green, and ethidium bromide, which only penetrates dead cells, in which membrane integrity is compromised, to stain their nuclei red. Panels in Figure 22A are representative fluorescent images from each treatment group with correlating to data analysis graphed in Figure 22B and exact percentages and significance listed below the graph. We observed a significant increase in live cells with CA treatment (74.99 +/- 8.59%) compared to untreated control islets (41.64 +/- 3.38%) and a substantial decrease in viability was recorded when islets were treated with STZ (20.02 +/- 0.65%). As we hypothesized, islets treated with STZ in the presence of CA were comparatively more viable (63.24 +/- 5.99%), demonstrating a significant increase in cell viability versus islets treated with STZ alone (Figure 22B). These data indicate that the addition of CA increases viability of human islets when used in the media alone, and even more significantly, when islets are treated with CA using STZ-treatment as a model of free-radical induced cell death.

A



B

% LIVE CELLS	P-VALUE
UNTREATED (41.64 ± 3.38) vs. CA (74.99 ± 8.59)	.0229
STZ (20.05 ± 0.65) vs. UNTREATED (41.64 ± 3.38)	.0103
STZ (20.05 ± 0.65) vs. CA STZ (63.24 ± 5.99)	.0089

Fig. 22. CA protects human islets from STZ-induced cell-death. Human islets (60 islets/group) were cultured in media alone, 68 μM CA, 11 mM STZ, or 68 μM CA + 11 mM STZ. A: Representative images of each group of islets after an 8-hour incubation, stained with acridine orange (green/live) and ethidium bromide (red/dead), then visualized under a fluorescence microscope at 10x magnification. B: The percentage of live and dead islet cells from A was assessed by Imagej software and the data is summarized in the table below B. Open bars = live cells ($n \geq 3$) and black bars = dead cells ($n \geq 3$). Data are presented as means (+/- SEM). Significance was tested using Student t tests (*$p<0.05$).

3.4.2 Systemic treatment with CA inhibits streptozotocin-induced diabetes

Streptozotocin (STZ) induces diabetes through a nitric oxide free radical mechanism resulting in DNA damage and islet cell death (127). Since CA can inhibit free radical damage (26, 48, 123, 128) we wanted to determine if systemic CA-treatment could protect islets from STZ-induced islet cell death *in vivo*. Five C57BL/6 mice were injected with 10 mg/kg CA daily for 7 days and 10 C57BL/6 mice were used as untreated controls. On day 2 all mice were injected with STZ. As shown in Figure 23, 90% of untreated mice (9/10) succumbed to STZ-induced islet cell death and subsequent diabetes (2.3 +/- .6 days) while none of the CA-treated mice (n=5) developed diabetes (>120 days) (Log-rank p=0.005). These results demonstrate that systemic treatment with CA protects islets from free radical damage to prevent STZ-induced diabetes.

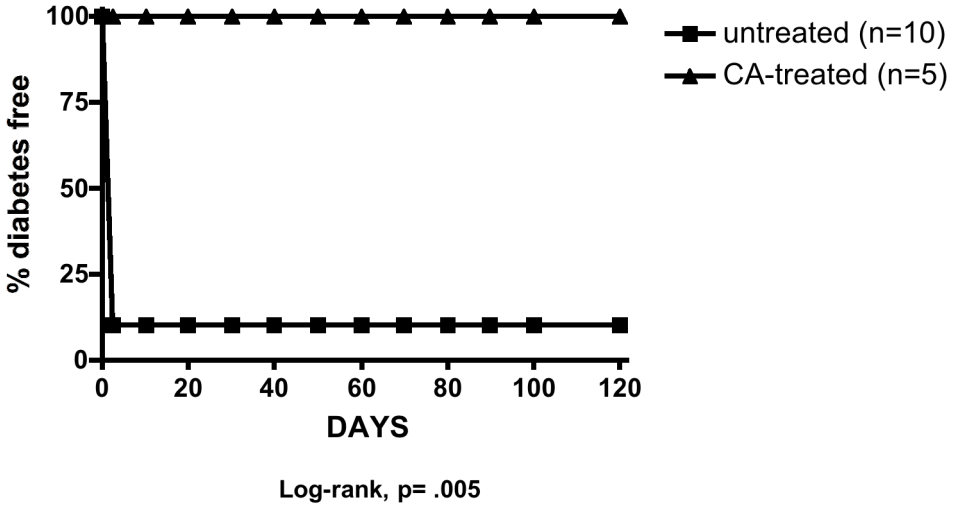


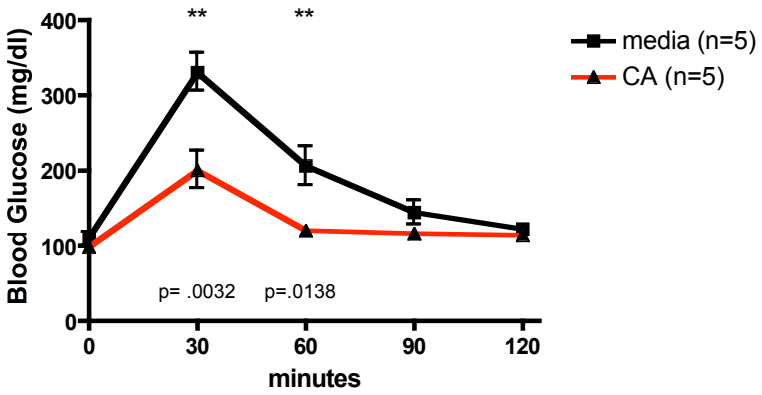
Fig. 23. Systemic CA-treatment inhibits STZ-induced diabetes. Mice received CA-treatment on days 1-7 and STZ was given on day 2. Black squares = untreated (n=9); Black triangles = CA-treated (n=5). Significance was tested using the Log-rank test (p=.0005).

3.4.3 Islets incubated with CA prior to syngeneic transplant exhibit increased function by intraperitoneal glucose tolerance test (IPGTT)

Based on previously published data demonstrating improved islet mass, viability, and function when human islets are treated with CA during isolation or in culture (38, 39), we wanted to determine if islet-directed CA can improve graft function in a syngeneic transplant model. Diabetic C57BL/6 recipients were transplanted with 175 syngeneic islets, which were previously incubated in the presence (n=5) or absence (n=5) of 68 μM CA for 24 hours. All animals were rendered euglycemic post-transplant and sustained long-term euglycemia. Post-nephrectomy all transplant recipients reverted to diabetes (data not shown). Fasting IPGTTs were performed on post-operative day 9 and day 70.

Recipients of CA-treated islets demonstrated significantly improved glycemic-control at 30 and 60-minute time points compared to untreated controls (Figure 24A), which correlates to a significant decrease in the area under the curve for recipients of CA-treated islets (Figure 24B). Specifically, the area under the curve for recipients of CA-treated islets was less than half (4,561.4 +/- 1054.2 min*mg/dl) of the area under the curve for recipients of untreated islets (10,828.8 +/- 1,336.1 min*mg/dl). To observe any long-lasting affect of the CA-incubation on islet function, mice from each group were monitored long-term and another IPGTT was performed on day 70. Although the 70-day IPGTT did not show statistical significance, the trend of improved glycemic control in recipients of CA-treated islets, namely at 30 and 60 minutes, was maintained (Figure 24C). These results are evidence that islet-directed CA-treatment is able to suppress antigen-independent ischemia-reperfusion injury as demonstrated by improved islet graft function.

A



B

p=.0034	Area Under Curve
Media alone	10,828.8 +/- 1,336.1
CA-treated	4,561.4 +/- 1,054.2

C

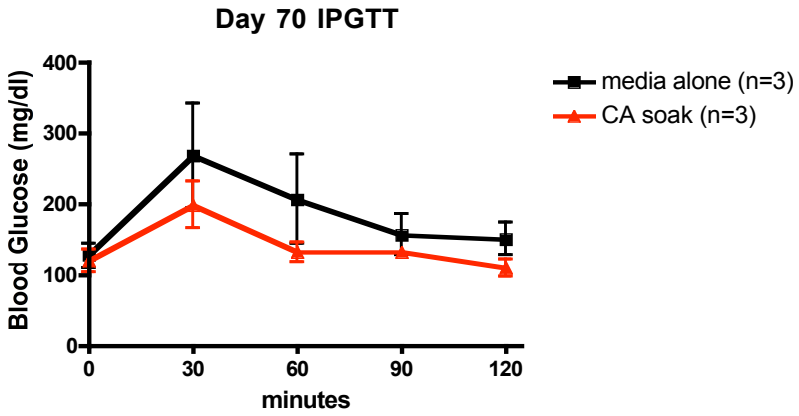


Fig. 24. Pre-treating islets with CA prior to syngeneic transplant improves islet function. Islets were incubated ± CA for 24 hrs then 175 untreated or CA-treated islets were transplanted into syngeneic recipients. A: Fasting IPGTTs were performed on post-operative day 9. Black squares/black line = untreated islets (n=5). Black triangles/red line = CA-treated islets (n=5). B: Area under the curve calculation for (A). C: Fasting IPGTT performed on post-operative day 70. Black squares/black line = untreated islets (n=3). Black triangles/red line = CA-treated islets (n=3). Significance was tested for A, B , and C using Student t tests (*$p<0.05$).

3.4.4 Islet-directed CA-treatment delays allograft rejection in an MHC-mismatched islet transplant model

Next, we tested the ability of islet-directed redox modulation to improve islet allograft survival. Since islet-directed CA-treatment can preserve islet function during antigen-independent ischemia-reperfusion injury in a syngeneic transplant model (Figure 24) and in human islet isolation (38, 39), we wanted to determine if islet-directed redox modulation using CA could reduce normalization blood glucose or delay antigen-dependent allograft rejection. These experiments were performed using BALB/c (H-2d) donor islets incubated in the presence or absence of 68 μM CA for 24 hours. After the 24 hour incubation 300 CA-treated or untreated islets were transplanted into diabetic C57BL/6 (H-2b) recipients.

Post-transplant, CA-treated islets normalized recipients (n=5) to significantly lower blood glucose levels (73 +/- 2.61 mg/dl) compared to recipients (n=5) of control treated islets (118.2 +/- 8.35 mg/dl) (Figure 25A), indicative of an increase in early islet graft survival ($p< 0.0001$). Though all allograft recipients were eugylcemic for over 2 weeks, the recipients of CA-treated islets retained euglycemia, and thus, a functioning allograft, significantly (Log-rank, p=.0246) longer (26 +/- 0 days) than the recipients of untreated islets (22.3 +/- 2.5 days) (Figure 25B**)**. Taken together, these data indicate that cytoprotection of islets using islet-directed CA-treatment alone can delay allograft rejection.

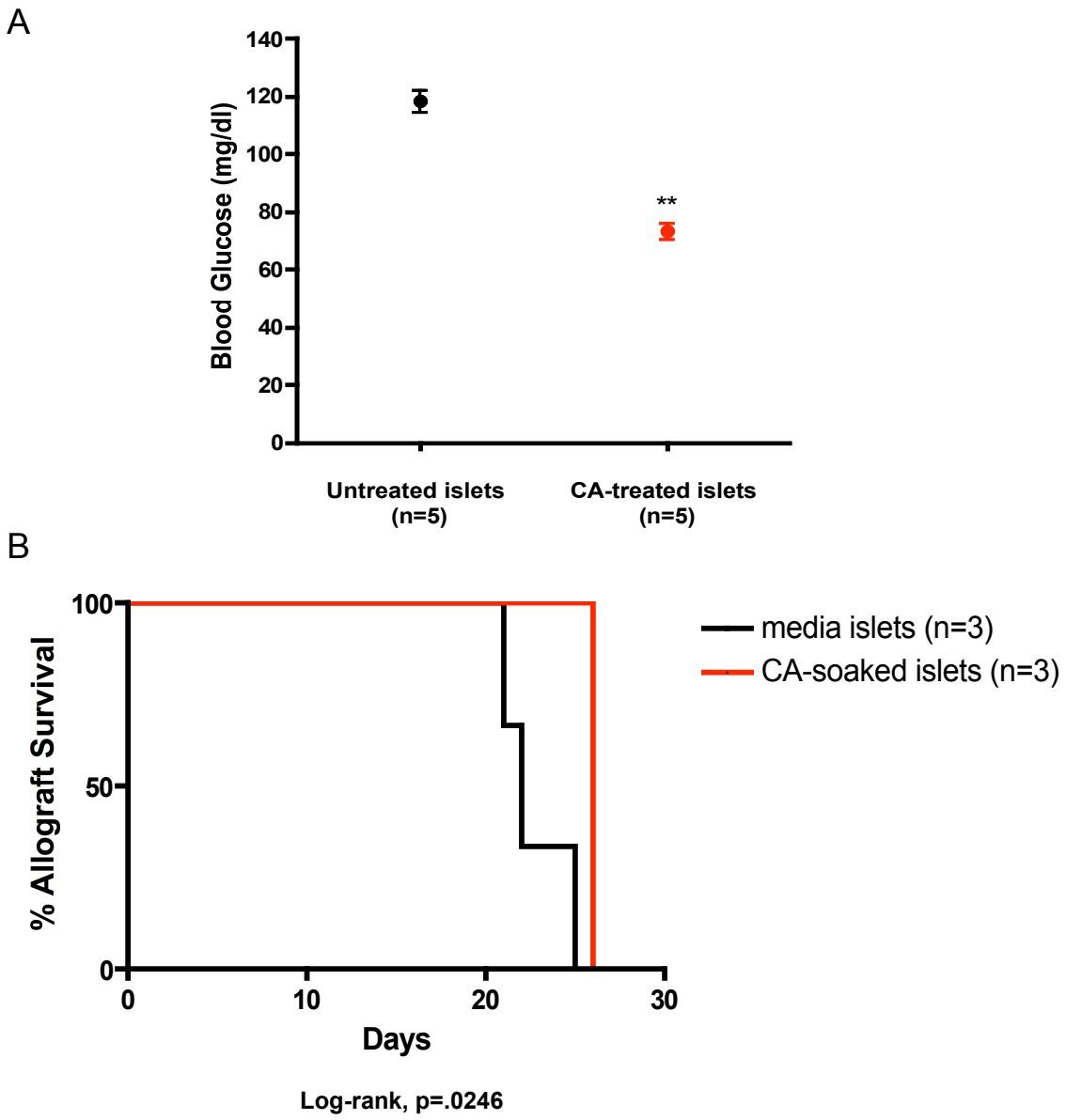
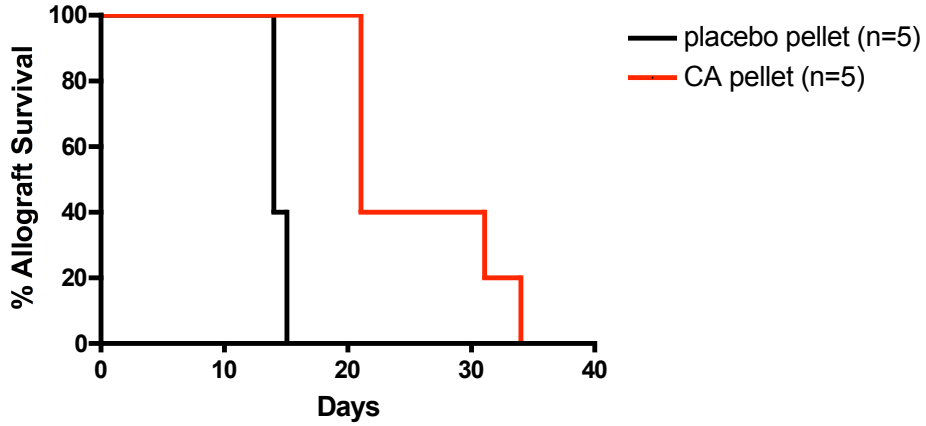


Fig. 25 Pre-treating islets with CA prior to allogeneic transplant improves islet function. Islets were incubated ± CA for 24 hrs then 300 untreated or CA-treated islets were transplanted into diabetic allogeneic recipients. A: Normalization blood glucose of recipients within 2 post-operative days; n of 5/group. Significance was tested using a Student t test (*$p<0.05$). B: Allograft survival of recipients; n of 3/group. Significance was tested using a Log-rank test ($p=.0246$). Black = recipients of untreated islets. Red = recipients of CA-treated islets.

3.4.5 Systemic delivery of CA prolongs allograft function in an MHC-mismatched islet transplant model

To determine if systemic administration of CA would equate to a more substantial delay in allograft rejection we transplanted 300 BALB/c islets into spontaneously diabetic C57BL/6-Ins2$^{Akita/+}$ mice (124). In our colony, 7-8 week old diabetic C57BL/6-Ins2$^{Akita/+}$ males have a blood glucose level of 580 +/- 19 mg/dl, which is comparable to the average blood glucose level (544 +/- 11 mg/dl) for the same mice housed at the Jackson Laboratory (Maine). Three days before transplant a CA (21-day, 5 mg/kg/day) or placebo pellet was inserted into diabetic recipients. All 10 recipient mice normalized post-transplant (125 +/- 16.94 mg/dl). CA-treated recipients (n=5) displayed a significant increase (Log-rank, p=.0023) in graft function (25.60 +/- 2.86 days) compared to untreated recipients (n=5) (14.40 +/- 0.25 days) (Figure 26A). H&E sections of islet-bearing kidneys post-rejection demonstrate a pronounced infiltrate in untreated recipients and a sparse infiltrate in CA-treated recipients (Figure 26B), suggesting the delay in graft rejection with CA-treatment is associated with decreased migration of immune cells to the site of the graft. These results demonstrate that systemic delivery of CA can delay graft rejection by depressing the cytotoxic free radical and inflammatory damage generated by the innate (antigen-independent) immune response and possibly by impacting the T-cell-mediated adaptive (antigen-dependent) immune response (26, 123) to hinder allograft rejection.

A



Treatment	Placebo pellet (n=5)	CA pellet (n=5)
Mean days to diabetes (p=.0107)	14.40 +/- 0.25 days	25.60 +/- 2.86 days

B

CA-treated | Untreated

Fig. 26. Systemic CA-treatment delays allograft rejection. On day 0 diabetic recipients were treated with a placebo or CA-pellet. 300 allogeneic islets were transplanted into all recipients on day 3. A: Allograft survival of recipients. Black = placebo pellet (n=5). Red = CA pellet (n=5). Rejection table below A. Significance was tested using a Log-rank test (p=.0023). B: Post-rejection H&E staining of representative sections of islet-bearing kidneys from untreated and CA-treated recipients.

3.5 DISCUSSION

Allograft acceptance can be achieved using immunosuppressive drugs, though most drugs that inhibit T-cell-mediated graft destruction have the unfortunate side effect of islet toxicity and do not significantly protect islets from ischemia-reperfusion insults (15, 23, 122) leading to primary non-function and β-cell death. Current literature in islet transplantation expresses a need for improved therapeutics that control rejection while preserving islet function through cytoprotection (7, 15, 38, 39, 44, 121, 129, 130). Since redox modulation affects the innate and the adaptive immune responses (26, 48, 123) and demonstrates cytoprotection during human islet isolation (38, 39), we hypothesized that it may be a useful approach in islet transplantation. Because only a fraction of transplanted islets survive ischemia-reperfusion injury (38, 39, 119), our current study utilized the well-described redox-modulator, CA, to determine if islet graft survival and function could be improved by inhibiting free radical and inflammatory damage.

In this study CA protects islets from STZ-induced free radical damage, antigen-independent inflammatory ischemic damage during syngeneic transplantation, and delays rejection in allogeneic transplantation. Specifically, we used STZ-induced diabetes as a model of islet cell death and found that systemic delivery of CA protected all mice from STZ-induced diabetes (Figure 23) likely by increasing antioxidant defenses in islets to render them more resistant to STZ-induced free radical damage. To isolate antigen-independent ischemia-reperfusion injury in a transplant setting, we used a syngeneic transplant model to analyze the

effects of pre-treating islets with CA. Data from the IPGTTs indicate that islet-directed CA protects islet function during antigen-independent inflammatory injury (Figure 24A-C).

Antigen-independent injury by the innate immune system plays a larger role in allograft rejection than previously thought correlating to the activation state of the powerful redox-dependent transcription factor, NF-κB. NF-κB shapes the innate and adaptive immune responses (29, 41, 42, 60, 63, 65) by controlling a myriad of pro-inflammatory and pro-apoptotic genes in multiple cell types, including β-cells (131-133). Our previously published work indicates that redox modulation using CA can hinder apoptotic and necrotic pathways by inhibiting NF-κB-DNA binding, PARP activation, and the production of chemokines and cytokines in human islets (39). In this study we expanded our previous work with human islets (38, 39) using STZ-treatment in the presence or absence of redox modulation to mimic islet cell damage incurred during ischemia-reperfusion injury. Our data demonstrates that redox modulation is protective in a robust setting of ischemia-reperfusion injury (Figure 22), likely by hindering the previously mentioned apoptotic and necrotic pathways. Other studies have also sighted NF-κB in islet death, demonstrating that manipulating components of the NF-κB pathway can hinder its activation, and imminent inflammatory damage, to protect islets from apoptosis in autoimmunity and islet transplantation (133-135). Since redox modulation using CA can enter mitochondria *in vivo* to impart antioxidant protection (128), preservation of mitochondrial function, and therefore ATP, is a potential mechanism by which CA protects from β-cell death (123). Simultaneous use of systemically delivered redox modulation to treat the recipient (Figures 23 and 26) and islet-directed CA to treat the donor islets (Figures 22 and 24) would limit β-cell exposure to cytokine and NO damage, thus hindering the hypoxic and inflammatory onslaught endured by islets during isolation and ischemia-reperfusion injury post-transplantation.

Moreover, Contreras et. al. notes that increasing islet yield post-isolation is paramount in order to propel the success of clinical islet transplantation, as the Edmonton protocol requires 1 to 3 pancreata per recipient (14). Currently, the demand for islets far outweighs the supply (14), especially since more than one islet infusion is frequently required to eliminate the need for exogenous insulin (12). Since CA-treatment increased islet viability in human (*in vitro*) (Figure 1) (38, 39) and murine (*in vivo*) (Figures 23-25) models of ischemia-reperfusion injury, CA-treatment may allow a reduced number of islets (islet equivalents) needed to normalize a recipient. The ability to decrease the number of human pancreata/islet infusions required to achieve long-term insulin-independence would overcome a major hurdle in islet transplantation (12, 14, 25). Additionally, it has been reported that rodent islets suffer from decreased protection from oxidative stress compared to human islets (136). This evidence suggests that our favorable results using redox modulation in murine models of transplantation may translate successfully to clinical transplantation.

In addition to reducing primary islet non-function (Figure 24), islet-directed CA-treatment also demonstrated a protective effect in antigen-dependent allogeneic islet transplantation (Figure 25). Comparatively, systemic delivery of CA **(**Figure 26**)** extends allograft rejection beyond islet-directed CA-treatment to support islet engraftment, viability, and function because CA can also act as an immunomodulatory agent by inhibiting APC activation (48), CD4 (26) and CD8 T cell effector function (123) and CTL target cell lysis by decreasing cytolytic effector molecule production in transgenic and alloreactive models (123). However, in this study, we are not certain if the delay in allograft rejection is due to increased cytoprotection and preservation of islets by CA during ischemia-reperfusion injury (Figures 24 and 25) or as a result of immune-modulation correlating to decreased activation of the alloimmune-response.

Both scenarios resulting in a subsequent decrease in migration of immune cells to the sight of the graft in CA-treated recipients (Figure 26) and most likely, the delay in allograft rejection is a combination of the two.

CA is known to promote islet (38, 39) and cell survival while inhibiting effector function (26, 123), however, studies examining the effects of redox modulation on β-cell proliferation and genes that regulate proliferation are yet to be performed. Because CA inhibits primary immunogenic proliferation (26, 123) and sustains disruption of effector function, but does not significantly inhibit secondary expansion (26), redox modulation could be of benefit when coupled to non-calcineurin or mTOR targeted anti-proliferative drugs, like mycophenolate mofetil (MMF). Additionally, if islets are treated with CA prior to transplant, they may be afforded increased protection from toxic immunosuppressives currently used in transplant protocols thereby increasing transplant success by limiting graft loss. Redox modulation may also allow for the dose and duration of immunosuppressants to be weaned over a transplant recipient's lifetime since CA protects from the inflammatory damage associated with islet isolation and transplantation. Taken together, redox modulation is a favorable therapeutic to add to the currently administered immunosuppressives since CA is a nontoxic, non-immunogenic small molecule compound that offers the flexibility of systemic and/or tissue-specific treatment, ease of application, and a history of reproducible outcomes (26, 38, 39, 48, 49, 123, 128). The reported benefits of CA-treatment for islets, autoimmunity and alloimmunity suggest redox modulation as a clinical approach, especially for diabetic recipients of islet allografts.

4.0 CHAPTER 4: AN ANTIGEN-SPECIFIC NEGATIVE VACCINATION USING APOPTOTIC DONOR CELLS ADMINISTERED IN A NON-INFLAMMATORY ADJUAVANT INDUCES LONG-TERM ALLOGRAFT TOLERANCE

4.1 ABSTRACT

The many complications involved in islet transplantation yield poor outcomes limiting the procedure to adults with a history of extreme glucose dysregulation. The main impediments restraining islet transplant success include islet primary non-function and repercussions on islet and recipient health resulting from nonspecific immunosuppression. In this study we examined two methods to delay/prevent allograft rejection with the goal of reducing the previously mentioned pitfalls of islet transplantation. Redox modulation, using a well-characterized small molecule catalytic antioxidant (CA), FBC-007, is the first approach we used. CA improves islet function and viability, suppresses innate and adaptive immune activation, and is capable of achieving antigen-specific hyporesponsiveness. We determined that this strategy is not harshly immunosuppressive and decreases activation and migration of immune cells to the site of the

islet allograft thus delaying allograft rejection. The second strategy is a negative vaccination. We administered apoptotic donor splenocytes in a non-inflammatory adjuvant prior to islet transplantation to induce donor-specific tolerance. This method proved even more successful than systemic CA-treatment as the negative vaccination tolerized recipients to allogeneic donor islets. These results demonstrate viable therapeutic approaches using islet-sparing and antigen-specific strategies to delay/prevent allograft rejection in an effort to minimize difficulties with islet transplantation.

4.2 INTRODUCTION

Currently, islet transplantation is limited to brittle diabetics (11) due to complications from harsh immunosuppressive therapy and a lack of long-term glycemic control post-transplant (12). Though immunosuppressive drugs are efficient at inhibiting the T-cell mediated destruction of the donor islet graft, they are not cytoprotective, are associated with islet toxicity, and very rarely protect from nonspecific inflammation due to ischemia reperfusion injury (15, 23, 122). The current nonspecific regimen of immunosuppression can lead to complications including infection and malignancy requiring the recipient to stop immunosuppressants, upon which the recipients succumb to allograft rejection (13). The outlook of islet transplantation in its present form is grim. Only 5/36 subjects remained insulin-independent 2 years post-transplant as reported in a trial of the Edmonton islet transplantation protocol (13). In agreement with the literature, solutions to hurdles in islet transplantation can be found in therapies that are islet-sparing and/or antigen-specific to improve post-transplant outcomes (7, 15, 38, 39).

In this study we analyzed the capabilities of two novel approaches in islet transplantation. The first approach focuses on cytoprotection for islets and immunomodulation of the alloimmune response using redox modulation. Redox modulation by the catalytic antioxidant (CA), FBC-007, has proven islet-sparing and immunomodulatory as demonstrated in our previous *in vitro* studies which detail the ability of CA to impair NF-κB activation in APCs (48) and to decrease CD4 (26) and CD8 T cell responses (123). Additionally redox modulation enhances human islet function post-isolation (38, 39) and murine islet function versus nonspecific ischemia reperfusion injury post-transplant (Figure 24). We have previously observed a delay in allograft rejection using systemic redox modulation (Figure 26), however the mechanism(s) responsible for *in vivo* protection from the alloimmune response have not been elucidated. A focus of this study is to investigate the mechanism of CA's immunomodulatory abilities in an *in vivo* model of allogeneic islet transplantation.

The second approach aims to treat transplant recipients in the absence of nonspecific immunosuppression using an antigen-specific negative vaccination approach. The negative vaccination consists of apoptotic donor cells administered prior to allograft transplant in incomplete freund's adjuvant (IFA). It is well-known that apoptotic cells induce tolerance and evade immunity (29, 137, 138). By educating the recipient immune system with apoptotic donor cells, in the absence of inflammation, DCs remain in the steady-state resulting in tolerance versus activation in response to donor alloantigen (5, 36). Antigen is detectable for a minimum of one week when given in IFA (subcutaneously or intraperitoneally), suggesting that unresponsiveness is a result of a slow, persistent depot release of antigen compared to the fleeting persistence of antigen administered in PBS (139, 140). Additionally, subcutaneously administered antigen is detectable longer than antigen given IV due to the route of administration affording a slow

release of antigen into the draining lymph nodes. Subcutaneously delivered antigen can persist for several days allowing an extended period of time for the APCs to process and present antigen unlike quick clearance when antigen is in the circulation via an IV route of delivery (141). Other methods using donor cells to delay allograft rejection have been preformed, but these strategies use live, chemically-treated or manipulated cells, with most protocols requiring the use of some form of immunosuppressant therapy in conjunction with the donor cells to achieve any delay in allograft rejection (43, 142-145). Some groups have irradiated donor splenocytes while others give antigen in IFA (141), but never has there been an approach combining the two methods: using apoptotic donor cells administered subcutaneously in IFA to induce a depot delivery of apoptotic alloantigen in a non-inflammatory environment so the donor antigen can be processed as self-antigen by host APCs (5, 143) to induce tolerance. Warren et al (1986) theorizes that the depot release of antigen is an attribute specific to adjuvants to induce an antigenic response, however, when a non-immunogenic antigen, like apoptotic donor cells, are delivered subcutaneously in a non-inflammatory adjuvant (IFA), we hypothesize that long-term antigen-specific hyporesponsiveness will occur. In this study we analyze the affects of CA-treatment and negative vaccination on the immune system in naïve mice and in an islet transplant setting to elucidate the mechanisms underlying the delay in allograft rejection.

4.3 MATERIALS AND METHODS

4.3.1 Mice

Male 6-8 week old C57BL/6 (H2-b) and BALB/c (H2-d) mice were purchased from

Jackson Laboratories (Bar Harbor, ME). All animal studies were approved and trials were conducted in accordance with the University of Pittsburgh Animal Care and Use Committee.

4.3.2 Viability assays

Spleens from BALB/c mice were harvested, homogenized, and lysed. Half of the splenocytes from each spleen remained untreated and the remaining cells were exposed to 3500 rads using an x-ray machine. Both groups of splenocytes were incubated in Complete Splenocyte Medium (DMEM supplemented with 10% heat-inactivated FBS, 10 mM Hepes buffer, 4 mM l-glutamine, 200 μM nonessential amino acids, 1 mM sodium pyruvate, 61.5 μM 2-mercaptoethanol, and 100 μg/ml gentamicin (Invitrogen Life Technologies, Carlsbad, CA, USA)) under normal conditions (37°C at 5% CO_2 in a humid air incubator) for 16 hours as cell death does not occur immediately after x-ray irradiation. After incubation cells were harvested and stained with FITC-conjugated Annexin-V and Propidium Iodide according to the BD Annexin V-FITC Apoptosis Detection Kit I manufacturer's protocol (BD Biosciences). Fluorescence was measured on a FACSCalibur (BD Biosciences).

4.3.3 Mixed leukocyte reactions (MLRs)

Unidirectional MLRs were performed by co-culture of 10^6 BALB/c splenocyte stimulators (treated with 3500 rad) with 10^6 C57BL/6 splenocyte responders as described by (80) with minor modification. All MLRs were performed in 96-well, round-bottom plates in a final volume of 200 μl CM and incubated for 5 days under normal conditions.

4.3.4 *In vitro* analysis of the T_H1 response after treatment with the therapeutic strategies

C57BL/6 mice were used as "recipients" and BALB/c mice were used as "donors". To analyze the T_H1 response when mice are treated with redox modulation C57BL/6 mice were injected with PBS or 10 mg/kg CA per day for 7 days. On the 7^{th} day, CA-treated and sham PBS-treated mice were sacrificed and the spleens were prepared for a unidirectional C57BL/6 responder MLR as previously described. To analyze the T_H1 response when mice are treated with a negative vaccination 10^7 BALB/c splenocytes were irradiated at 3500 rads and mixed 1:1 in IFA and delivered subcutaneously in C57BL/6 mice at the base of the tail in a volume of 100 μl on day 1. On day 7, untreated and negatively vaccinated mice were sacrificed and MLRs were preformed as previously described. Supernatants of all MLRs were harvested on Days 1-5 and IFN-γ ELISAs were performed.

4.3.5 Cytokine measurements by ELISA

MLR assays were performed as described above and supernatants were harvested on days 1 through 5 to measure IFN-γ production by ELISA. IFN-γ produced in supernatants of 96-well MLR assays were measured using antibody pairs from BD Pharmingen (San Diego, CA). ELISA plates were read on a Spectromax M2 microplate reader (Molecular Devices, Sunnyvale, CA) and data were analyzed using Softmax Pro v5.0.1 (Molecular Devices).

4.3.6 Islet allograft transplants

8-12 week old C57BL/6 males were used as recipients and 8-12 week old BALB/c mice were used as islet donors. Recipients received an intraperitoneal injection of 240 mg/kg streptozotocin (Sigma) on day 1 to induce islet cell death and subsequent diabetes. Animals were tested for diabetes using urine-test strips (Bayer) on day 3. All diabetic animals received 1 unit of insulin (Lantus) and 500 μl Ringers solution on days 3-5. On day 5, islets were isolated from naive donor BALB/c mice as described in (125). The same day, islets were simultaneously picked, counted, and redistributed into petri dishes containing 150 islets each. Each dish of islets contained islet media (10% heat-inactivated FBS, 2% Hepes [1M], 1% Penicillin/Streptomycin [10,000 mg/ml], 1 % L-glutamine [200 mM], and 0.1% 2-β-mercaptoethanol [50mM] in RPMI sterile-filtered). Islets were incubated up to 24 hours at 37 degrees C and 5% CO_2. Non-fasting blood glucose levels were tested in recipient mice on day 6 by obtaining a small blood sample from the retro-orbital sinus. Only animals with blood glucose 400 mg/dl and above were used as recipients in this study. CA-treated recipients were injected with 100 μl of 2mM CA subcutaneously beginning three days before islet transplant and were injected daily throughout the duration of the experiment. Negatively vaccinated mice were injected subcutaneously at the base of the tail with 100 μl of a 1:1 mixture of 10^7 apoptotic BALB/c splenocytes to incomplete freund's adjuvant (IFA) 7 days before the islet transplant. Donor splenocytes were rendered apoptotic through exposure to 3500 rads using a γ-irradiator or x-ray machine and the negative vaccination was emulsified using a leur-lock syringe system (Hamilton) prior to injection. Three hundred untreated BALB/c islets were transplanted under the kidney capsule of diabetic C57BL/6 recipients as previously mentioned. All diabetic recipients became euglycemic post-

transplant. Blood glucose was checked every other day. Two consecutive blood glucose readings over 400 mg/dl were indicative of allograft rejection. Diabetic animals were sacrificed following the second consecutive reading. Some recipients, however, were sacrificed 14 days post-transplant. Their kidneys were used for IHC and H&E staining, and their spleens were used for recall MLRs, for cytokine analysis.

4.3.7 Immunohistochemical analysis

Mouse kidney samples were fixed in 4% paraformaldehyde for 3 hours then transferred to 30% sucrose. After being imbedded in frozen section medium (Richard-Allan Scientific, Kalamazoo, MI.), cryo-sections (10μm) were cut using a cryostat (Microm HM550, Germen) and mounted onto gelatin coated or pre-cleaned slides. Sections were fixed in 2% paraformaldehyde for 5 minutes on gelatin-coated slides and then blocked with 20% normal goat serum for 1 hour at room temperature. Primary antibodies included rat anti-F4/80 1:100 (Caltag Laboratories Inc, Burlingame, CA), rat anti-CD4 1:20, rat anti-CD8 1:20, hamster anti-CD11c 1:30 (BD Biosciences, San Diego, CA). Polyclonal rabbit anti-insulin (1:100) (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) was double stained on these sections. Slides were washed 5 times in PBS and incubated with an Alexa Fluor 488 goat anti-rabbit (Molecular Probes, Eugene, OR), Cy3-conjugated goat anti-rat or goat anti-hamster (Jackson Immuno Research Lab, INC. West Grove, PA) secondary antibody. Then, slides were washed 3 times in PBS and nuclear staining was preformed with Hoechst stain. Next, slides were mounted with cover glass and images were captured at 20x magnification using a Nikon confocal microscope (Nikon D-ECLIPSE C1, Japan).

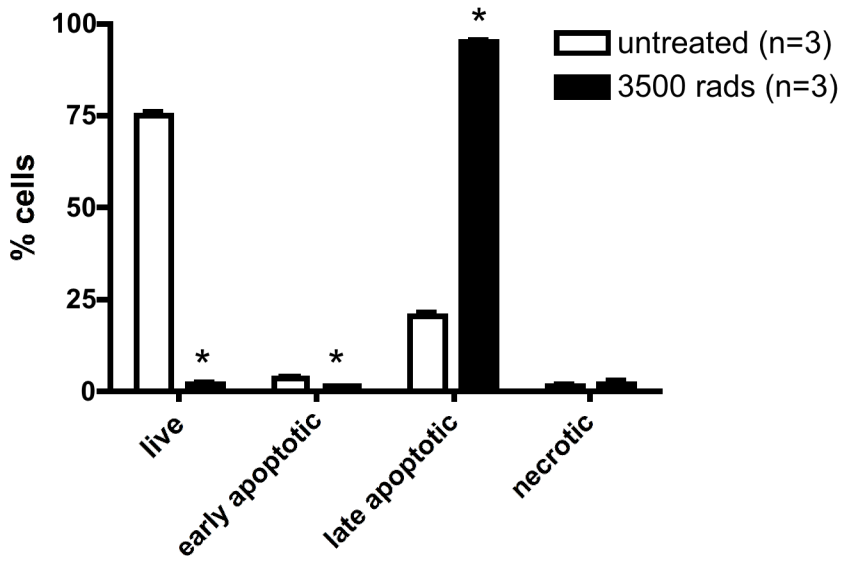
4.3.8 Statistical Analysis

Mean data are expressed as standard error of the mean (SEM). The difference between mean values was determined by a Student t test and the area under the curve was determined using the trapezoidal rule. Kaplan-Meier survival plots were analyzed by the Log-rank test. All statistical analysis was performed with the aid of PRISM (Graphpad, San Diego, CA) and JMP statistical software from the SAS institute using $p<0.05$ to achieve significance.

4.4 RESULTS

4.4.1 Treating splenocytes with 3500 rads induces apoptosis

It is well-documented that apoptotic cells can induce tolerance as they present antigen in the absence of inflammation (5, 36, 137, 138). To demonstrate that exposing cells to radiation induces apoptosis we performed a viability assay on untreated splenocytes or splenocytes which were exposed to a dose of 3500 rads (Figure 27). After 18 hours in culture, the majority of untreated cells were live (74.86 +/- 0.80), while the majority of cells exposed to radiation were late apoptotic (94.94 +/- 0.39). This data indicates that radiation induces apoptosis in over 90% of cells, making the cell preparation suitable for tolerance induction using irradiated donor cells, requiring no further manipulation or exogenous treatment with any reagent. This data also demonstrates the negligible presence of necrotic cells (2.05 +/- 0.61), which are known to induce inflammation (137, 138).



	Untreated	3500 rads
Live	74.86 +/- .80	1.87 +/- .23
Early apoptotic p<.0001	3.49 +/- .34	1.19 +/- .08
Late apoptotic p=.0096	20.36 +/- .85	94.94 +/- .39
Necrotic p<.0001	1.29 +/- .37	2.05 +/- .61

Fig. 27. Irradiation induces apoptosis in BALB/c splenocytes. BALB/c splenocytes were untreated (white bars) or treated with 3500 rads (black bars). Cells from both groups were plated at 10^6 cells/well in a 96 well plate. 18 hours later cells were harvested for a viability assay using Annexin V and PI. The top panel shows a distribution of the cell populations, which were assigned groupings based on classical Annexin V and PI staining. The lower panel details the graph in the top panel showing specific p values. Results are presented as the mean (± SEM) of three independent experiments. **, $p < 0.05$ between the respective treated and untreated (control) group.

4.4.2 The T_H1 response after treatment with the therapeutic strategies in naïve mice not receiving a transplant

The T_H1 response is the dominant component of the T-cell mediated alloimmune response that destroys donor allografts. For this reason, the effect of our therapeutic strategies on IFN-γ production was analyzed in naïve animals prior to attempting our strategies in allograft transplantation. We tested our therapeutic approaches by administering treatment to C57B/6 mice and then sacrificing the animal 7 days later to perform *in vitro* MLRs to measure the production of IFN-γ in the supernatants as an indicator of any effect on the T_H1 response. Animals were untreated, sham injected with PBS, treated daily with 10 mg/kg CA for 7 days, or received a negative vaccination of irradiated BALB/c splenocytes in IFA given subcutaneously 7 days prior to MLR (Figure 28). Compared to untreated control mice, mice receiving daily CA-treatment (Figure 28A) or the negative vaccination (Figure 28B) demonstrated a marginally significant decrease in IFN-γ production on day 5 of an MLR. These data suggest that our therapeutic strategies are not immunogenic or harshly immunosuppressive.

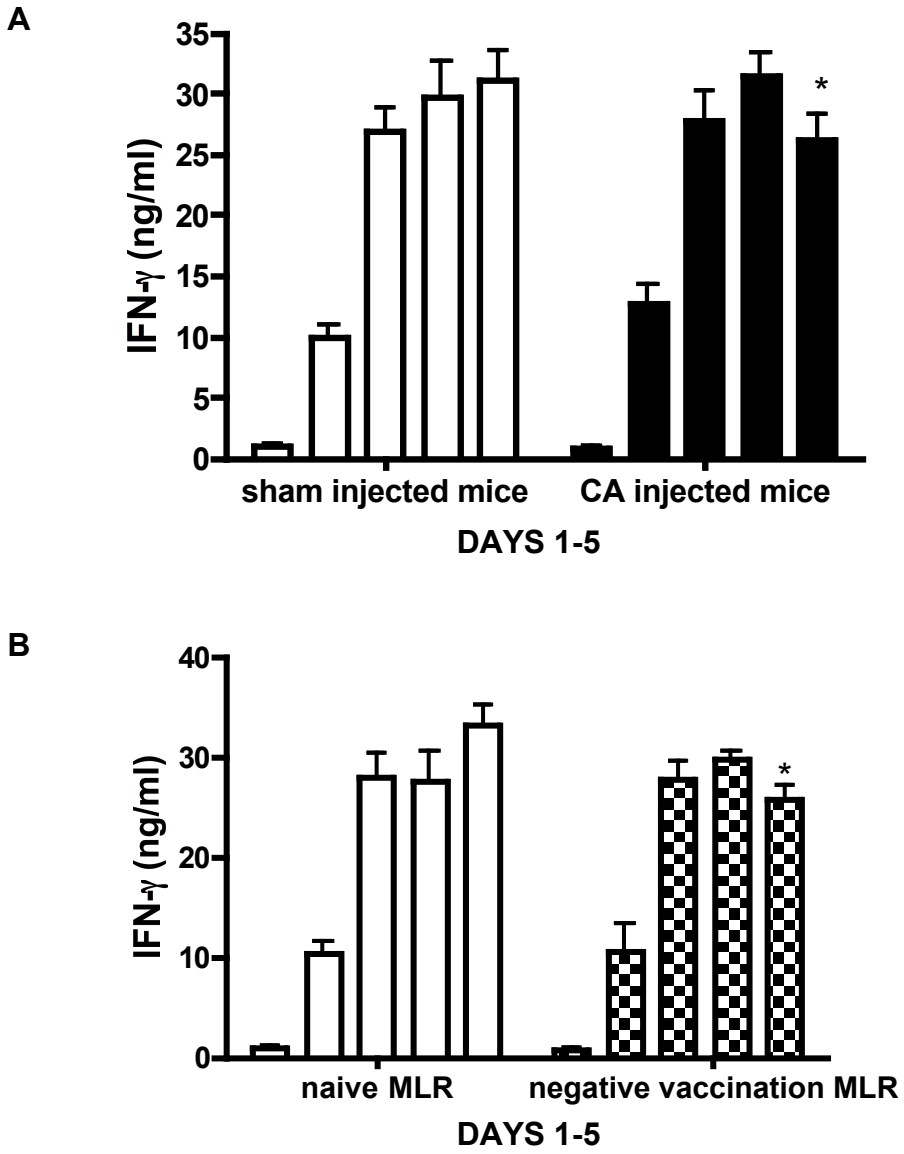
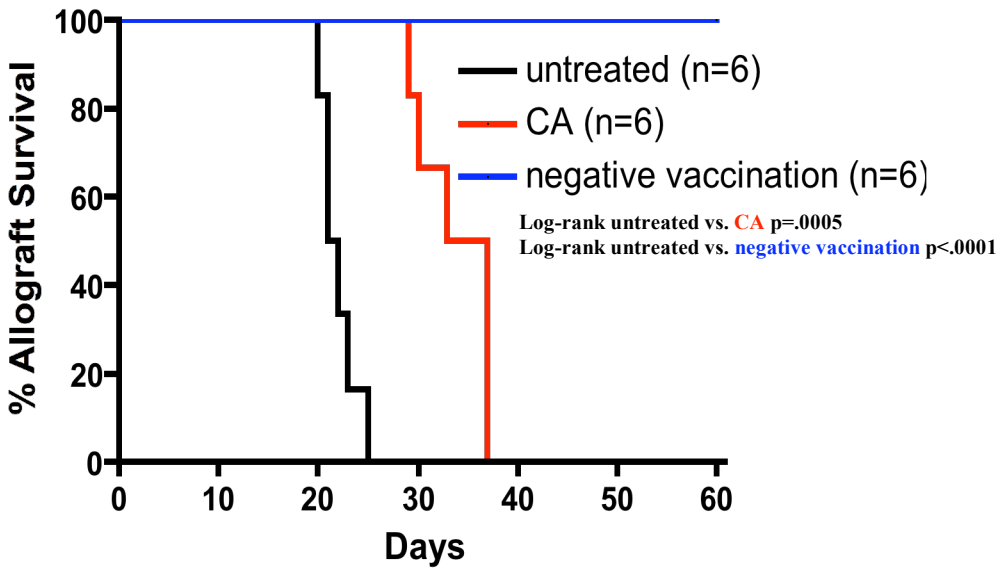


Fig. 28. ***In vivo*** **treatment with CA or a negative vaccination is not immunogenic or harshly immunosuppressive.** C57BL/6 mice were injected with PBS daily for 7 days (n=4) (white bars) (A), treated with CA at 10mg/kg/day for 7 days (n=4) (black bars) (A), given a single negative vaccination with apoptotic BALB/c splenocytes in IFA on day 1 (n=3) (checkered bars) (B), or untreated for 7 days (n=3) (white bars) (B). On the 8th day, all mice were sacrificed and MLR assays were performed in a 1:1 ratio of stimulators and responders using irradiated BALB/c splenocytes as stimulators. Supernatants were harvested on days 1-5 and IFN-γ ELISAs were performed. Results are presented as the mean (± SEM) of at least three independent experiments. **, $p < 0.05$ between the respective treated and untreated (control) group.

4.4.3 The negative vaccination strategy induces long-term allograft tolerance

Next, we performed allograft transplants using BALB/c (H-2d) donor islets and diabetic C57BL/6 (H-2b) recipients to determine if our therapeutic strategies were capable of delaying or preventing allograft rejection (Figure 29). On average, untreated control recipients rejected islet allografts in 22.0 +/- 0.73 days (n=6). Recipients receiving a daily injection of CA, beginning 3 days prior to transplant, demonstrated a significant delay (Log-rank, p=0.0005) in allograft rejection (33.83 +/- 1.51 days, n=6) compared to untreated controls. Recipients treated with the negative vaccination (10^7 BALB/c splenocytes exposed to 3500 rads and emulsified in IFA) 7 days prior to transplant reached the highest significance (Log-rank, $p<0.0001$) by maintaining islet allografts that sustained function in excess of 60 days (n=6). These results demonstrate that redox modulation can delay allograft rejection, though the negative vaccination appears to be the more effective strategy to induce long-term allograft tolerance.

A



B

Treatment	Mean Days to allograft failure	"t" test p value compared to untreated
Untreated (n=6)	22.0 +/- 0.73	-
CA (n=6)	33.83 +/- 1.51	<.0001
Negative vaccination (n=6)	60 +/- 0	<.0001

Fig. 29. CA delays allograft rejection and a negative vaccination induces long-term allograft tolerance. A: Diabetic C57BL/6 mice were untreated (n=6) (black line), systemically treated with CA daily, beginning 3 days before transplant (n=6) (red line), or given a single negative vaccination of apoptotic BALB/c splenocytes in IFA 7 days prior to transplant (n=6) (blue line). Log-rank was used to determine statistical significance between treated and untreated groups. B: This table details mean day to allograft failure for each group presented as means (± SEM). A "t" test was used to determine significance between treated and untreated groups and resultant p values are shown. **, $p < 0.05$ was used to determine significance between the respective treated and untreated (control) groups.

4.4.4 The T_H1 response in recipients of the therapeutic strategies 14 days post-transplant

In order to examine the effects of redox modulation or the negative vaccination on the T_H1 immune response in transplant recipients we used splenocytes from transplant recipients 14 days post-transplant as responders and irradiated BALB/c splenocytes as stimulators in recall MLRs. Upon secondary exposure to alloantigen in a recall MLR, untreated recipients produced significantly amplified levels of IFN-γ compared to a primary MLR (Figure 30). Recipients treated with CA responded to a secondary exposure of alloantigen with substantially decreased levels of IFN-γ compared to untreated recipients correlating with previously published work demonstrating the ability of redox modulation to induce antigen-specific hyporesponsiveness (26). Recipients of the negative vaccination produced IFN-γ levels comparable to those in a primary MLR, though this recall MLR is actually generating a tertiary response to alloantigen. Recipients of the negative vaccination were first exposed to alloantigen 7 days before allograft transplant with the negative vaccination and then for a second time with the allograft transplant, itself. Since the day 14 *in vitro* recall MLR is a tertiary response to alloantigen for recipients of the negative vaccination controlled IFN-γ levels served as predictors of long-term allograft tolerance for this treatment group.

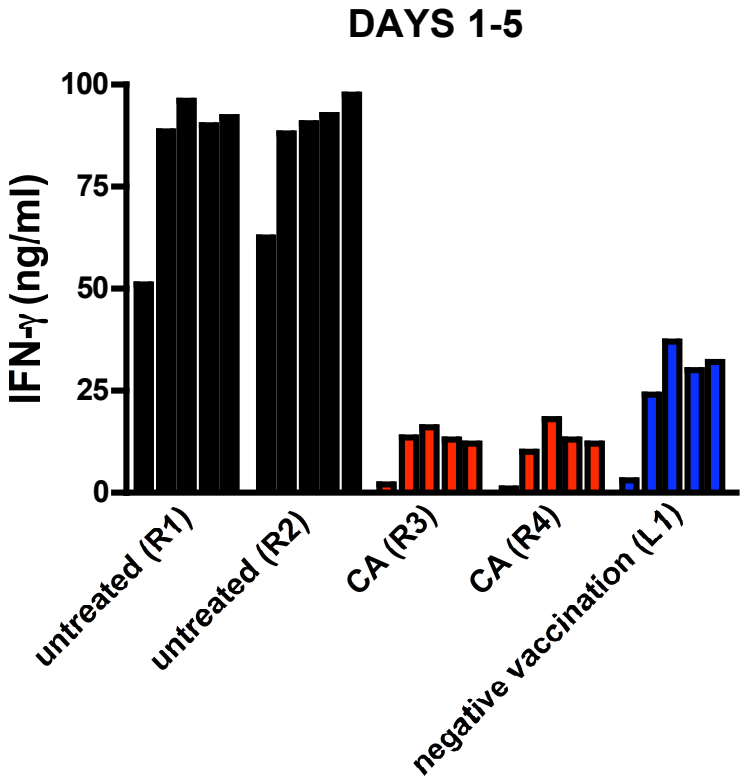


Fig. 30. CA-treatment or a negative vaccination can impair the T_H1 response in a recall MLR 14 days post-allotransplant. C57BL/6 recipients were untreated (n=2) (R1, R2) (black bars), treated with CA daily beginning 3 days prior to transplant (n=2) (red bars) (R3, R4), or given a negative vaccination of apoptotic BALB/c splenocytes in IFA 7 days prior to transplant (n=1) (blue bars) (L1). On post-transplant day 14, all mice were sacrificed and recall MLR assays were performed using a 1:1 ratio of stimulators and responders using irradiated BALB/c splenocytes as stimulators. Supernatants were harvested on days 1-5 and IFN-γ ELISAs were performed. Each set of bars represents an individual recipient. The solid blue line represents the maximum IFN-γ response from naïve MLRs (n=3).

4.4.5 Immunohistochemical analysis of islet-bearing kidneys 14 days post-transplant

To examine the cellular infiltrate at the site of the islet allografts, we performed immunohistochemical analysis on islet-bearing kidneys 14 days post-transplant. For all sections, insulin is stained green and nuclear staining is blue. In panel A (Figure 31), untreated recipients and recipients treated with the negative vaccination show a similar innate immune infiltrate of macrophages (F4/80), while stained sections of recipients treated daily with CA showed an overall decrease in peri-islet macrophage infiltrate. To examine the T-cell infiltrate, sections were stained with T cell specific antibodies. We observed the presence of a CD4 T cell population in untreated animals. Comparatively, recipients of the negative vaccination show a stronger presence of peri-islet CD4 T cells, and CA-treated recipients show the least amount of CD4 T cell infiltrate, as the only CD4 positive cells are on the periphery in the CA-treated sections (Figure 31B). To assess the infiltrate of cells responsible for direct damage to islets, sections were stained with a CD8 T cell antibody. Untreated recipients show the most pronounced presence and infiltrate of CD8 T cells, followed by a few CD8 T cells on the periphery for the negatively vaccinated recipients, and a barely detectable presence of CD8 T cells in recipients treated with CA (Figure 31C). Immunostaining for recipients receiving CA-treatment reveals a reduction in immune cell infiltrate, correlating with the delay in allograft rejection. The data also suggests a regulatory component for CD4 T cells surrounding islets in recipients of the negative vaccination as this CD4 T cell population is associated with allograft tolerance.

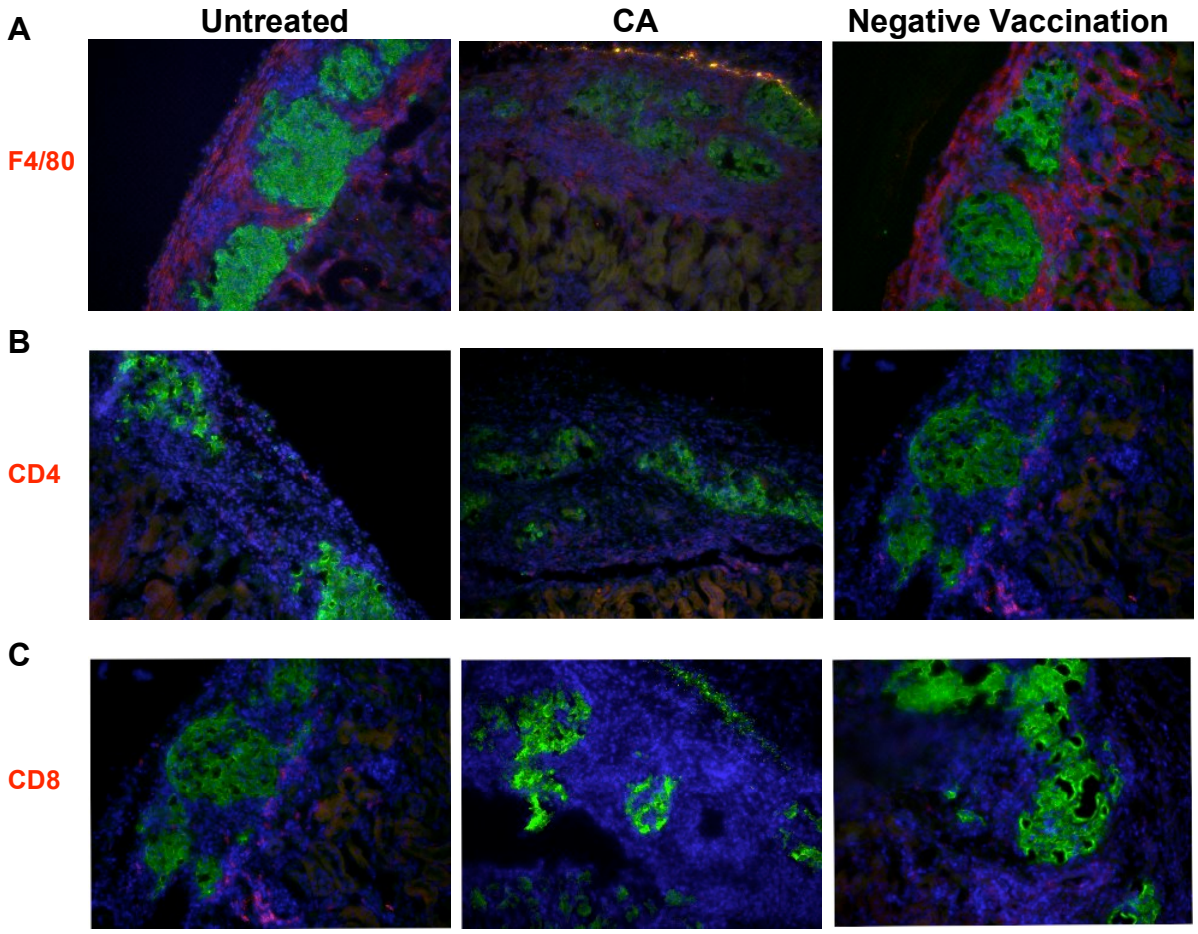


Fig. 31. Immunohistochemistry of islet-bearing kidneys 14 days post-transplant. C57BL/6 recipients were **untreated**, treated with **CA** daily beginning 3 days prior to transplant, or given a **negative vaccination** of apoptotic BALB/c splenocytes in IFA 7 days prior to transplant. For all slides green = insulin and blue = dapi. A: red = F4/80, B: red = CD4, and C: red = CD8. Sections shown are representative of respective groups.

4.6 DISCUSSION

Poor outcomes in islet transplantation forces the clinic to reserve islet transplantation only for brittle diabetics with severe blood glucose dysregulation because transplant recipients often suffer an increase in systemic complications post-transplant in excess of those attributed to type 1 diabetes (13). Current literature calls for improved cytoprotection for islets and antigen-specific immunosuppressive therapies, which can be employed in the absence of nonspecific immunosuppressive agents. The aim of this study was to introduce new approaches to old problems in islet transplantation in an attempt to improve islet transplantation protocols. By reducing challenges of islet transplantation including the side effects of harsh, nonspecific immunosuppressive drugs, it is possible to make the procedure more consistent with outcomes of long-term insulin-independence.

While the benefits of redox modulation have been widely reported (26, 38, 39, 48), the *in vivo* mechanism(s) responsible for immunomodulation by redox modulation have not been thoroughly examined in allograft transplantation. The approach of negative vaccination using our therapeutic protocol is novel in that IFA alone does not induce tolerance (141, 146-149) thus indicating that alloantigen-expressing apoptotic BALB/c splenocytes are the tolerizing component of our negative vaccination therapy. Irradiating donor splenocytes proved successful at achieving apoptotic donor cells (Figure 27) and our previous work with redox modulation characterizes its abilities to impair the immune response to alloantigen (123) and to augment islet

viability and function (38, 39). Figure 28A demonstrates that systemic treatment with CA is not severely immunosuppressive, suggesting that CA-treatment will not induce chronic infection or malignancy. Additionally, previous work with murine and human islets demonstrates that this agent is a nontoxic, cytoprotective, anti-inflammatory, and islet-sparing therapeutic (38, 39, 123), which is not the case with currently used immunosuppressive drugs, which specifically target adaptive immune function and can impair islet viability and function.

IFN-γ levels in the MLR assays from the negatively vaccinated mice (no transplant) are especially remarkable (Figure 28B), since the MLR is a secondary exposure to alloantigen as the first exposure is the negative vaccination. This result is evidence that our negative vaccination strategy is capable of controlling T_H1 immunity to donor alloantigen. Typically a secondary response to antigen produces a substantially elevated response compared to that of a primary response (Figure 30). After first using naïve animals to test our therapeutic strategies of systemic redox modulation and negative vaccination, these approaches were then tested in an *in vivo* model of transplant rejection where recipient mice were either untreated, injected with CA daily beginning 3 days prior to transplant, or treated with the donor-specific negative vaccination 7 days prior to transplant (Figure 29).

The negative vaccination strategy proved the most successful as none of the transplant recipients rejected their allogeneic islet transplant compared to recipients of redox modulation, which induced a delay allograft rejection for a significant period of time compared to untreated controls. To analyze *in vivo* treatments prior to graft failure, we performed recall MLRs on a cohort of transplant recipients treated with one of our therapeutic protocols14 days post-transplant to examine effects on the T_H1 response by measuring IFN-γ levels. The success rate of the allograft transplants did not correlate with the inhibition of IFN-γ (Figure 30) since CA-

treatment only afforded a delay in rejection yet produced the lowest T_H1 response. Surprisingly, recipients of the negative vaccination maintained long-term allograft tolerance despite higher levels of IFN-γ in the recall MLR compared to recipients treated with CA (Figure 30). The work of Markees, et al, and Konieczny, et al reveals that IFN-γ is required for graft acceptance, suggesting that our dosage of CA-treatment decreases IFN-γ levels to a range that may not be supportive of allograft tolerance (150, 151). Though redox modulation alone appears insufficient to entirely ablate allograft rejection, CA has much potential to be added to currently utilized transplant protocols to achieve long-term allograft tolerance while minimizing complications for the recipient.

The most notable difference in T_H1 analysis when using redox modulation (no allograft transplant) (Figure 28A) and in T_H1 analysis 14 days post-transplantation (Figure 30) is exposure to alloantigen (allograft transplant). As reported in our previous work (26), redox modulation can induce antigen-specific hyporesponsiveness. For example, when redox modulation is administered 3 days before transplant (exposure to alloantigen) a marked reduction in antigen-specific effector function is achieved (Figure 30). Taken together, *in vivo* treatment with CA prior to *in vivo* (exposure to alloantigen) induces antigen-specific hyporesponsiveness as demonstrated by a depressed T_H1 response in the secondary MLR in Figure 30. Comparatively, *in vivo* treatment with CA prior to a primary in vitro MLR does not result in robust immunomodulation to significantly suppress the T_H1 response as naïve mice treated with CA only yielded a slight decrease in IFN-γ levels on day 5 of the MLR (Figure 28A). Since the recipients of the *in vivo* negative vaccination (receiving no transplant) were exposed/tolerized to antigen with the vaccination itself, the in vitro MLR (Figure 28B) served as a secondary response. The day 14 post-transplant MLR using responder splenocytes from recipients of the

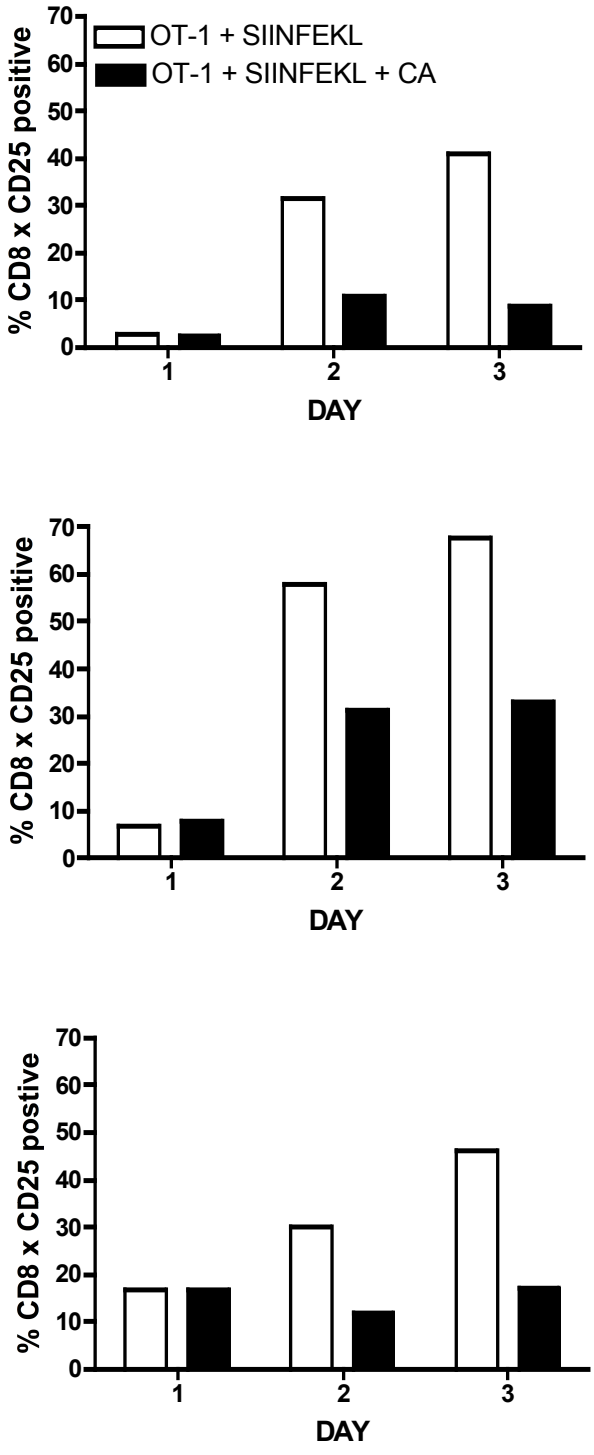
negative vaccination assays a tertiary response since the transplant is the second exposure to alloantigen and the negative vaccination is the first (Figure 29). In both secondary and tertiary MLRs for recipients of the negative vaccination, the T_H1 response was the same, which further confirms induction of long-term allograft tolerance.

More insight into the mechanisms responsible for the delay/prevention of allograft rejection using redox modulation and a donor-specific negative vaccination was presented with immunohistochemical analysis of immune cell populations (Figure 31A-C) 14 days post-transplant. A decreased macrophage infiltrate was observed in recipients of redox modulation, most likely due to impaired NF-κB signaling (48), while an increase in macrophage (F4/80) staining was seen in untreated and negatively vaccinated recipients (Figure 31A). These data demonstrate the ability of systemic CA to impair activation of the pro-inflammatory innate-immune response resulting in fewer macrophages trafficking to the site of the islet graft in CA-treated recipients. The CD4 T cell infiltrate is peri-islet in untreated and negatively vaccinated recipients. While the CD4 T cells are present in the CA-treated recipients, they appear to have migrated to a location a distance from the islet allograft (Figure 31B). We observed a noninvasive peri-islet CD4 T cell infiltrate, suggesting a regulatory function for these CD4 T cells found at the site of the allograft in negatively vaccinated recipients, which did not reject their allograft. The decreased infiltrate found in sections of CA-treated recipients suggests impaired activation and migration by immune cells. Lastly, direct effectors of islet cell death, the CD8 T cell population, appear to be most prevalent in untreated recipients, undetectable in CA-treated recipients and sparse in recipients of the negative vaccination (Figure 31C).

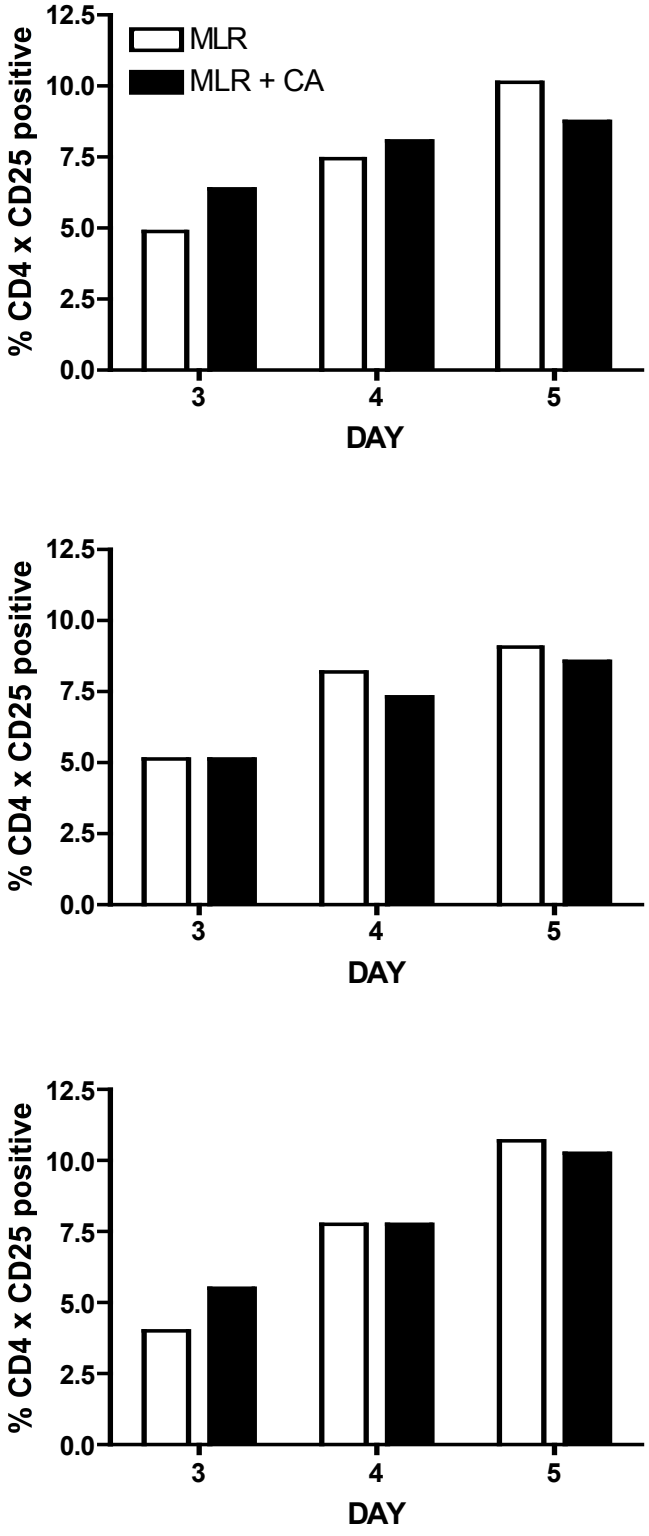
Taken together, the subcutaneous delivery of apoptotic cells in IFA in our protocol may be able to tolerize an increased number of T cells compared to IV delivery as Kearney et al

discovered that the total number of antigen-specific T cells in the lymph nodes following a subcutaneous injection were higher compared to IV or IP routes of delivery (141). Negative vaccination succeeds in alloantigen-specific hyporesponsiveness (Figure 30) and achieves islet allograft tolerance (Figure 29), though it does not bolster function of the islet allograft or impair autoimmunity like redox modulation. Based on previous studies, it is possible that systemic CA-treatment could depress autoreactive T cell effector function (49) in addition to offering protection from alloimmunity for islets post-transplant, as shown in this study (Figure 29). Ideally, recipients of islet transplants should receive islets that are functionally more robust and protected from nonspecific inflammation (38, 39) while avoiding complications of non-specific immunosuppression to achieve antigen-specific long-term allograft tolerance. A therapeutic protocol encompassing these requirements is especially applicable in islet transplantation using CA-treated islets from brain-dead donors and also in live kidney transplantation where peripheral blood leukocytes (PBL) can be easily harvested for the negative vaccination strategy, all in an effort to increase the probability of long-term islet allograft function.

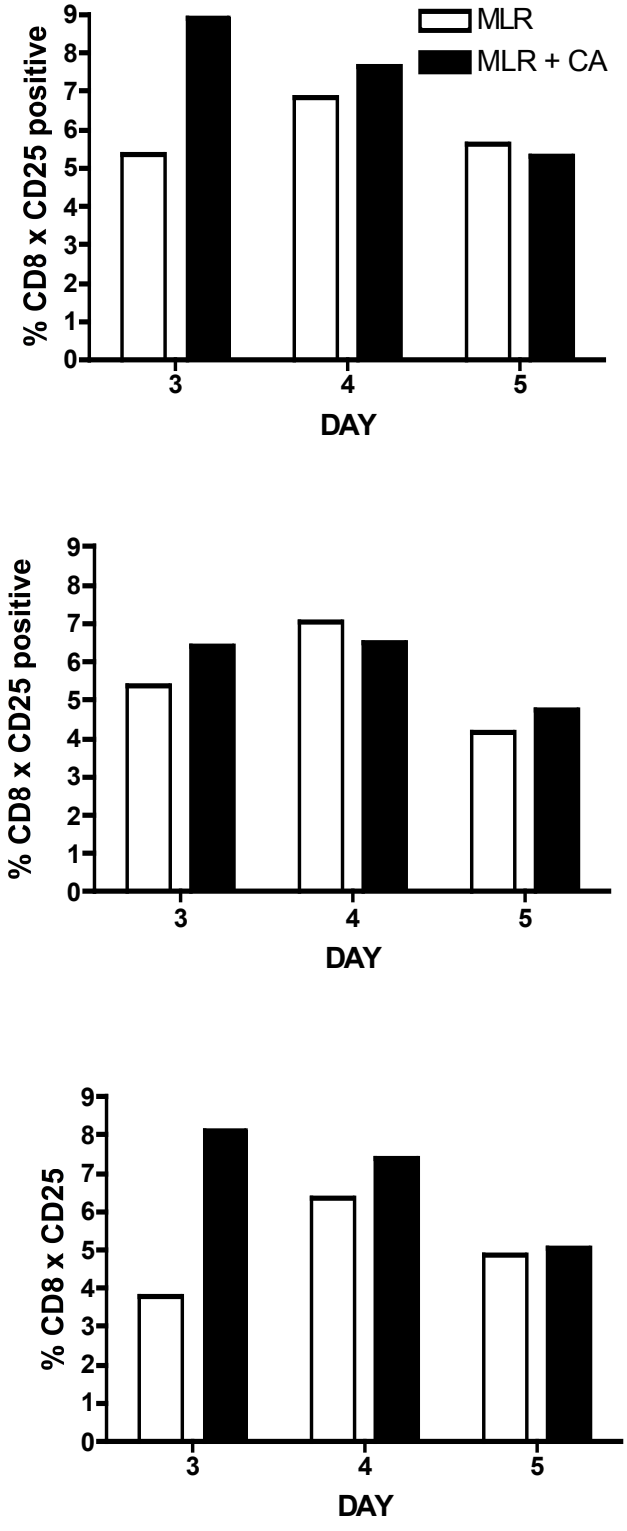
SUPPLEMENTAL DATA FOR FIGURES 13, 14, 17, 20 & 21



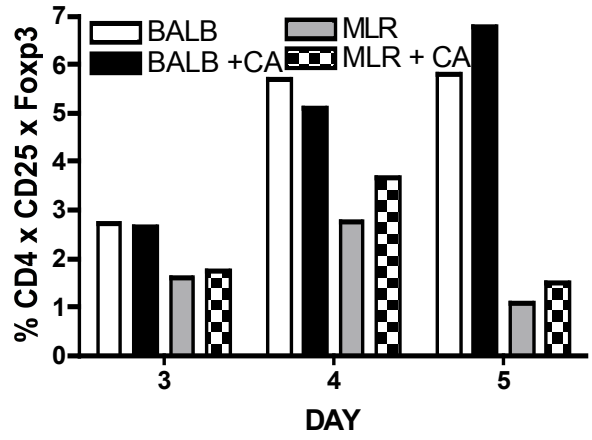
Supplement to Fig. 13. Redox modulation inhibits IL-2 receptor α-chain (CD25) expression on OT-1 Transgenic CD8 T cells. Supplemental FACS data showing three similar experiments representing the trend shown in Fig. 13.

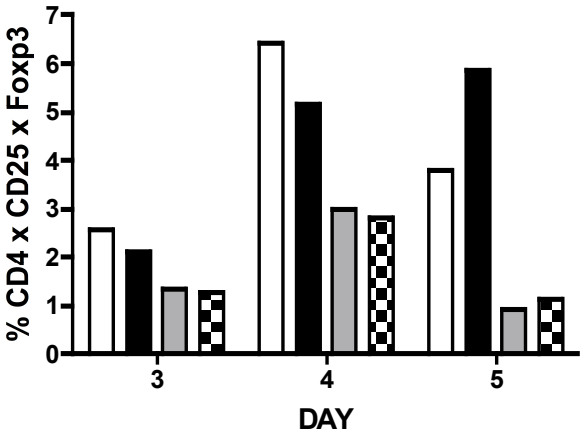


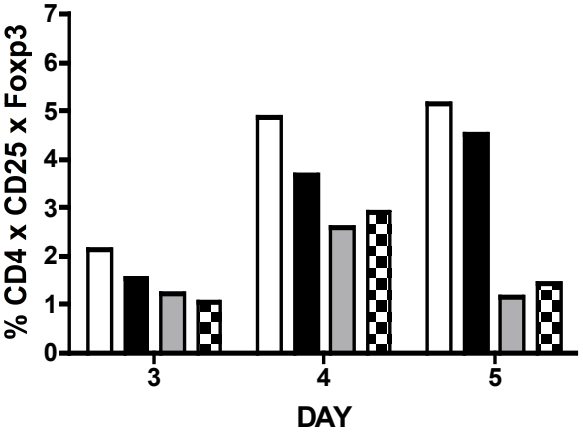
Supplement to Fig. 14A. Redox modulation has no effect on IL-2 receptor α-chain (CD25) expression on BALB/c T cells. Supplemental FACS data showing three similar experiments representing the trend shown in Fig. 14A.



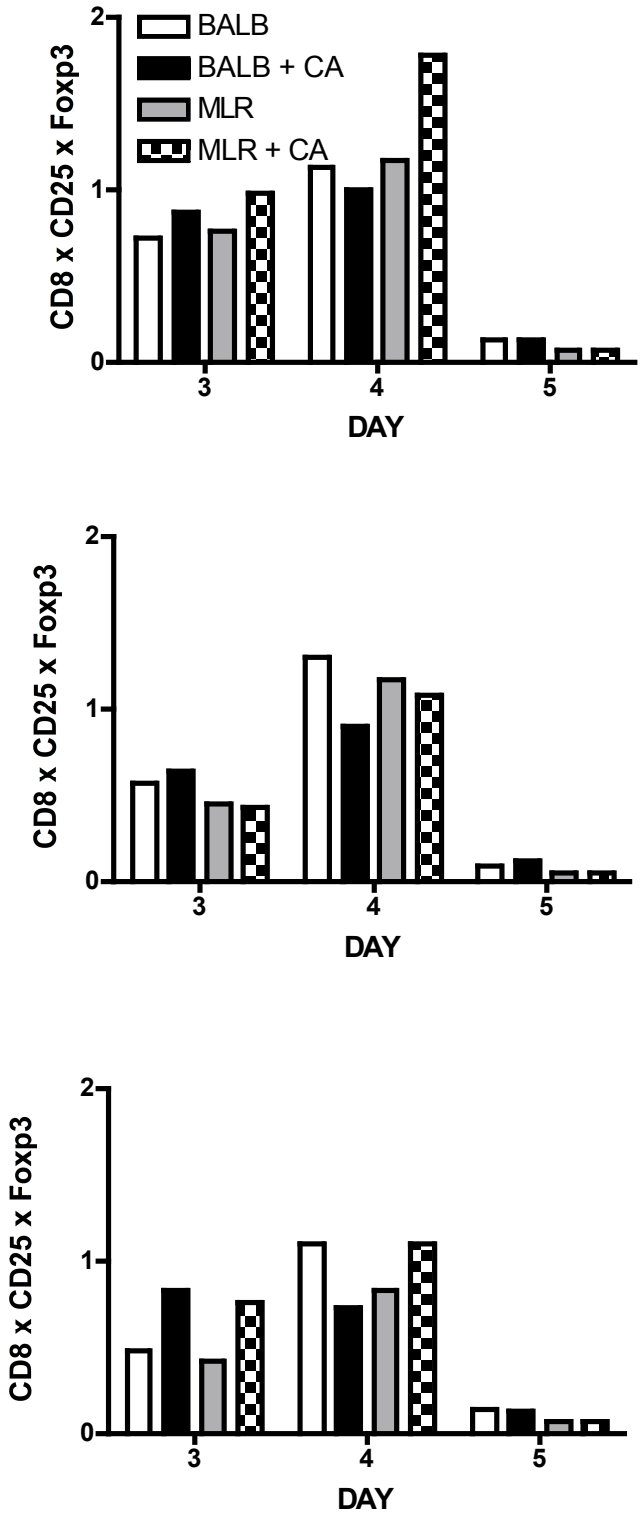
Supplement to Fig. 14B. Redox modulation has no effect on IL-2 receptor α-chain (CD25) expression on BALB/c T cells. Supplemental FACS data showing three similar experiments representing the trend shown in Fig. 14B.



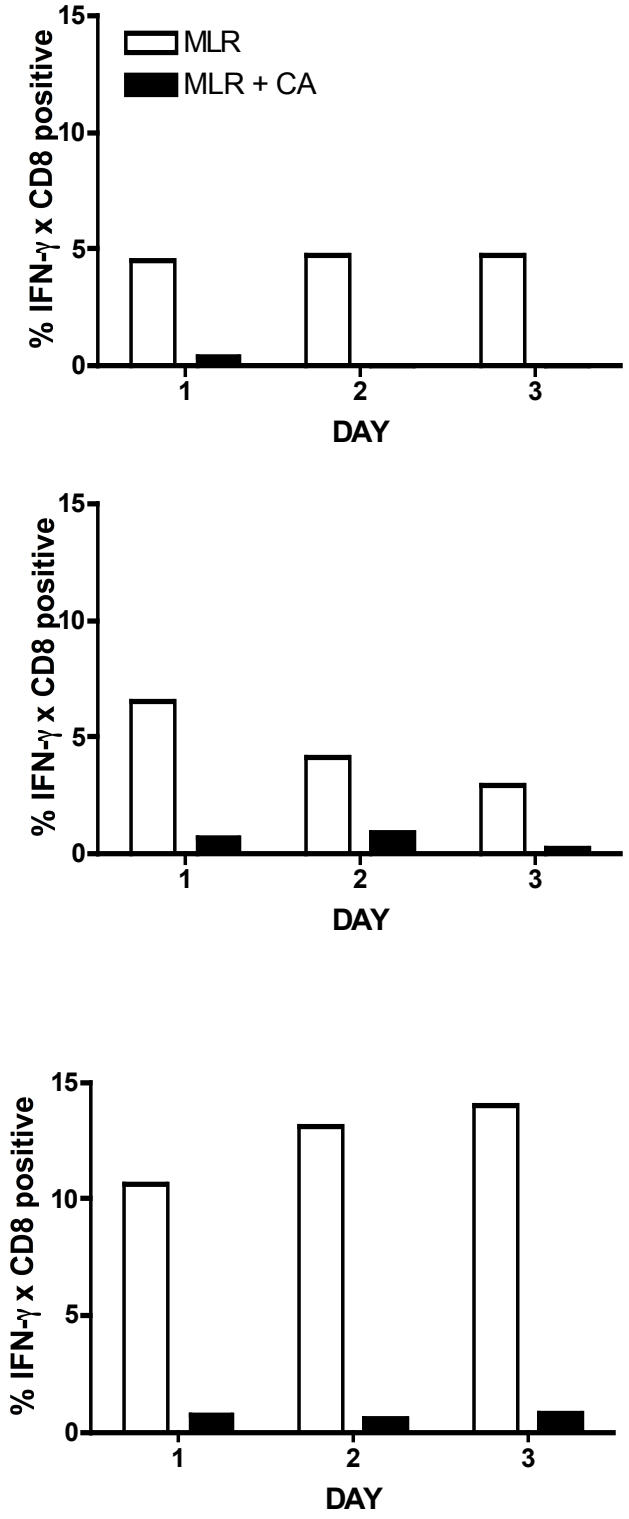




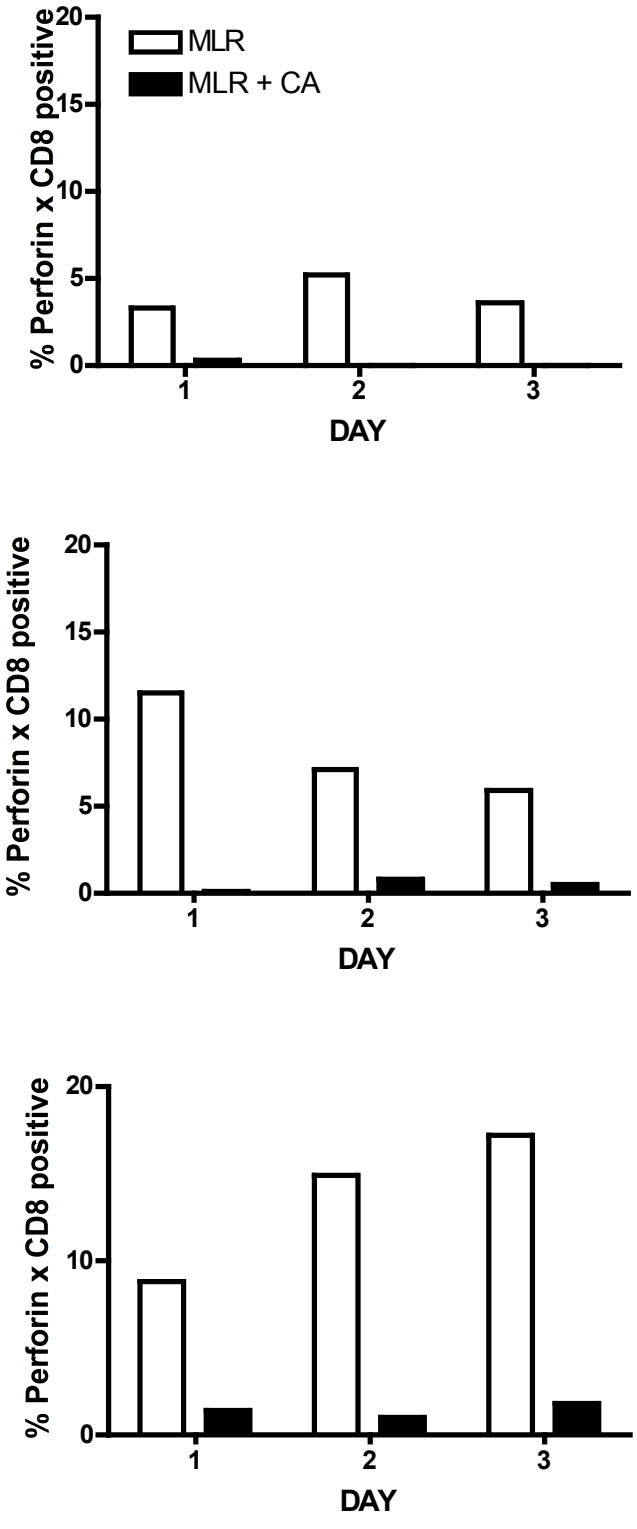
Supplement to Fig. 17. Redox modulation does not induce Foxp3 expression. Supplemental FACS data showing three similar experiments representing the trend shown in Fig. 17.



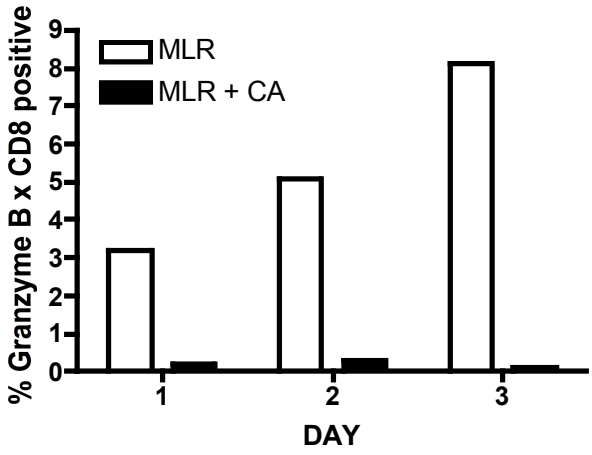
Supplement to Fig. 17. Redox modulation does not induce Foxp3 expression. Supplemental FACS data showing three similar experiments representing the trend shown in Fig. 17.

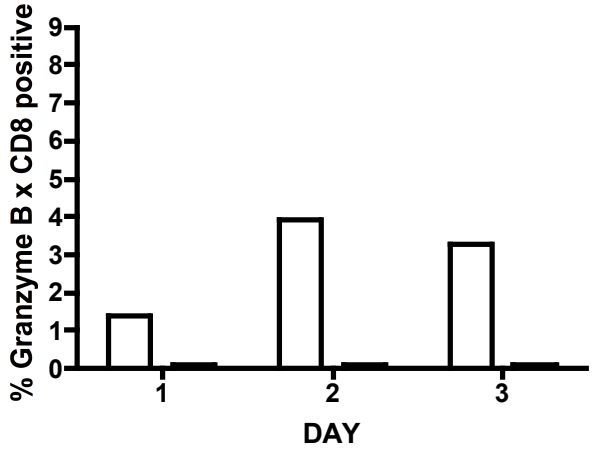


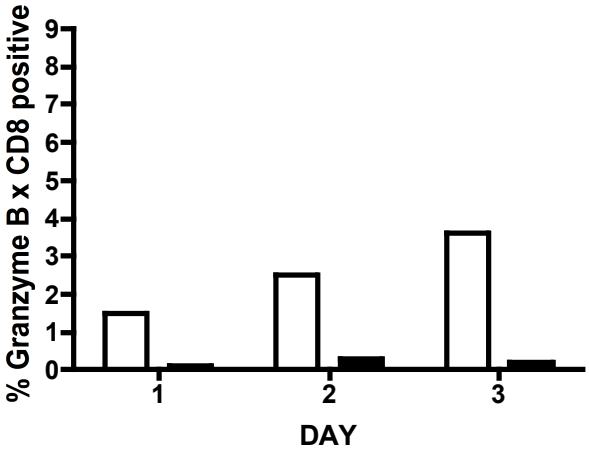
Supplement to Fig. 20A. Redox modulation decreases intracellular expression of IFN-γ in alloantigen-specific CTLs. Supplemental FACS data showing three similar experiments representing the trend shown in Fig. 20A.



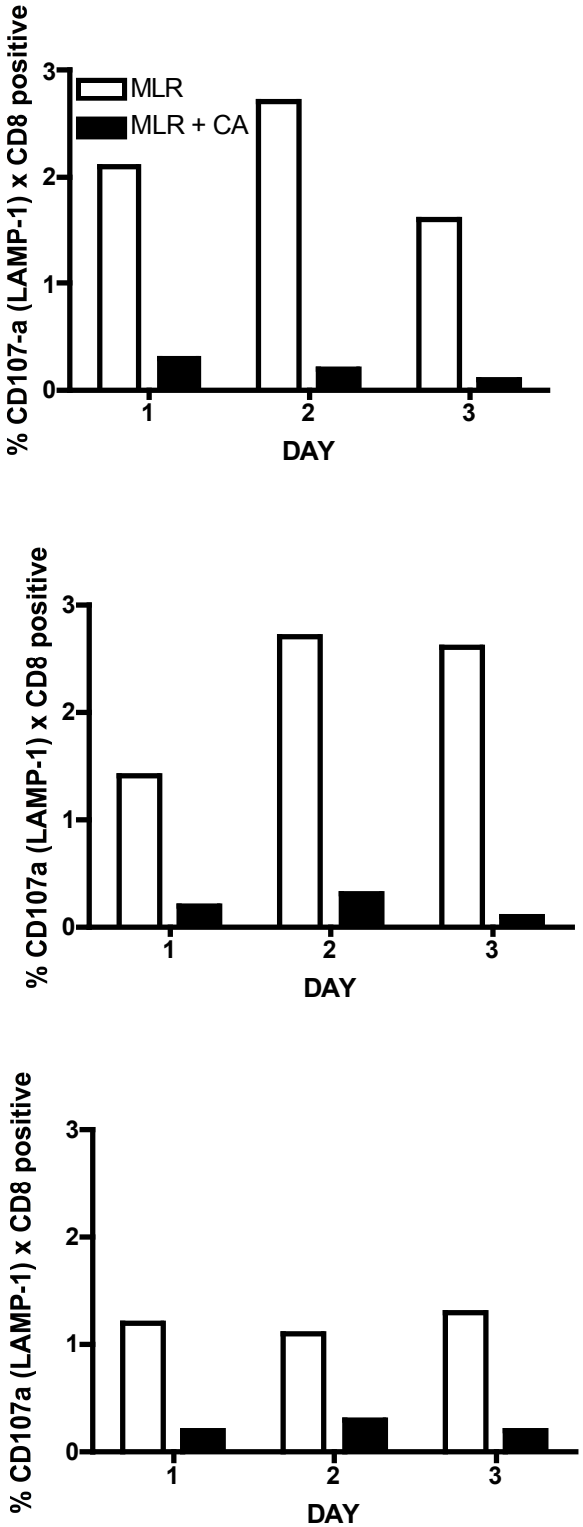
Supplement to Fig. 20B. Redox modulation decreases intracellular expression of perforin in alloantigen-specific CTLs. Supplemental FACS data showing three similar experiments representing the trend shown in Fig. 20B.



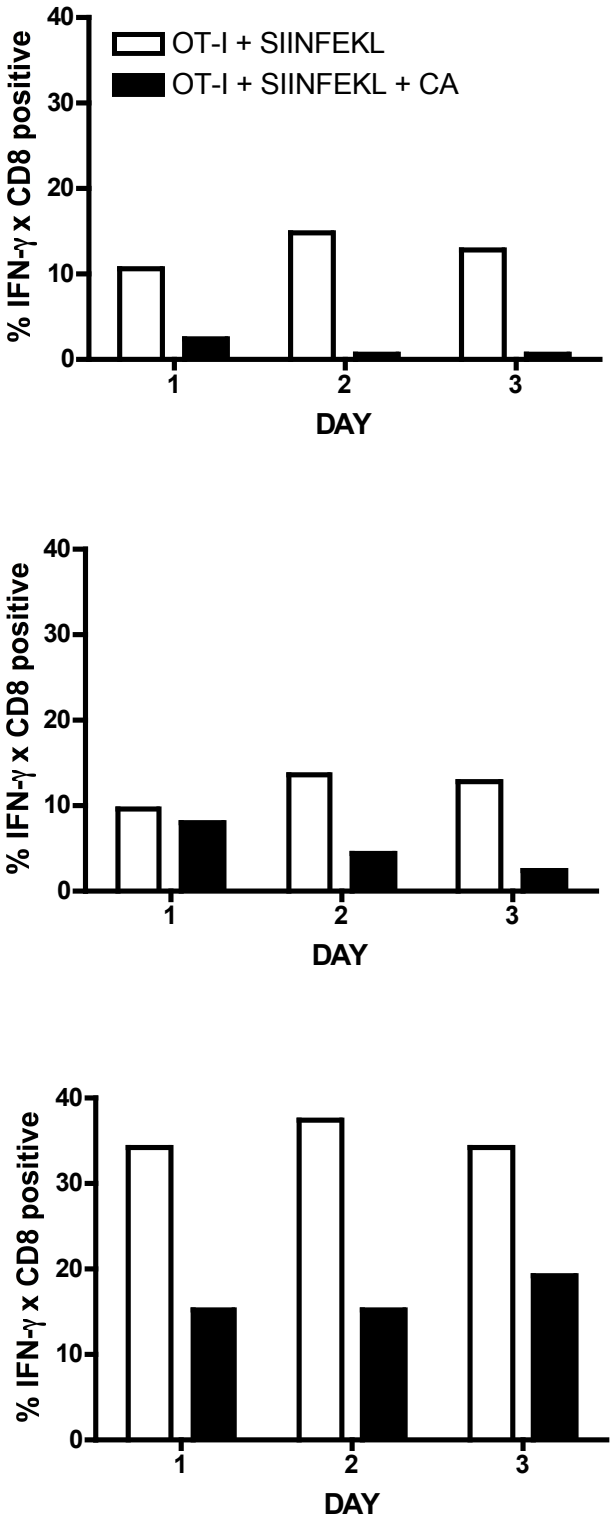




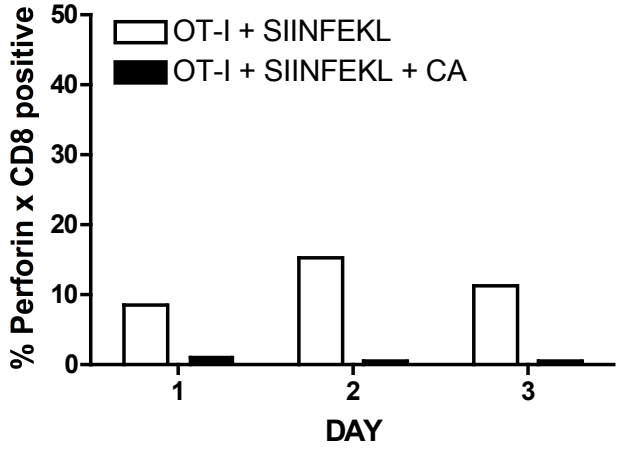
Supplement to Fig. 20C. Redox modulation decreases intracellular expression of granzyme B in alloantigen-specific CTLs. Supplemental FACS data showing three similar experiments representing the trend shown in Fig. 20C.

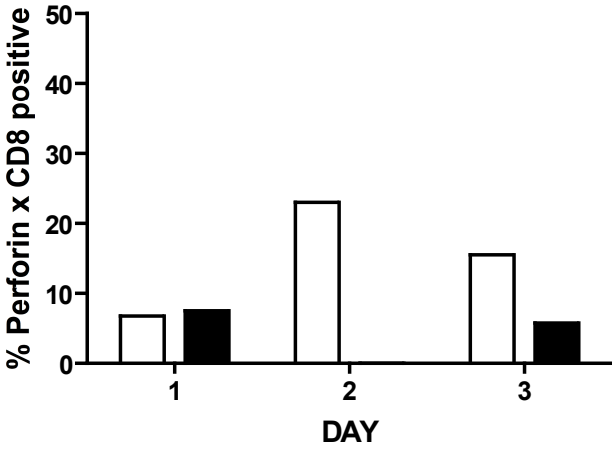


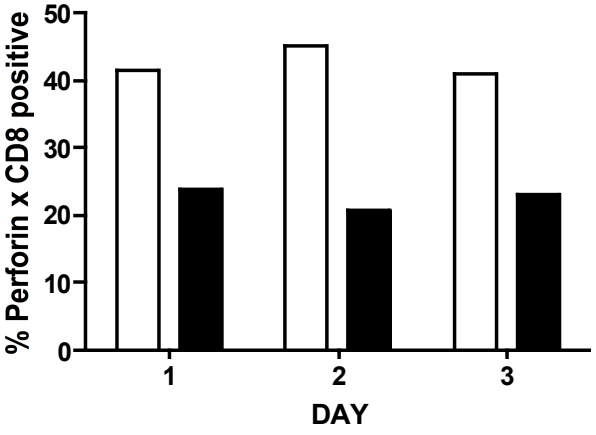
Supplement to Fig. 20D. Redox modulation decreases surface expression of CD107a (LAMP-1) on alloantigen-specific CTLs. Supplemental FACS data showing three similar experiments representing the trend shown in Fig. 20D.



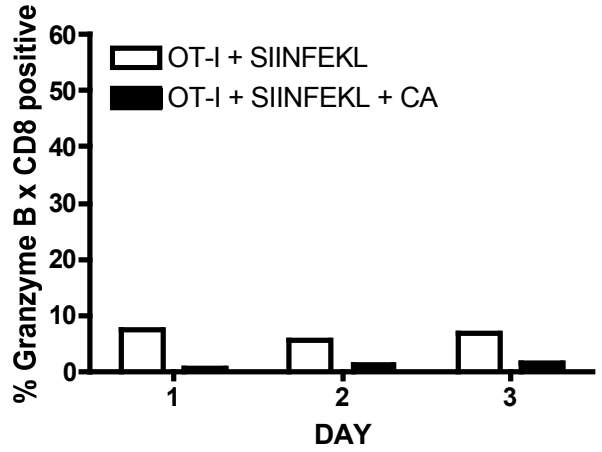
Supplement to Fig. 21A. Redox modulation decreases intracellular expression of IFN-γ in OT-1 CTLs. Supplemental FACS data showing three similar experiments representing the trend shown in Fig. 21A.

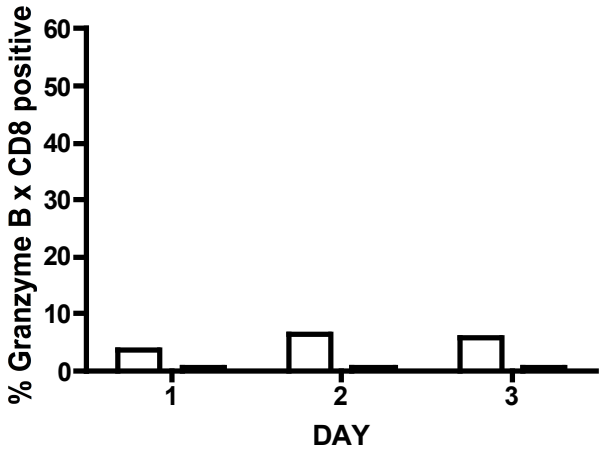


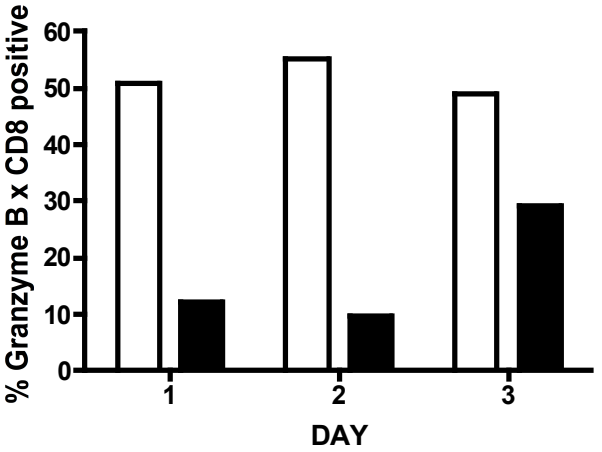




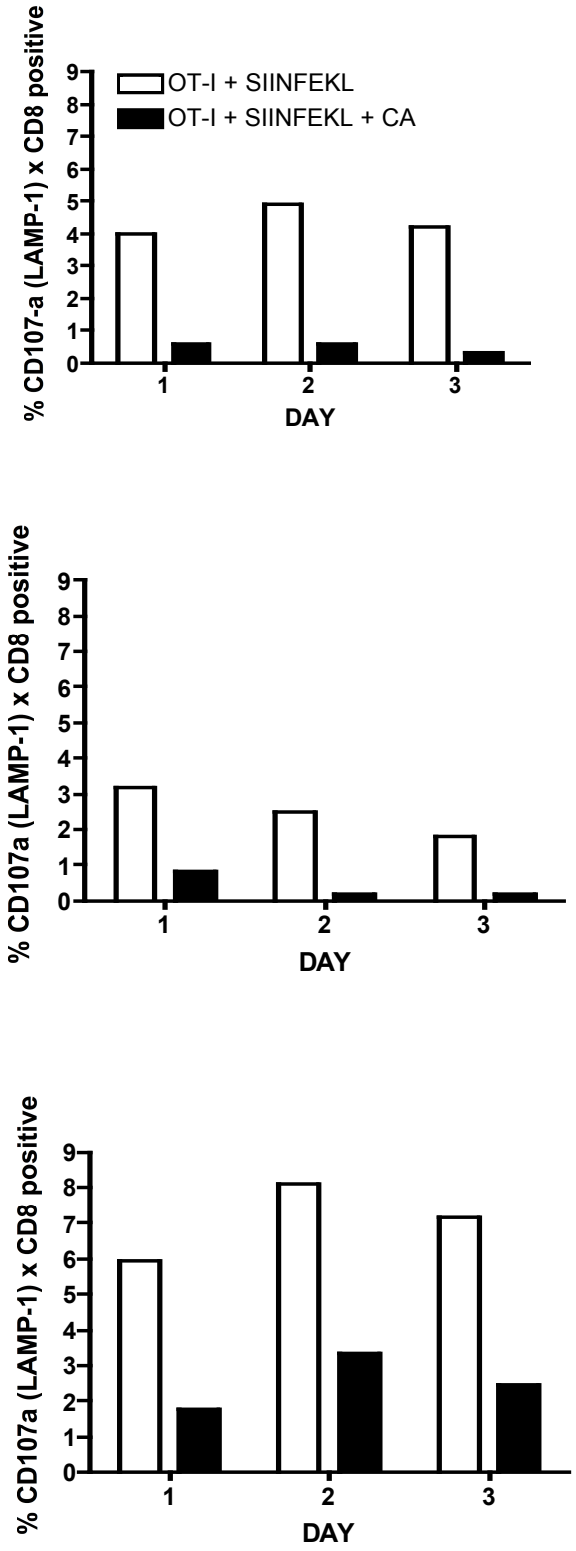
Supplement to Fig. 21B. Redox modulation decreases intracellular expression of perforin in OT-1 CTLs. Supplemental FACS data showing three similar experiments representing the trend shown in Fig. 21B.







Supplement to Fig. 21C. Redox modulation decreases intracellular expression of granzyme B in OT-1 CTLs. Supplemental FACS data showing three similar experiments representing the trend shown in Fig. 21C.



Supplement to Fig. 21D. Redox modulation decreases surface expression of CD107a (LAMP-1) on OT-1 CTLs. Supplemental FACS data showing three similar experiments representing the trend shown in Fig. 21D.

CONCLUSIONS

There have been several advances in islet transplantation over the last decade, however, many improvements must be made to ensure predictable long-term insulin-independence for allograft recipients. Our studies were performed in an effort to increase graft function post-transplant by refining the procedure of islet transplantation. While we have elucidated the benefits of using redox modulation in islet transplantation, we suspect that redox modulation can be advantageous in other autoimmune and inflammatory diseases. Redox modulation of immune function has great potential to reach the clinic for multiple uses including transplantation, autoimmune and inflammatory diseases, hypoxic injuries like stroke and heart attacks, cancer, and in degenerative disorders like Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis (ALS). Redox modulation has been well characterized in innate immunity and innate-immune signaling, however, the effects of CA on adaptive immune signaling must be pursued in disease-related models to implement systemic use in humans.

It is known that redox modulation induces "miseducation" of DCs by impairment of NF-κB and 3rd signal of activation (26, 48), which has downstream effects on T cell activation (26, 123). In the presence of redox modulation, decreases in STAT4 and T-bet activation in CD4 T cells (Tse, unpublished data) and depression of CD3 expression in CD4 and CD8 T cells have been observed (Sklavos, unpublished data). However, within the T cell, the effects of redox modulation on NF-κB activation and tyrosine phosphorylation, both essential for T cell activation, have not been thoroughly examined. Previously we demonstrated that BDC-2.5 T

cells are not deleted *in vivo* when upon adaptive transfer into young NOD.*scid* mice after CA-treatment (Piganelli, unpublished data). CA-treatment does not induce anergy in T cells as secondary proliferative responses are in tact (26) nor does it induce a regulatory population based on invariable Foxp3 expression in CA-treated cells compared to untreated controls (Sklavos, unpublished data). These data eliminate the possibility of an inducible T regulatory cell population arising in the presence of redox modulation. Ignorance is not a possibility, as exposure to antigen is uninhibited in our *in vitro* studies and T cells come in contact with antigen *in vivo*, as evidenced by *in vivo* recall assays (26); (Sklavos, unpublished data). Based on the aforementioned studies, redox modulation cannot be grouped under classical mechanisms.

Further analysis of the negative vaccination strategy is also required as the mechanism(s) of tolerance has not yet been identified. Anergy, regulation, and deletion of alloreactive T cells are all possibilities, though these mechanisms have yet to be tested. In review of this body of work, redox modulation holds potential as a clinical therapeutic. Since *in vivo* administration of CA depots in the liver, it would be an ideal therapeutic to use in islet transplantation as islets are infused into the portal vein to promote liver-engraftment. Presently, redox modulation is being used as cytoprotection for islets in trials of xenograft transplantation with non-human primates. Though in early stages, studies treating porcine islets with redox modulation prior to transplant into non-human primates mirror results from our murine transplant models. In both trials, recipients of CA-treated islets normalized to a lower blood glucose compared to recipients of untreated islets (Bottino, unpublished data). Based on the success in non-human primates, the next step for redox modulation is as a cytoprotective agent for whole organ cold storage/islet isolation/culture in clinical transplantation. Perhaps the first clinical work with redox modulation will be during islet isolation of autoislet transplants for patients with pancreatitis.

Based on our murine studies, redox modulation cannot be used as a monotherapy to inhibit allograft rejection. However, there is potential for redox modulation to be used systemically, in combination with a decreased dose and/or duration of immunosuppressive agents to protect islets from the toxic effects of harmful anti-rejection agents. This analysis of redox modulation using a small molecule catalytic antioxidant (CA) demonstrates that this strategy is effective as a cytoprotective and immunomodulatory approach that can be used in tissue-directed (islets incubated in CA), sustained-release pellet, or systemic form to improve islet function and impair allograft rejection of transplanted islets.

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