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Metallothionein Gene Dose and the Immune Response

Meaghan Roy-O-Reilly
University of Connecticut - Storrs, meaghanror@gmail.com

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Meaghan Roy-O'Reilly

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Research Advisor: Michael Lynes

Affiiliate: MCB Department, UConn

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Abstract

Metallothionein (MT) is a small, cysteine-rich protein with significant immunomodulatory activity. It has been shown to play a critical role in important cellular mechanisms including heavy metal detoxification, essential metal management and the inflammatory response. MT production can be induced by a number of cellular stressors and acts to lessen the harmful effects of oxidizing agents and heavy metal exposure. Previous studies have shown that the dose of the metallothionein gene present in an individual may have significant effects on the adaptive immune response, yet the mechanism behind this phenomenon remains unknown. We hypothesize that the gene dose of metallothionein will significantly affect the lympholiferative response between three mouse strains that produce different amounts of metallothionein due to genetic manipulation. Our results suggest that deviations from the normal gene dose of MT affect the speed and degree of a lymphoproliferative response; for example, MTKO produces a more robust response than either the MT-TGN or WT strains. Further investigation is needed to determine whether the hyperproliferation of the knockout (MTKO) murine strain is a result of increased oxidative stress and damage due to the lack of functional metallothionein. The means by which MTKO lymphocytes respond more robustly to proliferative stimulation may prove significant in contributing to the body of knowledge regarding the mechanisms of autoimmune disease development and it's therapeutic management. Insight into these MT gene disparities may help lay the groundwork for future treatments and therapies.

Introduction

The Mechanisms of Autoimmune Disease

Autoimmune diseases affect more than 23 million Americans, with direct healthcare costs totaling over 100 billion dollars annually [45]. These disorders are caused by an inappropriate immune response against self-antigens; the body begins mounting an immunological attack against it's own cells, often resulting in debilitating physiological conditions. The most common treatment for autoimmune disorders is immunosuppression, in which medication is used to decrease the immune response. However, traditional methods of immunosuppresion often involve chemicals and radiation that leave the patient immunocompromised and susceptible to a wide range of opportunistic infections, an outcome also seen in patients undergoing chemotherapy.

Dysregulation of the immune system can lead to both autoimmune and inflammatory disorders. In the case of autoimmunity, the immune system becomes activated by the body's own antigens whereas inflammatory disorders result from an inappropriately large reaction to non-self antigens and the downstream effects. The body has its own mechanisms for up-regulating or down-regulating the different facets of the immune system as needed. If we can elucidate some of the mechanisms by which the body naturally manages autoimmunity, we may discover novel therapeutic agents for autoimmune conditions.

During invasion by a foreign antigen, there are two different components of the immune system that react in order to protect the host. Innate immunity, the first response, discriminates between self and non-self. Activation of the innate immune system causes the expression of co-stimulatory molecules on phagocytic cells, which allows activation

of adaptive immunity. The adaptive part of the immune response is comprised of lymphocytes and macrophages, which can sense and eradicate foreign antigens like viruses, bacteria or improperly transformed host cells.

The immune response involves many signal cascade pathways and can be altered by a wide variety of mechanisms. Heavy metal exposure, radiation and stress can change the equilibrium of the immune system [6]. Depending on the circumstances and type of stress, prolonged periods can lead to immune suppression and others can lead to inappropriate immune activations like autoimmune disease.

Metallothionein as a Stress Response Protein

Metallothionein (MT) was first characterized in 1957 as a cadmium-binding protein [1]. There are four major isoforms of mammalian MT; MT1 and MT2 are present at basal levels in all mammalian tissues during all stages of development, while MT3 and MT4 are found largely in glutaminergic neurons and stratified squamous epithelial cells, respectively[2, 3].

Metallothionein is an unusually low molecular weight protein (~7 kDa) with an amino acid sequence roughly 60-70 amino acids in length. All known forms of MT contain approximately 20 conserved cysteine residues and five or more conserved lysine residues (MT-1 and 2 each have eight lysines) [4]. MT is most commonly found associated with Zn and it can also bind a wide variety of other essential and toxic metals, including copper, cadmium and mercury. Various cysteine-binding motifs are responsible for its strong affinity for these transition metals; NMR spectroscopy and X-ray crystallography show that the folded protein consists of an alpha and beta domain with cysteines organized in "clusters", enabling it to bind up to seven zinc atoms [5]. This high affinity for metals contributes to metallothionein's role as a stress response protein and enables it to protect cells from damage caused by toxic heavy metal cations [6].

Metallothionein is believed to play a role in several important "housekeeping" functions within the cell, including oxidation-reduction equilibrium, transcription factor activation and the regulation of essential metals within the cell [7]. Metallothionein has been shown to control the amount of zinc available in cells and participates in the transfer of essential metal ions to critical stress response proteins and transcription factors such as zinc-finger proteins, apoenzymes and other metalloproteins [8, 9].

Metallothioneins are often categorized as stress-response proteins because they can be induced by various cellular stressors including glucocorticoid stress, hormones, acute phase cytokines, endotoxin, heavy metals, interferon and reactive oxygen and nitrogen species [10-15]. During heavy metal exposure, divalent metal cations will react with components of host cells, causing widespread damage. Binding of MT to cadmium has been shown to increase cellular survival by sequestering heavy metals and maintaining it in a less toxic complex form [16]. Mice that overexpress MT show decreased morbidity and mortality when exposed to cadmium in their drinking water; conversely, mice with a truncated, non-functional MT gene were more sensitive to this heavy metal toxicity [16].

Metallothionein Plays a Crucial Role In Cellular Homeostasis

There is a high percentage of sequence similarity between human and murine metallothionein, indicating that the MT gene family is evolutionarily conserved and performs important housekeeping functions within the cell. Because of the rapid rate and varied induction of metallothionein up-regulation, we believe that the protein is involved in many homeostatic processes throughout the cell. While MT-null mouse mutants reproduce and develop normally, evidence suggests that MT synthesis impairment in humans may be linked to some neurological disorders [17, 18]. This link indicates that metallothionein might play an important role in metal management during human development.

While the absence of a functional MT gene complement can be survived in animals house in optimal conditions, research indicates that organisms that that possess functional MT have a genetic advantage during times of physiological stress [16]. The enhanced survival of organisms with an intact MT gene is believed to be due, in part, to metallothionein's role in the regulation of the immune system.

Metallothionein Regulates Zinc Bioavailability

MT's critical role in zinc bioavailability may indicate that it is an important regulator of the immune response. The presence of zinc is required for many facets of the immune response. Improper zinc management in humans has been associated with decreased proliferation of lymphocytes, improper ratios of CD4+ and CD8+ T cells, impaired natural killer cell activity and thymic atrophy [8]. All of these are critical components of the immune response and may lead to inappropriate immune activation and suppression. This leads us to believe that metallothionein may play an integral role in the development, differentiation and apoptosis of important populations of immune cells.

Bacteria require the incorporation of zinc for gene expression, virulence and metabolism, so it stands to reason that restricting available zinc might be an effective method to slow bacterial growth during infection [19]. Studies have shown that proteins that chelate or sequester zinc, like calprotectin and ZIP/Znt transporters can inhibit bacterial growth [19,20]. This suggests that metallothionein may also act to sequester essential zinc from microbial organisms during a bacterial infection.

Metallothionein and the Neutralization of Reactive Species

One important component of the innate immune response is a phenomenon known as a "respiratory burst", in which macrophages and neutrophils release reactive chemicals such as nitric oxide, hyderogen peroxide, hydroxyl radicals, hypochlorite and superoxide anions during infection [20-23]. Although this defense mechanism is directed at bacterial cells, it can also cause significant bystander damage to the host cells because these reactive species are small enough to pass through host membranes [24]. The thiol groups of metallothionein have the capability to bind and neutralize intracellular ROS, conferring a protective effect to the host cells [23].

Chemicals that induce the formation of ROS and RNS can also up-regulate the transcription of metallothionein genes. Current research indicates that the oxidation of cellular ligands by ROS causes the release of zinc, which activates MTF-1, which can then activate metallothionein gene transcription [25].This indicates that up-regulation of MT during ROS exposure may serve to prevent host tissue damage and apoptosis. In Alzheimer's disease, a common neurodegenerative disorder, inflammation can lead to the creation of reactive oxygen and nitrogen species that can damage neurons. Brain lesions in Alzheimer's patients have been linked to free radical bursts and it has been suggested that changes in MT expression may lead to this intermittent radical up regulation and the associated damage [26, 27]. MT has also been shown to have a protective role in neuronal wound healing [47].

The Immunomodulatory Functions of Metallothionein

The cysteines that are not involved in metal binding can participate in protein-protein interactions; MT can bind to proteins found on the surface of T and B lymphocytes, which may indicate a possible mechanism by which extracellular metallothionein stimulates proliferation. Extracellular metallothionein has been shown to stimulate modest levels of lymphoproliferation in murine splenocyte cultures and can act synergistically with other cellular mitogens Concanavalin A (ConA) and lipopolysaccharide (LPS) to produce a more robust proliferative response [28].

MT has been demonstrated to affect the apoptosis of cardiomyocytes by inhibiting the p38 map kinase, a key component in the inflammatory response; this indicates that MT may be involved in the regulation of inflammation and immune response signaling [29]. Metallothionein mediates p53 activity by managing intracellular levels of metal and reactive species [30]. High concentrations of MT decrease the movement of metals and inhibit apoptosis through p53, which induces anti-apoptotic effect and has been used to indicate cancer [31, 32]. MT may be a survival factor and may represent a potential therapy for cancer cells that overexpress MT.

Metallothionein has also been implicated in the indirect regulation of transcription factor activity. MT has been hypothesized to prevent the oxidation of IkB, modulating the activation of NF:kB; control like this could have downstream effects on genes involved in the immune response [33, 1]. The movement of MT into the nucleus during differentiation is believed to be an important part of zinc ion delivery so DNA transcription and replication can occur [35].

Given that we believe that MT plays a key role in managing stress and normal equilibrium within the body, it may also contribute to the normal immune response. If MT is involved in the immune response, it may prove to be the link to stress-induced changes in the immunity. Research has shown that mice with severe inflammatory disease have very high levels of MT bound to circulating leukocytes. Knocking out the MT genes for this mutation accelerates the disease and reduced the lifespan [36]. Patients with auto immune disease of the connective tissue (like scleroderma and systemic lupus erythematosus) and inflammatory rheumatic diseases like psoriatic arthritis, vasculitis and rheumatoid arthritis have low levels of circulating copper bound MT compared to control subjects. Injecting cortisones to increase levels of circulating Cu-MT produced both clinical improvement and decreased inflammation [37].

Experiments have also been done with rat experimental autoimmune encephalomyelitis (EAE) and MT-bound Zn II injections; results revealed a slowing of the clinical progression and lethality of the disease [38]. It was accompanied by decreases in IL-6 and TNF-a expression and leukocyte infiltration. The effects of MT injection on the development and progression of collagen-induced arthritis in rats was also analyzed. Injections of zinc (to induce MT) and MT-1 and 2 reduced the progression to arthritis; indicating that MT also regulates immune cell activation [39].

Metallothionein Gene Dose and the Immune Response

Several recent papers have explored the effects of MT gene dose on the immune response. Mice that possess a targeted disruption of the Mt-1 and Mt-2 genes (MTKO) were immunized with ovalbumin and were found to produce much higher levels of anti-OVA antibody than their wild type counterparts [1]. Flow cytometry analysis of spleen tissue from MTKO mice revealed an increase in the percentage of CD4+ and CD8+ T-cells, and these splenocytes were shown to be hyperproliferative in response to mitogen stimulation.

It has been hypothesized that the lack of functional MT in the MTKO model leaves the mouse less equipped to handle elevated levels of reactive oxygen species. This increase in intracellular ROS leads to activation of transcription factors like NF-kB, which augments the expression of immunomodulatory cytokines [1]. In essence, this lack of endogenous MT creates a primed immune response that could potentially lead to dangerous autoimmunities under stressful conditions.

Metallothionein has also been found to be a critical component of the pathogen-induced immune response [40]. Three congenic mouse strains were infected with Listeria monocyogenes; the wild type C57BL/6 strain, the B6-MTKO knockout, and the B6-MTTGN strain, which overexpresses metallothionein due to 112 extra copies of the Mt-1 gene. Surprisingly, both the transgenic and knockout strains were more resistant to infection than the control C57BL/6; there appears to be a significant advantage to both low and high gene doses of metallothionein when compared to the wild-type mice in this particular biological context. In addition, MT gene dose has also been found to suppress

the advance of rheumatoid arthritis in mice, pointing to the protein as a potential opportunity for treatment of certain autoimmune diseases [41]. If the means by which extreme levels of metallothionein affect the immune response can be characterized, it may enable the development of more targeted therapies that will allow augmentation of a patient's innate immunity and suppression of debilitating autoimmune disorders.

Proliferation as a Critical Catalyst in the Immune Response

For our research, we focused on the ability of metallothionein to scavenge free radicals as the potential mechanism responsible for the hyperproliferation of MTKO mice. The metal-thiolate clusters in metallothionein can be readily oxidized, thereby neutralizing ROS and metallothionein can be as much as 100 times more effective than glutathione in preventing cell damage by certain oxidation products [42].

We decided to examine the proliferative response because MT is believed to play a critical role in cellular signaling, which is required for cell proliferation. Because metallothionein has been shown as a reservoir for metals and a neutralizing force in reactive species management, it suggests that MT may help to play a role in the events leading up to and during an immune response. MT has been shown to facilitate the activation of certain transcription factors that are activated by zinc. When human macrophages where stimulated with LPS, it was found that MT expression was enhanced [43]. When MT was down-regulated with anti-sense mRNA, LPS could no longer induce a respiratory burst without harming cell viability [44].

Metallothionein may also act as a stimulation signal for lymphocytes. MT can induce lymphoproliferation in vitro and acts synergistically with lymphocyte mitogens like ConA and LPS to enhance proliferation. Interestingly, cells that are pre-treated with MT are not sensitive to anti-proliferative agent N-ethyl maleimide, indicating that the proliferative qualities of MT correlate with the levels of free thiols [28]. C3H/HeJ mice have a defect in protein kinase C translocation, rendering them unresponsive to stimulation with LPS. The addition of MT to these splenocytes in culture restores

proliferation [28]. This suggests that MT may interact directly with membrane surface proteins in order to effect signal transduction.

MT is believed to play a complex role in the immune response and normal immune function and may represent a novel target for autoimmune disease therapies. The experiments in this thesis focus on the immunological differences between animals that possess a targeted gene disruption of the Mt1 and MT2 gene loci, the wild type and the MT transgenic mouse that carries 112 extra copies of the extra Mt1 gene. The MTKO mouse has been shown to have a sensitive to heavy metal and free-radical exposure, but develops normally otherwise. In contrast, the MT-TGN mouse is less sensitive to heavy metals. We hypothesize that the targeted gene disruption of the Mt1 and M2 sequences will significantly alter the humoral immune response.

Materials and Methods

Mouse Strains

All experiments utilized primary splenocytes from age and sex-matched from C57BL6/J-MT1^{tm1Bri} (MTKO), C57BL6/J-Tgn(Tm1)^{174Bri} (MT-TGN) and C57BL/6 (WT) mice obtained from our own breeding stock kept at the AAALAC accredited University of Connecticut vivarium. Animals were housed separately from other colonies and were kept on a 12:12 hour light:dark cycle, with food and water available at all times. The sentinel animals in this colony are serologically screened regularly and were found to be free of common murine pathogens. The protocols for all of our proposed experiments were approved by The University of Connecticut Institutional Animal Care and Use Committee.

Media and Reagents

The M199 utilized for the cell proliferation assays was purchased from GIBCO/BRL (Grand Island, NY). This media was supplemented with Fetal Bovine Serum (FBS) from Hyclone (Logan, UT), 0.15% (w/v) sodium bicarbonate, 0.1mM non-essential amino acids, 1mM sodium pyruvate, 1mM L-glutamine and 50 ug/ml gentamycin. Concanavalin A (ConA), bovine serum albumin (BSA) and lipopolysaccharide (LPS, E.Coli) were purchased from Sigma Chemical (St. Louis, MO).

Splenocyte Preparation

To quantify the effects of differing amounts of endogenous metallothionein on proliferation, splenocytes were obtained from all three mouse strains (WT, MT-TGN and

MTKO). The spleens were aseptically prepared and placed in 3ml of M199 in a small Petri dish. Spleens were then gently dissociated with flame-sterilized frosted slides. The single cell suspension was the moved to a sterile tube and pelleted by centrifugation at 120 xg for 5 minutes. After decanting the supernatant, a hypotonic erythrocyte lysis was conducted. The cells were then centrifuged again and resuspended in a final volume of 2ml complete M199 media.

Proliferation Assays

The cells were then plated in a 96-well cell culture-treated white microfluorescence plate. 100ul of each cell suspension was added per well and the unfractionated cells were cultured in the presence or absence of a polyclonal activator (ConA or LPS). All samples in the proliferative assays were incubated at 37 degrees in a special gas mixture (10% CO2, 7% O2, 83% N2) in order to reduce oxidative stress and prolong cell survival. Proliferation was assayed at 24, 48 and 72 hours with the Promega Cell Titer-Glo Assay, which utilizes luminescence to quantify cellular ATP levels as an indication of cell population size. Luminescence was measured by a 96-well microplate reader.

Results

Optimization of Cell Quantification Assays

The proliferative assays were first attempted using Jurkat T-cells in order to optimize the concentration of cells and the cellular mitogens LPS and ConA. This optimization utilized the Promega 96 Aqueous Non-Radioactive Cell Proliferation Assay (MTS), a colorimetric method for determining the number of viable cells in culture. The MTS reagent is bioreduced by cells into a formazan product that can be measured at 490nm in a 96-well plate. This assay did not allow for the development of a satisfactory cell number standard curve and Jurkat T-cells did not show measurable levels of stimulation when dosed with Concanavalin A and LPS. An appreciable signal could not be obtained from any cellular concentration (data now shown).

The MTS assay is often not suitable for lymphocytes since less of the formazan product that is produced by mitochondria is produced in smaller amounts by lymphocytes. In contrast, the Promega Cell Titer Glo Assay, which quantifies cellular number based on the amount of available ATP, is compatible with lymphocyte biochemistry. The Cell Titer Glo Assay yielded an optimal cell concentration of cultured T-cells (100,000/well) and primary splenocytes (60,000/well); this cellular number was large enough to produce a luminescent signal significantly above the background reading, but still small enough that cellular viability was not negatively affected.

Optimization of Cellular Proliferation Assays

We next endeavored to find the optimal concentration of cellular mitogens (ConA or LPS) to treat cells. When the concentration was too low (2.5ug/ml ConA in PBS),

there was no appreciable difference between the luminescence of the control cells (treated with PBS only) and that of the treated cells. When the concentration was too high (10ug/ml ConA in PBS), there was a reduction in the level of luminescence; we hypothesize that this is due to the cross-linking activation of the mitogens, which may have caused cellular clumping and restricted nutrient availability, causing cell death. Dosing the cells with 5ug/ml of ConA or LPS for 24 hours seemed to yield optimal results and was chosen as the standard stimulation concentration.

The Proliferative Response of Mouse Strains with Different Numbers of MT1 and MT2 Gene Copies

In these experiments, splenocytes from each strain were isolated and stimulated with Concanavalin A (ConA) during a three-day incubation, in which proliferation was assayed at different time points (Fig 3). Twenty-four hours post-stimulation, MTKO cells showed the highest levels of proliferation, yet after forty-eight hours, the MTKO proliferative response had fallen far behind the other strains, indicating that their lack of metallothionein may put them at a disadvantage after the initial primed response. In addition, when the ConA dosage was increased, only the transgenic cells were able to maintain a response to the higher dosage.

Proliferative Response Is Dependent Upon Culture Conditions

Splenocytes from each strain (varying MT gene dose) were isolated and stimulated with lipopolysachharide (LPS) for 36 hours, after which proliferation was assayed. Cells that had been cultured in a reduced oxygen environment (37 degrees, 10%

CO2, 7% O2, 83% N2) displayed the same relationship as seen during Concanavalin A stimulation; the KO splenocytes showed higher levels of proliferation than the MT-TGN or WT. Yet when cultured under normal oxidative conditions in 5% CO2 (where the atmosphere consists of a greater amount of oxygen), the MT-TGN cells fared significantly better than the WT or KO splenocytes. This may indicate that the over-expression of metallothionein confers some protection against a more oxidative environment because the MT is able to sequester and neutralize harmful reactive species.

Discussion

The immune response is a complex cascade of events triggered in response to many different stressors and antigens. A mechanism this intricate requires precise regulation that simultaneously allows for recognition and activation against a pathogen while avoiding an undesirable hyper-reactivity or hypo-reactivity. If metallothionein plays an important role in immune regulation, then it may provide to be a key therapeutic target in the treatment of diseases that result from inappropriate immune activation or suppression, like autoimmune disorders and cancer.

Metallothionein is a stress protein that is known to confer protection against toxic exposure to metals and oxidizing agents. Yet recent work has shown that MT also plays an integral role in the regulation of immune response. The experiments described here were intended to investigate the differences in the immune response caused by different levels of metallothionein. Proliferation is one of the most important phenomenona of the early immune response and elucidating the mechanism by which a low gene dose of metallothionein causes hyper-proliferation of MT-KO spleen cells may help us to better understand and manipulate these mechanisms.

In conclusion, MTKO cells are primed by their lack of metallothionein to respond more quickly to polyclonal activation. In addition, over-expression of MT in transgenic cells may confer protection and augment cellular survival. MT may regulate lymphocyte responses through several different mechanisms. Metallothionein is known to neutralize reactive intermediates within the cell. These reactive intermediates are generated by the ligation of lymphocyte membrane receptors and go on to activate transcription factors. In an organism with little functional metallothionein, it is possible that the higher threshold

levels of reactive species cause enhanced transcription factor activation; MT is known to decrease TNF activation of NF-KB by inhibiting IkB degradation.

MT may also act by regulating the activity of kinases that are necessary for transcription factor acivity, such as p38 map kinase. MT may also alter transcription my altering the availability of copper and zinc at the nucleus. The formation of ROS in lymphocytes may stimulate the release of zinc from metallothionein; the absence of zn-MT may cause repressors of transcription to remain inactivated, leading to inappropriate activation and disease states.

Further studies are needed to determine the molecular mechanisms of the MTKO "primed" response and whether it is due to constant low level oxidative stress (which cellular metallothionein is believed to reduce in normal cells). Planned experiments include manipulation of the cellular redox environment with antioxidants, incubation in different oxidative stress conditions and addition of zinc in order to upregulate MT levels in mice that can synthesize the protein. We also plan to conduct ELIspot assays of the three strains in order to quantify the number of active B-cells secreting antibody in the presence or absence of antioxidant in order to explore the role of oxidant in B-cell differentiation. Using this data, we endeavor to elucidate the role of endogenous metallothionein in the suppression of oxidative stress and cellular protection. If we can characterize the means by which extreme MT levels affect the immune response, it may enable the development of more targeted therapies that will function to augment innate immunity and suppress debilitating autoimmune and inflammatory disorders.

Figures

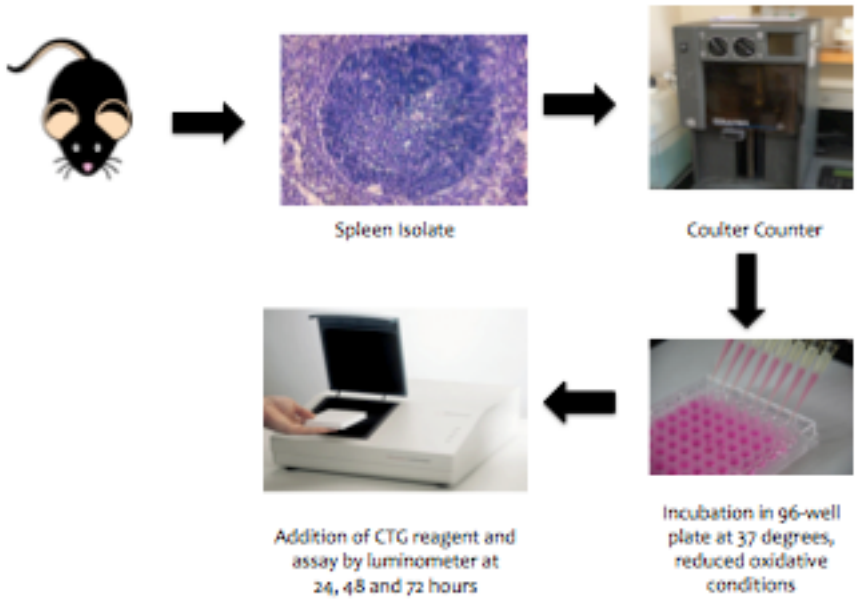
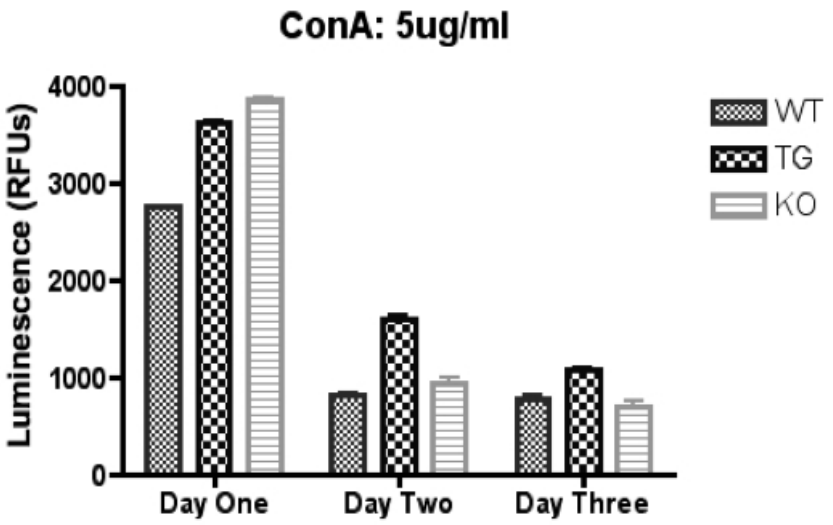


Figure 1. Experimental Outline. Primary lymphocytes from the three mouse strains were re-suspended in complete M199 media (5% FBS, .1mM non-essential amino acids, 1 mM sodium pyruvate, 1 mM l-glutamine, 50 ug/ml gentamycine and sodium bicarbonate) and cell number was determined by a particle counter. They were incubated at 37 degrees in a special gas mixture (10% CO2, 7% O2, 83% N2) in order to reduce oxidative stress and prolong cell survival.

Figure 2. Luciferase Reaction. Mono-oxygenation of luciferin catalyzed by luciferase in the presence of Mg2+, ATP and molecular oxygen.

A



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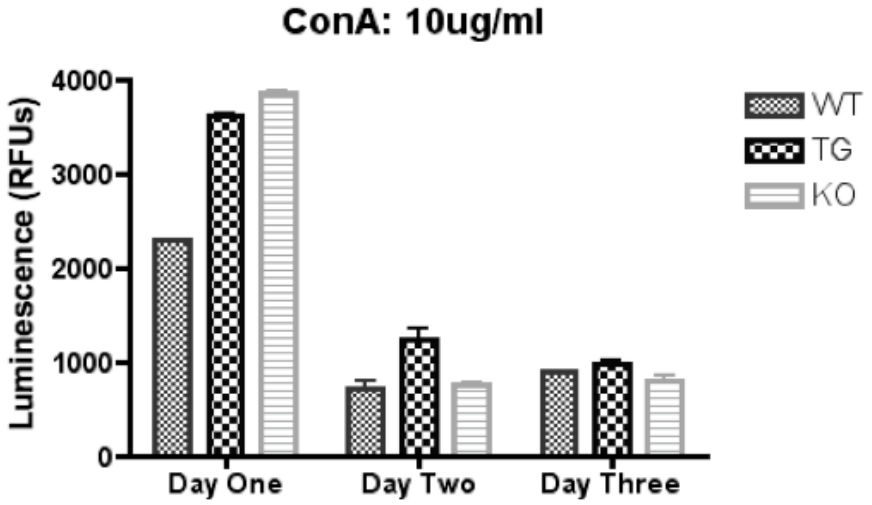

Figure 3. 040511 **Dosage curve of WT, KO and TG mouse strains treated with Concanavalin A (ConA).** The primary splenocytes isolated C57BL6/J (denoted B6-WT), C57BL/6J-TgN(Mt1)^{174Bri} (denoted B6-MTTGN), and C57BL/6J-Mt1^{tm1Bri}Mt2^{tm1Bri} (denoted B6-MTKO) mice were cultured in the presence of 5ug/ml (a) or 10 ug/ml (b) ConA. The cultures were aliquoted into 96-well plates and incubated at 37 degrees in a reduced oxidative environment. The Cell Titer Glo assay was used to quantify proliferation at 24h, 48h and 72 hours post-stimulation. RFU= Relative Fluorescence Units. $P < .05$ for all points.

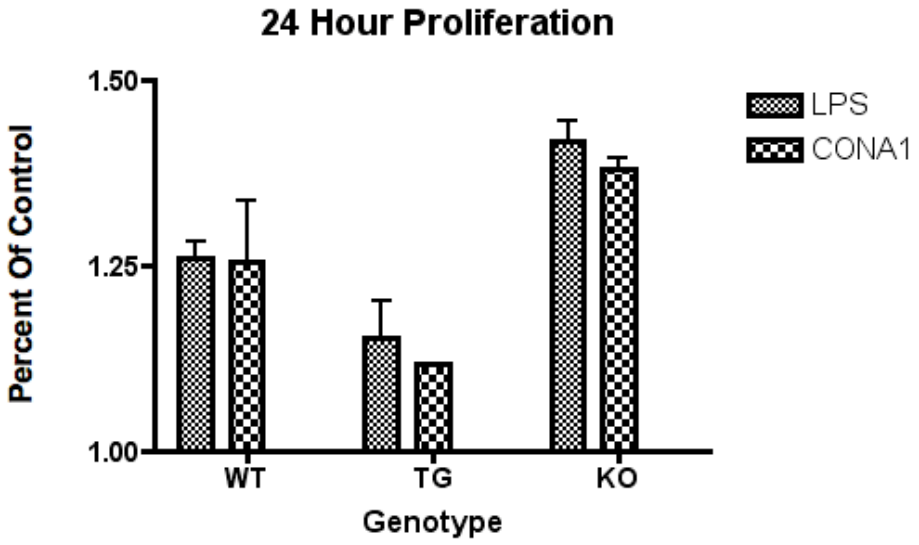

Figure 4. 060611 **Dosage curve of WT, KO and TG mouse strains treated with Concanavalin A (ConA) or Lipopolysaccharide (LPS).** The primary splenocytes isolated C57BL6/J (denoted B6-WT), C57BL/6J-TgN(Mt1)^{174Bri} (denoted B6-MTTGN), and C57BL/6J-Mt1^{tm1Bri}Mt2^{tm1Bri} (denoted B6-MTKO) mice were cultured in the presence of 5ug/ml ConA or LPS. The cultures were aliquoted into 96-well plates and incubated at 37 degrees in a reduced oxidative environment. The Cell Titer Glo assay was used to quantify proliferation at 24h post-stimulation. $P < .05$ for all points.

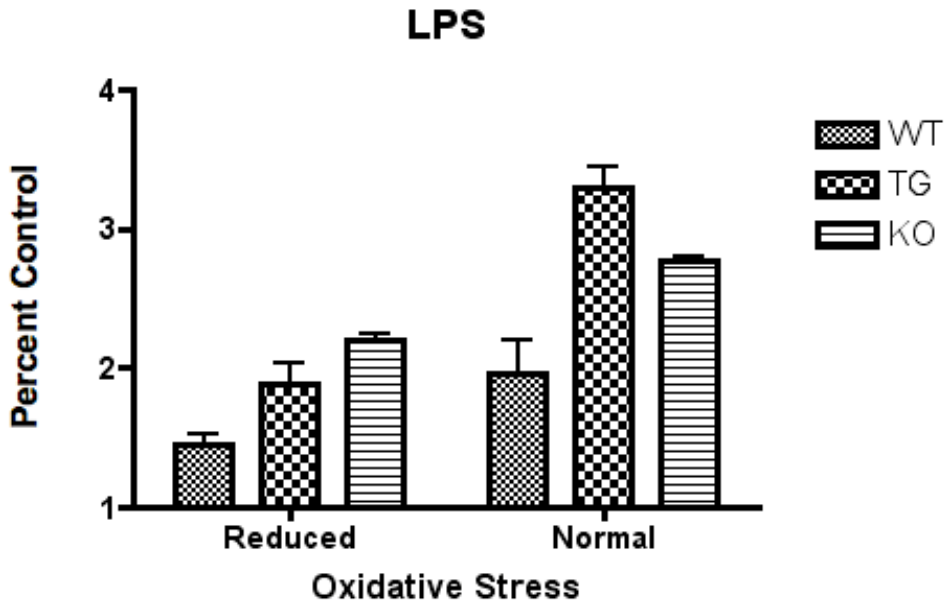

Figure 5. 110511. **Dosage curve of WT, KO and TG mouse strains treated with LPS under normal or reduced oxidative culture conditions.** The primary splenocytes isolated C57BL6/J (denoted B6-WT), C57BL/6J-TgN(Mt1)^{174Bri} (denoted B6-MTTGN), and C57BL/6J-Mt1^{tm1Bri}Mt2^{tm1Bri} (denoted B6-MTKO) mice were cultured under reduced oxidative conditions (See Materials and Methods) or normal oxidative conditions in 5% CO2. The cultures were aliquoted into 96-well plates and incubated at 37 degrees in a reduced oxidative environment. The Cell Titer Glo assay was used to quantify proliferation at 24hr post-stimulation. $P < .05$ for all points.

Acknowledgements

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