Classically Conditioning the Up-Regulation of TNF-α and IL-2 for Overall Immune System Enhancement in Sprague-Dawley Rats

Senior Comprehensive Thesis

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Abstract:

The purpose of this experiment was to analyze an alternative treatment for immune system disorders that can bypass activation of the "Negative Feedback Loop" of immunity, alleviating the risk of immunosuppression. This alternative method is the use of "Classical Conditioning" to demonstrate that the effects of a drug can be conditioned, resulting in a similar but lessened physiological response to treatment. This lessened physiological response improves overall immune system function while avoiding the immunosuppressive effects of sustained drug treatment. Eighteen Sprague-Dawley rats served as the subjects. The Control Group received saccharin water paired with saline injections (3.20 mg/kg); the Antagonist Group received saccharin water paired with nor-BNI injections (3.20 mg/kg); and the Agonist Group received saccharin water paired with U69,593 injections (0.32 mg/kg). The Agonist and Antagonist Groups were further subdivided into Classical Conditioning (CC) and Sustained Drug Treatment (SDT) conditions. The SDT received drug treatment for the entirety of the experiment, while the CC received drug treatment for only the first half and saline injections for the remainder of the experiment. The level of TNF-α present in rat blood serum at the conclusion of the experiment served as the independent variable. The results indicate that the lessened physiological effect demonstrated in the CC Antagonist group was beneficial to overall immune system function, suggesting that the successful avoidance of "Negative Feedback Loop" activation was achieved. The alternative treatment method put forth in this experiment should be tested in other experiments to compile evidence supporting the beneficial effects of "Classical Conditioning" treatments.

Introduction:

The human immune system is a complex and highly developed system involving balanced signaling between the neuroendocrine and immune systems. Proper modulation of immune function depends on the sensitive balance of neuroendocrine-immune system "crosstalk" that macro-manages the release of cytokines needed to fight pathogens. Due to the sensitive nature of immune system function, modulation can be corrupted with even the slightest malfunction, and the results can be devastating (Taub, 2008). Immune system disorders affect millions of Americans. Despite the efforts of scientists to unravel the complexities of immune system function, all attempts thus far have stopped short of offering an all-encompassing explanation of the mechanisms modulating immune system function. The existence of the "Negative Feedback Loop" of immunity serves to suppress immune system function when it is not needed. However, scientists have discovered that the most commonly employed method for treating these disorders, sustained drug treatment, activates the "Negative Feedback Loop", resulting in overall immunosuppression. Although some disorders of the immune system are caused by an overactive immune response, immunosuppression does not cure the disease as one might assume. Other disorders of the immune system are caused by a hypo-reactive immune response and further immunosuppression acts to worsen the symptoms (Taub, 2008). In either case, immunosuppression is never a preferred outcome and has dangerous side effects.

Further research needs to be conducted involving the pathologies of immune system disorders because not much is known thus far. For example, in Crohn's Disease the body mistakes food and bacteria as foreign invaders. The immune system then proceeds to attack the

"invaders", resulting in a hyperactive immune response. However, scientists do not yet know if this phenomenon is a cause of the disease or a result of the disease. Scientists debate over whether the disease is caused by genetic factors, the immune system itself, or the environment (Marketon & Glaser, 2008). Even using what is known, immune system disorders are still very difficult to treat because of the complexities and sensitivities of the immune system. Modulation of the immune system involves reaching the perfect balance of immune-endocrine interactions, because too little and too much modulation of immune system function both result in devastating consequences (Marketon & Glaser, 2008). Crohn's Disease is currently treated with corticosteroids and immunosuppressant drugs to prevent chronic inflammation of the intestine lining, ulcerations, and bowel injury (Marketon & Glaser, 2008). However, these medications have negative side effects, such as high blood pressure, osteoporosis, and increased risk of infection. The medications suppress the immune system to alleviate symptoms of the disease, but long-term use of immunosuppressant drugs results in low immune function, increased susceptibility to illness, and illness characterized by longer durations and more severe symptoms (Marketon & Glaser, 2008).

Other immune system disorders emerge as a side effect of drug treatments for other illnesses. For example, cancer patients undergoing chemotherapy may also become immuno-compromised. Chemotherapy results in the paralysis of bone marrow, the site of origin of T-cell production. The result of this is a decreased availability of white blood cells able to fight infections. In severe cases, chemotherapy may even destroy all of the bone marrow stem cells, thereby preventing the generation of new white blood cells. Someone undergoing this type of treatment is then much more susceptible to illness, but in their case a simple illness such as the common cold may result in severe illness or even fatality (Marketon & Glaser, 2008). With the

prevalence of cancer in the United States and the massive number of people undergoing chemotherapy, it is of the utmost importance to develop a method of treatment without such adverse side effects as chemotherapy.

One alternative treatment currently being studied is the possibility of using classical conditioning to treat immune system disorders. Classical conditioning is defined as "a learning process where something that has not previously produced a particular physiological response (conditioned stimulus) is paired and becomes associated with something that has produced a particular physiological response (unconditioned stimulus). As a result the conditioned stimulus begins to elicit the same physiological response that the unconditioned stimulus previously elicited." (Ader & Cohen, 1982, p. 1534). As mentioned previously, sustained drug use results in immunodeficiency in Crohn's Disease, cancer, and many more examples, along with a cadre of other detrimental side effects. However, using classical conditioning procedures could result in an individual's physiological response to a drug being conditioned, eliminating the need for sustained use of that drug. By associating the drug (unconditioned stimulus) with some other stimulus (conditioned stimulus), it should be possible to elicit the same physiological response with only the presentation of the conditioned stimulus and the removal of the unconditioned stimulus. Ader & Cohen (1982) demonstrated with their laboratory rats that this type of association could be made and that the effects of conditioning lasted for six weeks before extinction occurred. This finding suggests that the use of classical conditioning procedures can be long-lasting enough to rationally be considered as an alternative to sustained drug treatment in immune system disorders. This theory is the substance of the specific research question at hand here: Can classical conditioning be used to improve overall immune system function?

Some scientists present the argument that the immune system is too complex to be reliably conditioned. However, this statement has already been proven wrong by two classical conditioning researchers at the University of Rochester. Ader & Cohen (1982) were the first researchers to classically condition immune system function, but instead of conditioning enhancement, they conditioned suppression. They paired the immunosuppressant drug, cyclophosphamide, with saccharin water and continuously presented the pair to lab rats over the course of several weeks. After the cyclophosphamide was taken away for half of the lab rats and they were only presented with saccharin water, they still continued to show the same immunosuppressant effects as the other half of the lab rats who were still given the drug. All rats showed a decrease in the number of T cells and a decrease in the number of antibodies and some showed fatalities after pairing the saccharin water with the cyclophosphamide. Ader & Cohen thus demonstrated the first scientifically proven example of conditioned immunosuppression in laboratory rats and have since then reliably repeated their results in subsequent experiments (Ader & Cohen, 1982). The goal of the current study is to demonstrate classically conditioned immune system enhancement in laboratory rats.

As mentioned earlier, immune system modulation involves a complicated network of neuroendocrine and immune system signaling. In this network, cytokines released in an immune response signal the release of hormones that then signal for the body to cease the immune response. High levels of stress hormones depress immune system function and the absence of these hormones facilitates immune system function (Saurer, Ijames, Carrigan & Lysle, 2008). Hormones and cytokines interact in a complicated balancing act to modulate immunity. Thus, it must be possible to condition immune system function somehow through the manipulation of this signaling. This study will use a manipulation directed towards the kappa-opioid receptor on

immune system cells. It has recently been shown that immune system cells express opioid receptors and that opioid receptors control antibody production, Natural Killer (NK) cell production, proinflammatory cytokine and proinflammatory cytokine receptor expression, and the development and maturation of T cells in the thymus (Chao, Gekker, Hu, Kravitz, & Peterson, 1998). By working specifically with the kappa-opioid receptor, it should be possible to classically condition the release of proinflammatory cytokines to activate the Hypothalamus-Pituitary-Adrenal (HPA) axis and stimulate the differentiation and maturation of naïve T cells in the thymus into mature $CD4^+$ cells (Helper T cells) that are able to fight pathogens.

Recent research has shown that pharmacological manipulations of the kappa-opioid receptor results in changes in the levels of two proinflammatory cytokines, TNF-α and IL-2. Both of these cytokines play an important role in modulating the immune system. In a Finley et al. study (2008), the kappa-opioid receptor agonist U50,488H in concentrations as low as 1 nM inhibited the release of TNF-α and IL-2 and inhibited the maturation of T cells in the thymus. The decreased expression of TNF-α and IL-2 was shown at the transcriptional level (Finley, Happel, Kaminsky, & Rogers, 2008). This theory that kappa-opioid receptors are important in the process of T cell maturation in the thymus is supported by the increased rates of kappa-opioid receptor expression on cell surfaces of mature T cells in the thymus (Finley et al., 2008). In addition, the inhibiting effects of U50,488H on TNF-α and IL-2 expression are completely blocked by pre-treatment with the kappa-opioid receptor antagonist nor-BNI. Intraperitoneal injection of nor-BNI in concentrations as low as 0.56 mg/kg increased rates of TNF-α and IL-2 by 2.5 times compared to control mice (Parkhill & Bidlack, 2006). In addition, kappa-opioid receptors are also located on cells within the paraventricular nucleus, and thereby have the ability to activate the HPA axis (Finley et al., 2008).

Although nor-BNI is technically the antagonist (which generally results in decreased activity at the receptor site) of the kappa-opioid receptor, its effect has been proven to increase activity at the receptor site. This phenomenon is explained with the activation of the JNK pathway. The binding of nor-BNI to the kappa-opioid receptors on immune system cells activates the JNK pathway, a secondary cascade of mitogen-activated protein (MAP) kinases which are responsive to stressful stimuli (such as cytokines) and inflammatory signals (such as the binding of nor-BNI to the receptor). Therefore, the binding of nor-BNI to the kappa-opioid receptor on an immune system cell activates the secondary cascade of MAP kinases in the JNK pathway. JNK, by phosphorylation, then modifies the activity of numerous proteins that reside in the mitochondria or nucleus of an immune system cell. The overall effect is to decrease the release of the cytokine IL-1, which usually inhibits the release of the proinflammatory cytokines IL-2 and TNF-α. By inhibiting the release of IL-1, the JNK pathway increases release of IL-2 and TNF- α (Bruchas et al., 2007). Specifically, the JNK pathway is involved in T cell differentiation, proliferation and apoptosis. By stimulating the kappa-opioid receptor with an antagonist, the overall effect is to increase levels of proinflammatory cytokines released, resulting in improved T cell differentiation and proliferation in the thymus (Bruchas et al., 2007).

The Bruchas et al. (2007) study showed that the end result was better control over the T cells exiting the thymus. Other studies have shown that certain autoimmune disorders can be caused by the accidental release of “double-negative” T cells, those that are lacking both the $CD4^+$ and $CD8^+$ MHC complexes, from the thymus. MHC complexes are necessary for T cells to bind self-antigens and for recognition of foreign invaders. Without an MHC complex a T cell has no inhibition and attacks every cell that crosses its path, including healthy cells that the body needs. The thymus generally weeds out the “double-negative” T cells and signals them to die,

but sometimes they are released into the bloodstream and cause autoimmune disorders (Bruchas et al., 2007). The binding of a kappa-opioid receptor antagonist to the receptor site stimulates thymus function and enhances its ability to discriminate between double-negative T cells and T cells with an MHC complex. Although it may seem counterproductive to treat a hyper-immune response with a drug that will increase immune function even more, the antagonist could possibly cure the disease by enhancing thymus function and decreasing the likelihood that double-negative T cells are released into the bloodstream. However, it is important to remember that sustained treatment with the antagonist will activate the "Negative Feedback Loop" and lead to immunosuppression, so it is vital to investigate the applicability of Classical Conditioning treatment to this disease.

The kappa-opioid receptor also has additional relevance to immune system modulation because it is intimately related to the release of TNF-α and IL-2, both proinflammatory cytokines implicated in the development of cancerous cells. Increased rates of TNF-α have been shown to increase apoptosis of cancerous cells and decrease the proliferation of cancerous cells (Butts & Sternberg, 2008). Increases in rates of IL-2 increase Natural Killer cell activity; increase antibody production; and increase both memory T cell and cytotoxic T cell function (O'Connor et al., 2008). The interaction of TNF-α and IL-2 synergistically activate corticotrophin-releasing dendritic cells in the paraventricular nucleus, thereby activating the HPA axis. These cells then release cortotropin-releasing hormone from the pituitary gland to activate the thymus, leading to the proliferation of T cells in the thymus and enhancing the transformation of naïve T cells into mature $CD4^+$ cells that are able to fight pathogens (O'Connor et al., 2008). This fact is supported with evidence from a recent study, showing that injection of xylazine (an α-2 receptor agonist) in high concentrations in the thymus inhibits the proliferation, differentiation, and

maturation of T cells, but this effect is completely reversed with the exogenous administration of both IL-2 and TNF-α (Bellinger et al., 2008). This suggests that IL-2 and TNF-α are necessary for the neuroendocrine signaling involved in the process of T cell maturation in the thymus.

However, the crosstalk between the neuroendocrine and immune systems is very sensitive and needs to remain in balance. As Ader & Cohen (1982) demonstrated, disturbances in immune system modulation may result in fatalities of lab rats. Overstimulation of the HPA axis results in IGF-1 and cortisol release from the adrenal gland, the last component of the HPA axis (O'Connor et al., 2008). IGF-1 and cortisol are both stress hormones involved in a negative feedback loop to suppress inflammation. It has been proven that sustained stress results in overall immune system suppression and that cancer growth is intimately connected with IGF-1 receptor activation (O'Connor et al., 2008). This could explain why in the previously mentioned study with xylazine, the α-2-Androgen receptor agonist, had suppressing effects on T cell maturation in high concentrations but not in low concentrations (Bellinger et al., 2008). It is possible that low concentrations of xylazine are not powerful enough to release the elevated levels of cortisol and IGF-1 needed to suppress immune function through the negative feedback loop. This suggests that small increments of elevated TNF-α and IL-2 levels are beneficial for immunity, and are low enough to avoid the detrimental effects of the HPA negative feedback loop on immunity.

This theory is supported with the recent discovery that TNF-α and IL-2 are both involved in IGF-1 resistance (O'Connor et al., 2008). Large increases in TNF-α and IL-2 result in mass tyrosine phosphorylation, leading to IGF-1 expression and cancer cell growth. However, small and interspersed elevations in the cytokines are not enough to result in tyrosine phosphorylation

and IGF-1 is never expressed. Thus, small and interspersed elevations in IL-2 and TNF-α lead to IGF-1 resistance, resulting in tumor cell apoptosis and decreased proliferation of tumor cells (O'Connor et al., 2008). Thus, it is possible and has been recently demonstrated that by carefully calculating the doses of drugs, scientists can avoid lethal consequences by manipulating the phenomenon of IGF-1 resistance.

The current study demonstrates that the up-regulation of proinflammatory cytokines, specifically TNF- α in this experiment, can be classically conditioned through pharmacological manipulation of the kappa-opioid receptors expressed on immune system cells and cells located within the paraventricular nucleus that activate the HPA axis. Laboratory rats were placed in a classical conditioning paradigm that paired saccharin water with a specified kappa-opioid-receptor-selective agent. The goal of this study was to explore the theory that classical conditioning can be used to enhance overall immune system function by resulting in a similar, but lessened physiological effect, when compared with sustained drug treatment, and that the lessened effect can bypass activation of the "Negative Feedback Loop".

METHODS:

Animal Subjects

This study used 18 Sprague-Dawley rats housed individually in the Animal Colony located in Carnegie Hall. They were then split into three groups consisting of six rats each. Each group consisted of half males and half females. The further division of the Agonist and Antagonist groups between the CC and SDT conditions was done by counterbalancing the number of males and females in each condition, so as to eliminate the possibility of gender

differences affecting the results of the experiment. The laboratory rats were housed under a 24-hour illumination schedule for the duration of the experiment and given free access to Purina Rat Chow and water. Their weights ranged from 234.50 grams to 554.00 grams, with the average weight being 411.72 grams. The history of these rats was unknown. However, all were greater than one year of age. One female rat from the Agonist Group was found dead after the first experimental session. The rat may have been sick already due to the fact that two other rats acquired gastrointestinal illnesses and were replaced before the start of the experiment. However, this could also be due to the fact that the rat was the smallest of all of them and was in the Agonist Group receiving an injection of U69,593, the drug hypothesized to inhibit the release of proinflammatory cytokines and suppress immune system function. This rat was not replaced, leaving the Agonist Group with only five rats at the time of analysis. Two more rats, one male and one female, from the Control Group were found dead the day after the conclusion of the experiment. This is believed to be due to the stress caused by the tail vein procedure used to acquire the blood samples. Both rats had bruised and scabbed tails, suggesting that an infection was acquired after the needle puncture.

Variables

The independent variable in this experiment was the drug condition with three levels: control (n=6), agonist (n=6), and antagonist (n=6). In addition the agonist and antagonist groups were further subdivided into four levels: Classical Conditioning (CC) Agonist (n=3), Sustained Drug Treatment (SDT) Agonist (n=3), CC Antagonist (n=3), and SDT Antagonist (n=3). The Sustained Drug Treatment groups received saccharin water and the specified kappa-opioid-

receptor-selective agent for the entire duration of the experiment. The Classical Conditioning groups received saccharin water paired with the specified kappa-opioid-receptor-selective agent for the first three experimental sessions, but their injections were changed to saline paired with saccharin water for the fourth to the seventh experimental sessions. The dependent variable in this experiment was the measured level of TNF- α present in the rats' blood serum at the conclusion of the experiment. Blood was collected using a tail vein procedure and the level of TNF- α present in the blood was measured using an ELISA assay.

Drug Preparation and Saccharin Water Solution Preparation

The dose of U69,593 used was 0.32 mg/kg. The U69,593 powder was dissolved in 50% Ethanol, isotonic saline, and 10 microliters of glacial acetic acid. This solution was then placed in a hot water bath for thirty minutes and vortexed to dissolve the drug completely. The dose of nor-BNI used was 3.2 mg/kg. The nor-BNI powder was dissolved in isotonic saline. Saline administration was also delivered at 3.2 mg/kg. The saccharin water solution was prepared using four sugar packets for every 20 cc's of distilled water.

Apparatus

This experiment was conducted in the Animal Colony located in the basement of Carnegie Hall at Allegheny College. Each rat was removed from its individual cage and given a saccharin water solution through a curved stainless steel gavage attached to a 12-gage needle, which was placed at the back of the rat's tongue and designed to efficiently administer the

solution down the rat's esophagus. Afterwards, each rat was injected with its assigned drug (or non-drug) treatment using a 12-gage needle and placed back into its individual caging before the next rat received its treatment. Collection of the blood samples was performed in the Animal Physiology Lab located in the basement of Carnegie Hall using a 14-gage needle. Samples were immediately frozen at -20 degrees Celsius and transported on ice to Professor Tricia Humphrey's microbiology lab located on the second floor of Steffee Hall at Allegheny College, where the ELISA assay (BD OptEIA Rat TNF- α Kit, Cat. No. 558535) was performed. A standard microplate reader (set at 450 nm) located on the third floor of Steffee Hall and owned by Allegheny College was then used to display the results of the assay.

Procedure

Three groups of Spraque-Dawley laboratory rats were individually housed in the Animal Colony. The Agonist and the Antagonist group received saccharin water solution preceding their assigned drug (or non-drug) treatments. The control group received saccharin water solution paired with a saline injection. The Agonist group received U69,593. The Antagonist group received nor-BNI. All drugs were administered using single intraperitoneal (i.p.) injections. U69,593 was administered at 0.32 mg/kg and nor-BNI was administered at 3.20 mg/kg. Rats received this treatment for three experimental sessions. After the completion of three experimental sessions, the control group continued to be tested in the same manner as above, but the Agonist and Antagonist groups were split into either the CC groups or the SDT groups. One half of the Agonist group and one half of the Antagonist group continued to receive saccharin water paired with the respective drug, while the other half of each group received saccharin

water paired with a saline injection. This lasted for the duration of four experimental sessions. Afterwards, blood samples were collected using a tail vein procedure. As much blood as possible was extracted from each rat, but not exceeding ½ mL. Each sample was stored in non-heparinized holding tubes and frozen at -20 degrees Celsius. The samples were then thawed to room temperature and analyzed using the BD OptEIA Rat TNF- α ELISA Kit (Cat. No. 558535). The assay was run through a microplate reader at 450 nm and analyzed.

Data Analysis

A one-way ANOVA and a two-way ANOVA were used to analyze the data. These statistical designs were implemented because they were able to provide information about the differences between drug treatment and also the differences between CC and SDT groups. The one-way ANOVA was executed to determine differences between the mean concentrations of TNF- α in the blood serum between drug groups. The two-way ANOVA was used to determine differences both between-groups and within-groups for the Agonist and Antagonist groups. This served to analyze the effects of drug type, treatment type, and their interaction.

Results:

The results of this experiment were not statistically significant. However, all of the analyses used showed a trend towards significance. The post-experimental mean concentrations of TNF- α in the rat blood serum showed the hypothesized relative differences. The Antagonist group exposed to nor-BNI (3.20 mg/kg) displayed the highest TNF- α concentrations with a mean concentration of 10,005.16 pg/mL. The Agonist group exposed to U69,593 (0.32 mg/kg)

displayed the lowest TNF- α concentrations with a mean concentration of 3,502.99 pg/mL. The Control group landed in the middle, displaying a mean TNF- α concentration of 5,817.15 pg/mL. A graph displaying the differences among means between these groups is shown in Figure 1.

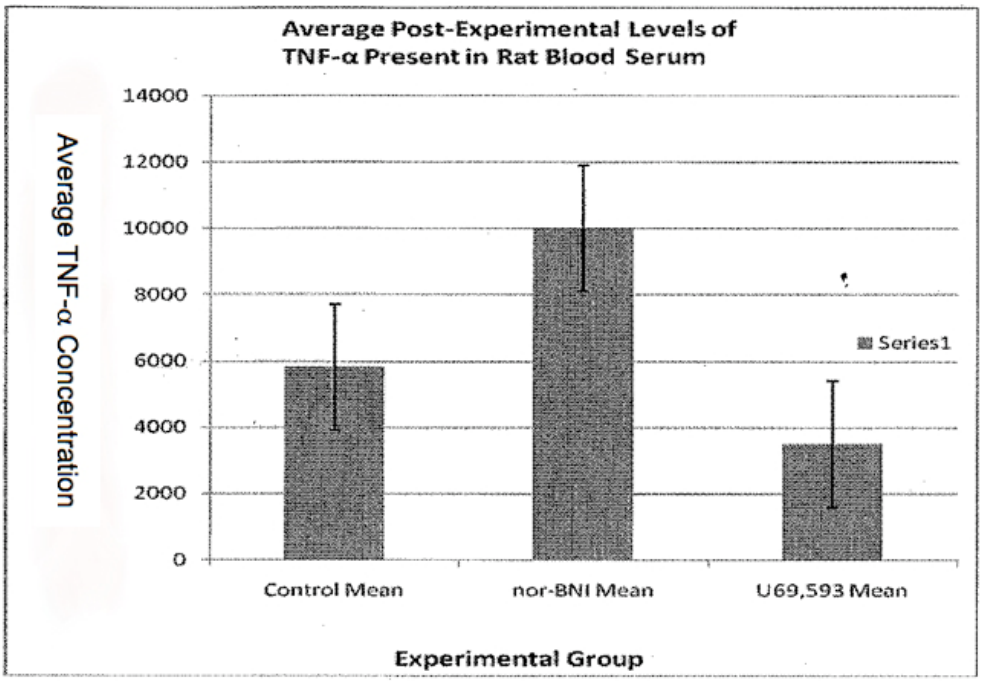

Figure 1. Mean concentrations of TNF- α present in rat blood serum based on Drug Type. Error bars reflect standard error of the means. F (2, 13) = 0.779, p = 0.479. Control n = 5, nor-BNI n = 6, U69,593 n = 5.

A one-way ANOVA that compared average TNF- α concentrations across the Control, Antagonist, and Agonist groups did not result in significance across the variables [$F(2,13)= 0.779$, $p<0.05$]. This indicated that the drug treatment type did not significantly affect differences among TNF- α concentration between groups.

In order to determine differences between the SDT and CC conditions, a two-way ANOVA was used. As hypothesized, the Antagonist SDT group showed the highest mean TNF- α concentration with 11,353.97 pg/mL. The Antagonist CC group had the second highest mean

concentration with 8,656.35 pg/mL. The Agonist SDT was next in line with 4,418.37 pg/mL and the Agonist CC had the lowest concentration with 3,502.99 pg/mL. The differences between means of the Antagonist CC and Antagonist SDT groups are shown in Figure 2. The differences between means of the Agonist CC and Antagonist SDT groups are shown in Figure 3.

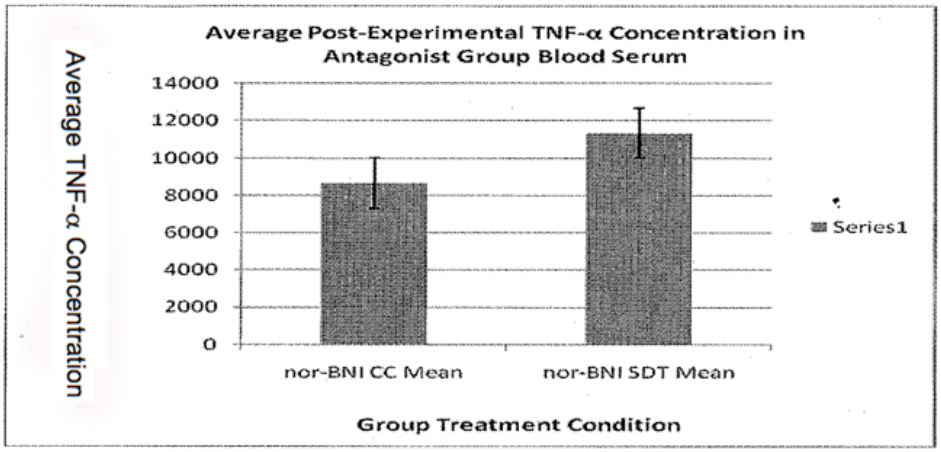

Figure 2. Mean concentrations of TNF- α present in Antagonist group blood serum based on treatment type. Error bars reflect standard error of the means. nor-BNI CC n = 3, nor-BNI SDT n = 3.

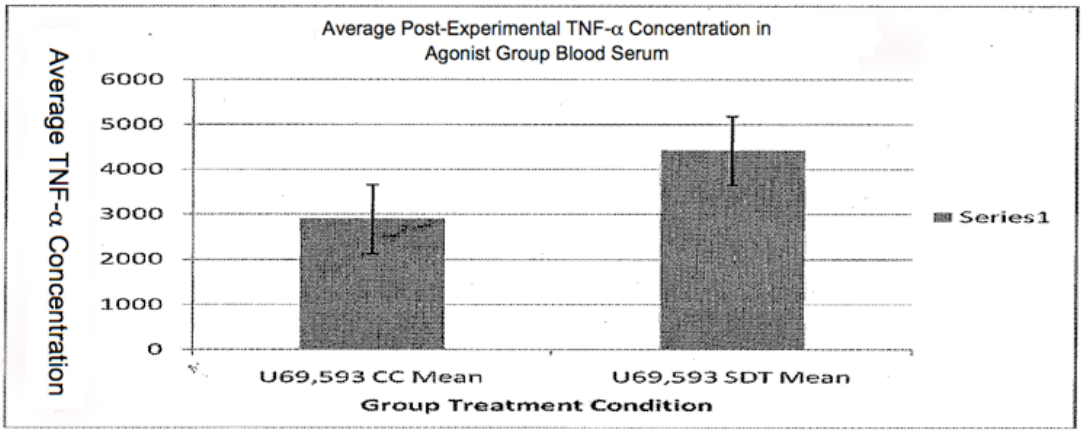

Figure 3. Mean concentrations of TNF- α present in Agonist group blood serum based on treatment type. Error bars reflect standard error of the means. U69,593 CC n = 3, U69,593 SDT n = 2.

Although these charts display noted differences in mean TNF- α concentration between treatment types, the results of the two-way ANOVA were not significant. The test analyzed the differences between drug type (nor-BNI, or U69,593) and treatment type (SDT or CC) and the strength of the interaction between these two factors. Isolating the drug type factor, the test yielded the following results: [$F(1,1)=1.808$, $p=0.221$]. Isolating the treatment type factor, the test yielded the following results: [$F(1,1)=0.200$, $p=0.668$]. Looking at the interaction effects, the test yielded the following results: [$F(1,1)=0.015$, $p=0.905$]. This indicates that the differences in mean TNF- α concentrations both between-groups and within-groups were not significant.

Discussion:

This experiment serves as evidence that classical conditioning procedures may be useful in the treatment of immune system disorders and that with further research done on the issue, it may be possible to use classical conditioning procedures in replacement of sustained drug treatment for immune system disorders. The results of this experiment show a trend towards significance and display noted differences in the mean TNF- α concentrations between treatment types. The results suggest that classical conditioning with the kappa-opioid receptor antagonist, nor-BNI, may increase levels of TNF- α to improve immune system functioning, while bypassing the "Negative Feedback Loop" of immunity. The non-significance of the results could be due to the small sample size. A larger sample size would add more strength to the statistical analysis. Future experiments should replicate the lay-out of this experiment utilizing a larger sample size.

Other possibilities for the non-significance of the results include the large error margin involved in performing the ELISA assay and the importance of acquiring the required amount of blood serum from the rats. The ELISA assay is a sensitive procedure and should only be performed by a trained microbiologist to eliminate the risk of large potentials for error. Future experiments using this method of analysis should note the importance of having a trained microbiologist perform the assay. In this experiment, the experimenters were unable to collect equal amounts of blood serum from all of the rats and the highest amount able to be collected was still much smaller than the required amount for the assay, which was 100 microliters. Although the math was corrected before the ELISA analysis was completed, the variance in the volume of blood serum collected for each rat likely had a large effect on the non-significance of the results and the large variance of concentration within treatment groups. Future experiments of this type should utilize a trained expert in tail vein procedures.

Overall, the general hypothesis of this experiment was supported with the results. The nor-BNI administrations were predicted to enhance overall immune system functioning, while the U69,593 administrations were predicted to suppress overall immune system functioning. The nor-BNI group displayed the highest mean TNF- α concentrations, while the U69,593 groups displayed the lowest mean TNF- α concentrations. In addition, one rat fatality was observed in the U69,593 group, adding weight to the claim that U69,593 suppressed immune system functioning. One might presume from these results that nor-BNI enhances immune system functioning by acting on the kappa-opioid receptor to increase levels of TNF- α. This experiment also hypothesized that the SDT groups would display a higher TNF- α concentration than the CC groups. The results also supported this claim, albeit the results were nonsignificant. The nor-BNI SDT group had a higher level of TNF- α concentration compared to the nor-BNI CC group,

while the U69,593 SDT group displayed a higher level of TNF- α concentration compared to the U69,593 CC group. One might presume from these findings that the lower levels of TNF- α concentration in the CC groups adds weight to the claim that classical conditioning procedures may be helpful in treating immune system disorders because of their ability to avoid stimulation of the "Negative Feedback Loop" of immunity.

Future directions for this sort of research should also be set up to analyze levels of IL-2 concentration. This experiment was limited by monetary constraints, so only one of the proinflammatory cytokines involved in immune system functioning could be analyzed. Future experiments should also utilize flow cytometry to count the number of T cells present in rat blood serum after this experimental manipulation. Again, this experiment was limited by monetary constraints and had no affordable source to utilize the method of flow cytometry. The experiment should also be lengthened to provide information about how long the effects of classical conditioning may last.

Appendix

Table 1

ANOVA Summary Table for the Between-Groups Effects of Drug Type on TNF- α Concentration

ANOVA

TNF- α					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.205E8	2	6.026E7	.779	.479
Within Groups	1.006E9	13	7.740E7		
Total	1.127E9	15			

Table 2

ANOVA Summary Table for the Between-Subjects Effects of Treatment Type and Drug Type on TNF- α Concentration

Tests of Between-Subjects Effects

Dependent Variable:TNF

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	1.290E8	3	4.300E7	.723	.569	.237
Intercept	4.976E8	1	4.976E8	8.370	.023	.545
Drug	1.075E8	1	1.075E8	1.808	.221	.205
Treatment	1.189E7	1	1.189E7	.200	.668	.028
Drug * Treatment	915696.622	1	915696.622	.015	.905	.002
Error	4.162E8	7	5.945E7			
Total	1.092E9	11				
Corrected Total	5.452E8	10				

a. R Squared = .237 (Adjusted R Squared = -.091)

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