Lankenau Institute for Medical Research

Annual Progress Report: 2009 Formula Grant

Reporting Period

January 1, 2010 – June 30, 2010

Formula Grant Overview

The Lankenau Institute for Medical Research received $241,281 in formula funds for the grant award period January 1, 2010 through December 31, 2010. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Tirapazamine and a Novel Drug in the Control of Hypoxic Human Colon Cancer Cells – Low oxygen (hypoxia) is prevalent in most solid tumors. These types of hypoxic cells are up to three times less responsive to cancer therapy that involves radiation and chemotherapeutic agents. Previously several approaches have been tested to target hypoxic cancer cells in vitro. These approaches used drugs such as tirapazamine that produced "damages" in low oxygen tumors. However, these damages are easily repairable by repair molecules since tirapazamine does not target cancer cell's repair capacity, which play a major role in the survival of cancer cells. Our recent studies have shown that a novel drug studied in our lab inhibits repair molecules in human colon cancer cells. In this project, we will test whether this drug increases hypoxic cancer cell response to tirapazamine under conditions found in solid tumors.

Anticipated Duration of Project

1/1/2010 – 12/31/2010

Project Overview

A vast majority of rodent and human solid tumors exhibit hypoxia. The role of hypoxia in the resistance of cells to γ radiation and certain chemotherapeutic agents has been clearly demonstrated in cells and animal studies. Clinical evidence also exists for the role of hypoxia in the poor outcome of therapy. Over the last three decades, hypoxic specific cytotoxins have been synthesized and tested for their effects on hypoxic cells. One such drug, tirapazamine that induces oxidative stress has been tested to target hypoxic cancer cells in vitro and in vivo. However, most tissue culture studies have demonstrated that tirapazamine is mostly effective as a hypoxic cytotoxin at higher concentration. The lack of toxicity by tirapazamine as a single agent therapy at low doses even in vitro may be related to the compound producing "repairable damages" while not targeting cellular repair molecules such as glutathione. Glutathione plays a major role in the survival of cancer cells against oxidative stress induced cell death. Here, we propose to improve the efficacy of tirapazamine for hypoxic cancer cells by targeting

glutathione, a major intracellular antioxidant, with a novel drug developed in our laboratory. Our recent results have demonstrated that this drug increases the response of cancer cells to oxidative stress induced by radiation and certain chemotherapeutic agents by oxidizing glutathione (GSH) and proteins in a microenvironment found in solid tumors. In this project, we will determine the combined use of non-toxic doses of this novel drug and tirapazamine in controlling the growth of hypoxic human colon cancer cells in vitro.

All of our studies will be carried out in two human colon cancer cells (HCT116, HT29) with various concentrations of tirapazamine and the new drug. In order to mimic the tumor microenvironment related to oxygen and glucose concentrations, we will test this approach in these cells at various concentrations of glucose under hypoxic and normoxic conditions in vitro. Using this approach, we will first determine (specific aim 1) whether this drug increases the cytotoxicity of tirapazamine or tirapazamine increase the cytotoxicity of this new drug in human colon cancer cells. Second, using high pressure liquid chromatography and electrochemical detection and/or biochemical assays, we will determine (specific aim 2) whether tirapazamine itself can enhance or inhibit the detoxification of this new drug by glucose deprived cancer cells in the presence and absence of oxygen. Third, we will determine (specific aim 3) whether glutathione and total protein thiols are affected by the combined treatment of this new drug and tirapazamine under these conditions.

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Expected Research Outcomes and Benefits

Radiation and chemotherapeutic agents that damage tumor cell DNA are widely used to treat tumors. However, hypoxia plays a major role in the poor outcome of radiation and chemotherapy. Several hypoxic sensitizers, which are mostly non-toxic by themselves in normoxic cells, are more effective when combined with radiation or chemotherapeutic agents than either agent alone in vivo. One such drug, Tirapazamine, is currently used in clinical trials in the US and other countries. This compound has shown a slight but significant improvement in the outcome of certain patients in combination with radiation and platinum compounds. However, short term clinical studies reported a wide variety of acute toxicities such as nausea and vomiting, myalgia, muscle spasms, fatigue and hematologic toxicity when given a combination of tirapazamine/cisplatin. Studies to determine the efficacy of low doses of tirapazamine, which was used to avoid toxicity, demonstrated that low dose tirapazamine caused

only little changes in tumor doubling time in mouse tumor models. Consistent with these animal studies, tissue culture studies have also demonstrated that tirapazamine alone at moderate concentrations was not successful in killing hypoxic cancer cells. We hypothesize that the repair molecules such as glutathione and proteins, which are involved in preventing oxidative stress and/or the repair of lesions induced by tirapazamine, are responsible for its failure to kill hypoxic cells at low concentrations of tirapazamine in vivo and in vitro. We have recently demonstrated (unpublished), for the first time, that a novel drug studied in our lab inhibits the repair molecules in human cancer cells by inhibiting the function of glutathione and proteins via oxidation of functional thiols in a microenvironment found in solid tumors. This new compound is expected to improve the cytotoxic effects of tirapazamine in hypoxic cancer cells. The successful outcome of these studies will be beneficial for the better treatment of cancer patients.

Summary of Research Completed

Specific Aims

The goal of this project is to determine the efficacy of hydroxyethyl disulfide (HEDS) in improving the therapeutic benefits of tirapazamine in human cancer cells deprived of glucose and oxygen in vitro. HEDS is expected to inhibit the function of glutathione (GSH) and proteins in glucose depleted cells. Sensitization of cancer cells to tirapazamine by inhibition of repair molecules such as glutathione would thereby increase the therapeutic benefits of tirapazamine under low glucose and low oxygen microenvironment. Our approach is aimed at simultaneously oxidizing GSH and proteins since changes in redox homeostasis of GSH and proteins is expected to decrease the survival of hypoxic cancer cells during tirapazamine treatment. We proposed to test our novel and unique hypothesis in human colon cancer cells HCT116 and HT29, which are known to be sensitive and resistant respectively to oxidative stress induced by radiation.

In the first six months of the funding period, we have initiated several studies to test the efficacy of HEDS in improving the response of colon cancer cells to tirapazamine.

Studies and Results

Our major hypothesis is that cellular (PSH & GSH) redox modifier (HEDS) can be used to sensitize cancer cells to tirapazamine. This hypothesis is based on our previously published data that demonstrated HEDS mediated sensitization (cell death) of human cancer cells to γ radiation.

Specific Aim 1:

In specific Aim 1, we have proposed to determine whether HEDS increases the cytotoxicity of tirapazamine in human colon cancer cells in low glucose and low oxygen. In addition, we have proposed to determine whether HEDS also increases the cytotoxicity of tirapazamine in human colon cancer cells in normal glucose and normal oxygen. Our results have demonstrated that HEDS alone decreased the survival of glucose deprived human colon cancer cells (HCT116) both under hypoxic and normoxic conditions (Figures 1 and 2). Additionally, HEDS sensitized the response of these cells to tirapazamine in both hypoxic and normoxic conditions (Figures 1 and 2). These unexpected sensitizing effect of HEDS to tirapazamine even in normoxic cells

suggested that HEDS in combination with tirapazamine could be used to target both normoxic and hypoxic cancer cells that are deprived of glucose in solid tumors. However, the sensitizing effect is observed only in glucose deprived cells and not glucose rich cells (Figures 1 and 2).

Specific Aim 2:

As part of specific aim 2, we have proposed to determine whether low glucose is effective in inhibiting the detoxification of HEDS by human colon cancer cells in the presence of tirapazamine under hypoxia. In addition, we have also proposed to determine whether tirapazamine itself has any inhibitory effect in the presence of glucose on the detoxification of HEDS by human colon cancer cells under normoxic and hypoxic conditions. The results have demonstrated that HEDS detoxification was inhibited by low glucose under both normoxic and hypoxic conditions suggesting that hypoxic cancer cells are not resistant to low glucose mediated inhibition of HEDS detoxification (Figures 3 and 4). The inhibition of HEDS detoxification by low glucose was maintained even in the presence of tirapazamine suggesting that tirapazamine does not affect the pharmacological effects of HEDS. However, glucose rich cells maintained HEDS detoxification under normoxic and hypoxic conditions either in the presence or absence of tirapazamine (Figures 3 and 4).

Specific Aim 3:

In this specific aim, we have proposed to determine whether HEDS mediated depletion of GSH and protein thiols in glucose deprived cancer cells can occur under hypoxic conditions. In addition, we have also proposed to determine whether tirapazamine itself has any effect in the presence and absence of glucose on the depletion of glutathione by HEDS in human colon cancer cells under normoxic and hypoxic conditions. The results have demonstrated that HEDS decreased the glutathione level in both normoxic and hypoxic HCT116 cancer cells in the absence of glucose (Figure 5 and 6). Additionally, the results have demonstrated that the efficacy of HEDS in decreasing the glutathione level is not affected by tirapazamine in HCT116 cells (Figure 5 and 6). However, HCT116 maintained the GSH level fairly well in the presence of tirapazamine and HEDS both under normoxic and hypoxic conditions when glucose was present (Figures 5 and 6).

Significance

The results from human cancer cells HCT116 demonstrated the application of HEDS to sensitize colon cancer cells to tirapazamine. We are on target in carrying out the experiments proposed in our project. Our preliminary results with human colon cancer cells demonstrated that pharmacological effects of HEDS is maintained even under hypoxic conditions and sensitized the human cancer cells to tirapazamine.

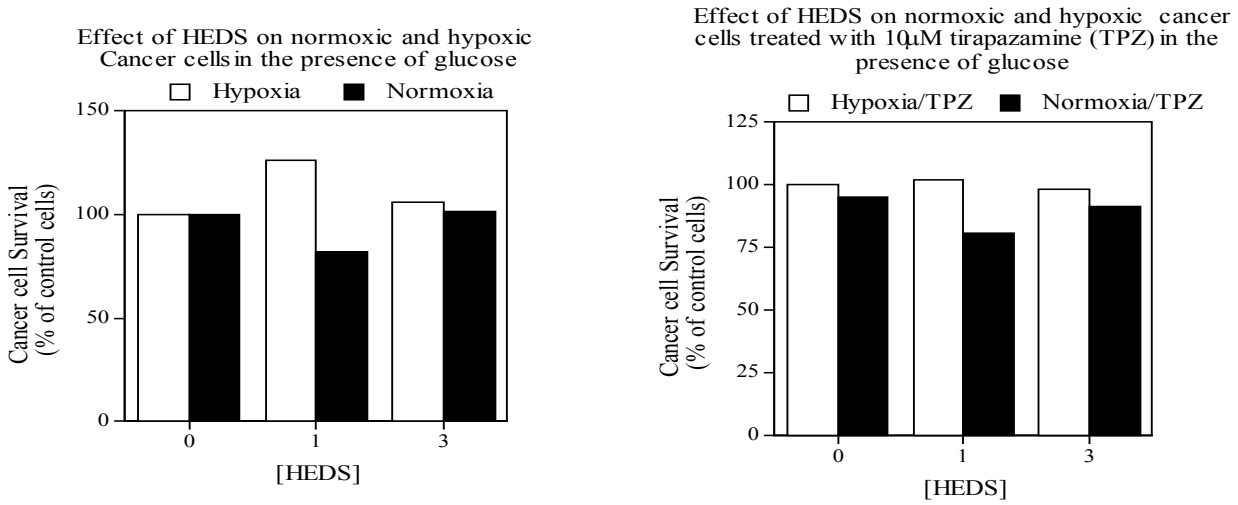

Figure 1: Effects of HEDS (0, 1, 3mM) and tirapazamine (0, 10μM) on the survival of HCT116 human colon cancer cells in the presence of glucose. The results have demonstrated that HEDS did not inhibit the survival of human colon cancer HCT116 cells either under normoxic or hypoxic conditions in the presence of glucose. These results also suggested that HEDS is not toxic to cells with normal glucose either in the presence or absence of tirapazamine.

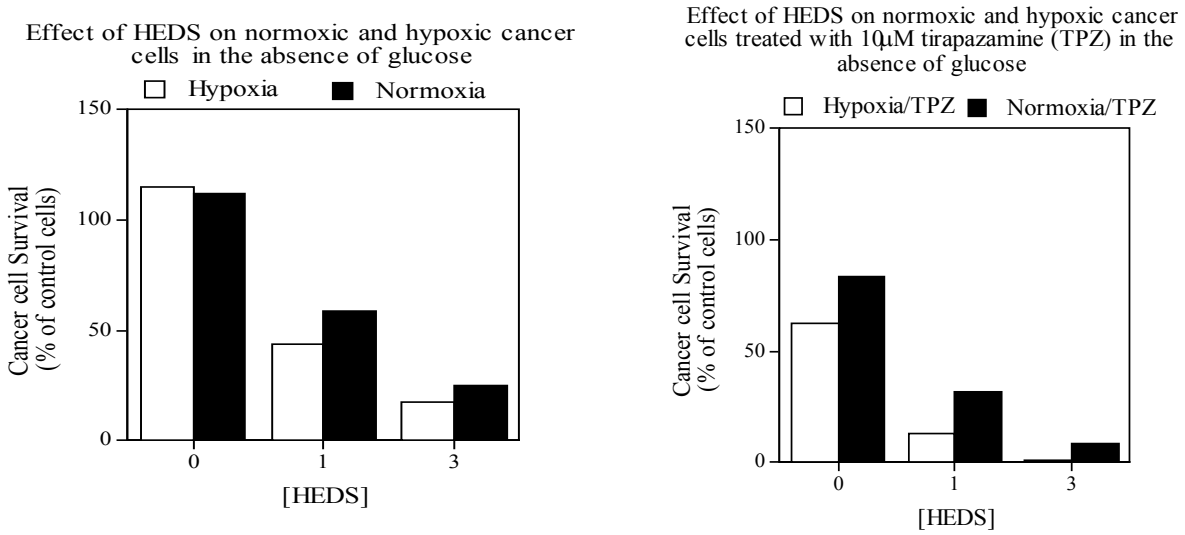

Figure 2: Effects of HEDS (0, 1, 3mM) and tirapazamine (0, 10μM) on the survival of HCT116 human colon cancer cells in the absence of glucose. The results have demonstrated that HEDS inhibited the survival of glucose deprived human colon cancer HCT116 cells under normoxic and hypoxic conditions. However, the HEDS and tirapazamine combination is more effective than the tirapazamine alone both in normoxic and hypoxic conditions.

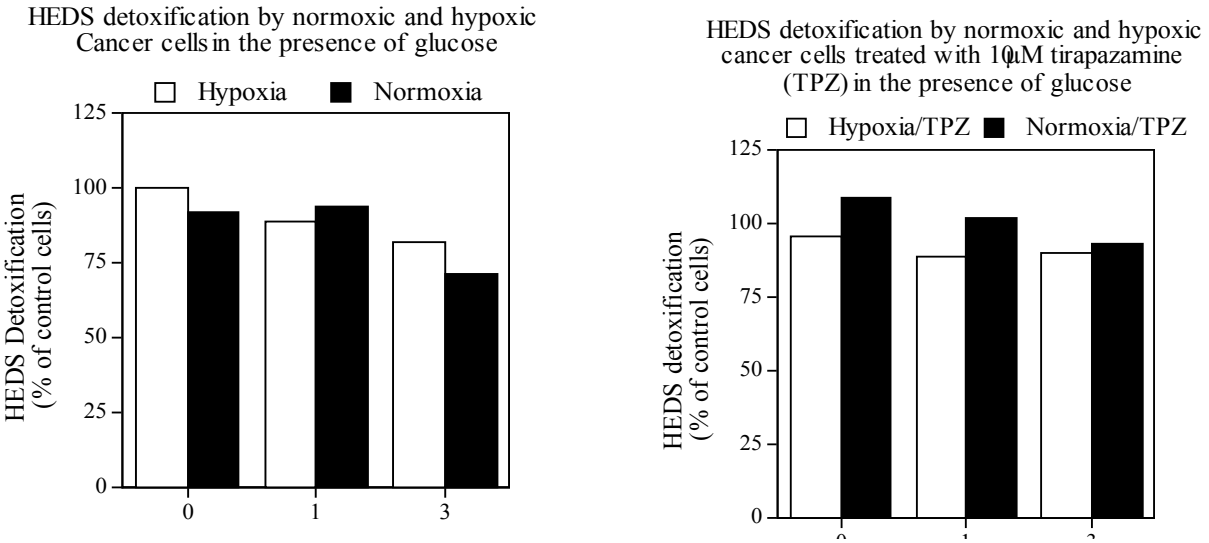

Figure 3: HEDS detoxification by human colon cancer cells HCT116 measured 3 days after treatment with HEDS (0, 1, 3mM) and tirapazamine (0, 10μM) in the presence of glucose. The results have demonstrated that the detoxification of HEDS occurred both in normoxic and hypoxic conditions in the presence of glucose. Tirapazamine either alone or in combination with HEDS did not affect the bioreductive capacity of these cells in hypoxic or normoxic conditions.

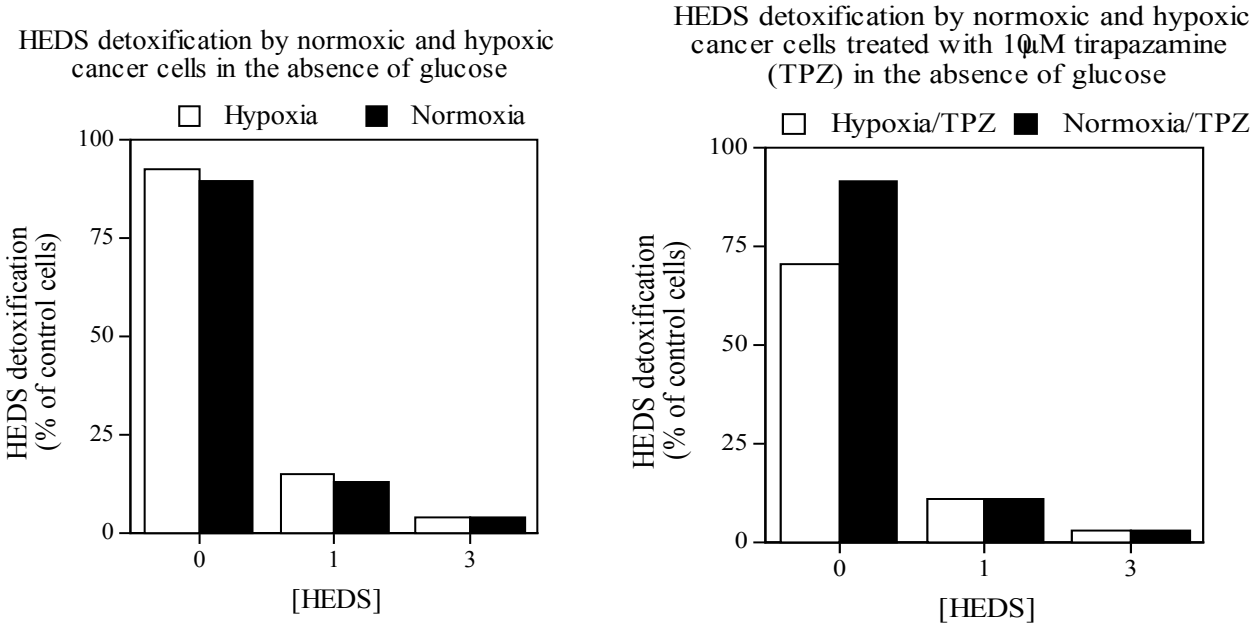

Figure 4: HEDS detoxification by human colon cancer cells HCT116 measured 3 days after treatment with HEDS (0, 1, 3mM) and tirapazamine (0, 10μM) in the absence of glucose. The results have demonstrated that the detoxification of HEDS is inhibited both in normoxic and hypoxic conditions by cells treated with HEDS in the absence of glucose. Tirapazamine did not affect the pharmacological effects of HEDS either alone or in combination with HEDS under hypoxic or normoxic conditions.

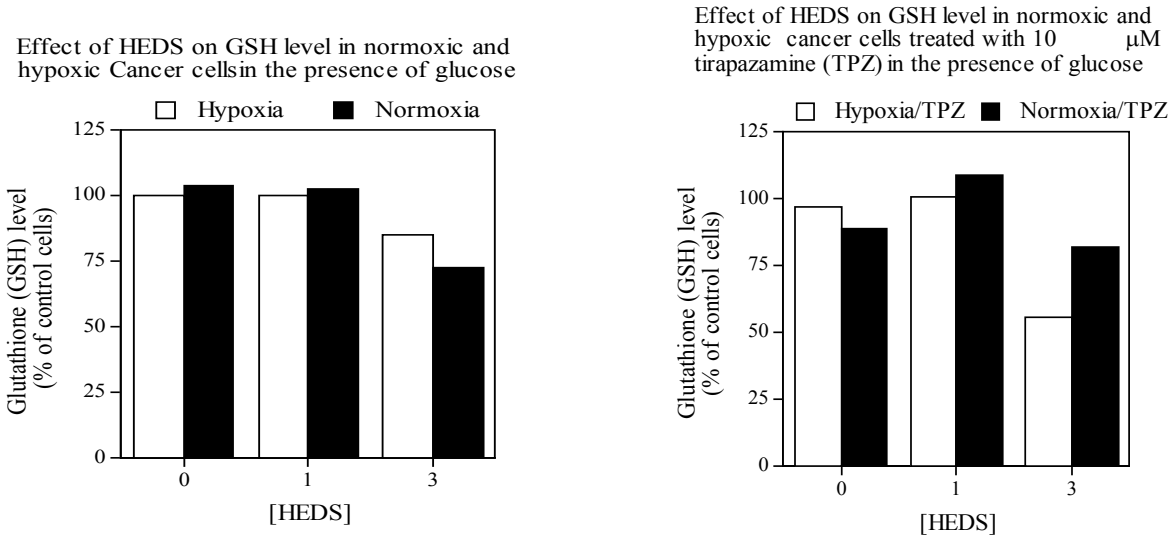

Figure 5: Effects of HEDS (0, 1, 3mM) on the intracellular glutathione in human colon cancer cells HCT116 measured 3 days after treatment with HEDS (0, 1, 3mM) and tirapazamine (0, 10µM) in the presence of glucose. The results have demonstrated that HEDS did not affect the glutathione level in human colon cancer HCT116 cells in the presence of glucose under hypoxic and normoxic conditions. Glutathione levels remained unaffected by tirapazamine either alone or in combination with low concentrations of HEDS in the presence of glucose. However, HEDS at higher concentration slightly decreased the glutathione level even in the presence of glucose

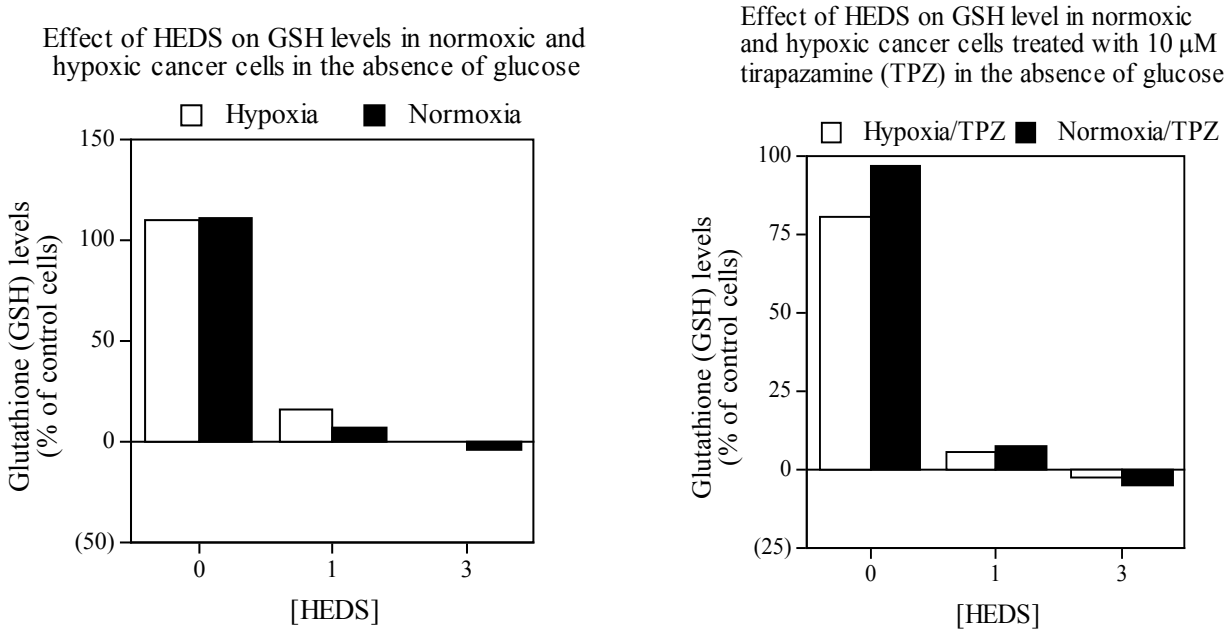

Figure 6: Effects of HEDS (0, 1, 3mM) on the intracellular glutathione in human colon cancer cells HCT116 measured 3 days after treatment with HEDS (0, 1, 3mM) and tirapazamine (0, 10µM) in the absence of glucose. The results have demonstrated that HEDS decreased the glutathione level in human colon cancer HCT116 cells in the presence of glucose under hypoxic and normoxic conditions. Hypoxia or tirapazamine did not alter the pharmacological effects of HEDS.

Research Project 2: Project Title and Purpose

The Role of IDO in B Cell Activation and Memory - Patients with the autoimmune disease rheumatoid arthritis have an elevation in the level of activity of the immunoregulatory enzyme IDO. IDO elevation correlates with disease activity, suggesting that IDO may contribute to the disease process. In support of this, we have evidence that inhibiting IDO activity interferes with arthritis development in mice. This alleviation of arthritis resulted from a diminished autoreactive B cell response, suggesting for the first time an important role for IDO in directing B cell responses. In this project, we will use model systems of B cell activation to explore the importance of IDO in driving B cell activation. The overall goal of this project is to define the mechanism by which IDO modulates B cell responses and provide new insights into strategies that can be used to manipulate this pathway to reduce or prevent disease.

Anticipated Duration of Project

1/1/2010 – 12/31/2010

Project Overview

Rheumatoid arthritis (RA) is a chronic and debilitating inflammatory autoimmune disease of unknown etiology. As with other autoimmune disorders, elevated tryptophan catabolism has been detected in RA patients. This is indicative of activation of the immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO); however, the role that IDO plays in the disease process is not well understood. The current paradigm for IDO function is immunosuppressive, providing an important inhibitory role to limit effector T cell responses. In contrast, the role of IDO in B cell-mediated responses has not been addressed. The widely held belief that IDO acts solely in a T cell immunosuppressive role has led to the general assumption that inhibition of IDO activity should exacerbate autoimmune disorders. However, using the K/BxN mouse model of RA and 1-methyl-tryptophan (1MT), an inhibitor of IDO, we found that inhibiting IDO activity had the unexpected consequence of ameliorating, rather than exacerbating arthritis symptoms. This alleviation of joint inflammation was not due to an altered T cell response, but rather resulted from a diminished autoreactive B cell response. We have preliminary data demonstrating that 1MT is also able to inhibit B cell responses to immunization with model antigens, suggesting for the first time an important role for IDO in directing B cell responses to both self and foreign antigens. *Our working hypothesis is that IDO is not simply an immunosuppressive enzyme, but rather plays a more complex role in modulating inflammatory responses by directing the immune profile of B cell responses.*

<u>We will investigate this hypothesis with the following specific aims:</u>

Aim 1. Determine the role of IDO in the development of B cell memory.

Aim 2. Define the mechanism by which IDO drives B cell activation in response to foreign antigens.

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

IDO is associated with the pathological inflammation and immune escape necessary for tumor growth and there is a substantial body of preclinical evidence that small molecule inhibitors of IDO will provide a therapeutic benefit as anticancer agents. Contrary to established notions of IDO activity simply being immunosuppressive, our preliminary data also indicate that targeting IDO activity may be therapeutically beneficial for suppressing the autoimmune symptoms associated with the spontaneous development of autoantibody-mediated arthritis. We demonstrate that this alleviation of arthritis is due to an inhibition of the autoreactive B cell response, suggesting that IDO plays an important role in driving the autoreactive B cell response. In this project, we will employ genetic knockout mice and a set of specific small molecule inhibitors of IDO to probe the involvement of this enzyme in B cell-mediated immune responses. The overall goal of this project is to define the mechanism by which IDO modulates B cell responses and provide new insights into strategies that can be used to manipulate this pathway to reduce or prevent disease.

Summary of Research Completed

In this project we use the model antigen system (4-hydroxy-3-nitrophenyl) acetyl (NP) – conjugated to keyhole limpet hemocyanin (KLH) or chicken gamma globulin (CGG) in combination with different adjuvant systems to investigate the role IDO plays in the development of B cell activation and memory. Experimentally, adjuvants have been used to define critical components of pathways required for T and B cell immune responses by overcoming activation defects in knockout mouse strains. In this update, we describe experiments using mice genetically deficient in IDO1 together with three different adjuvant systems to define the pathways that require IDO to drive B cell activation.

Loss of IDO activity inhibits early B cell responses to NP-KLH in Titermax

In preliminary experiments, immunization of mice with NP-KLH in alum demonstrated a deficiency in anti-NP antibody production in IDO1 deficient mice. This suggested that IDO1 is necessary for the generation of a productive B cell response. However, it was not clear from these studies what role IDO1 was playing. Therefore, to begin to define the conditions under

which IDO1 deficiency affects B cell antibody responses, we used different model antigen/adjuvant systems.

To start, we used the titermax adjuvant system, a particulate adjuvant that is similar to the adjuvant alum used in our preliminary experiments. Titermax, like alum, induces chemokine production by macrophages and monocytes, leading to cell recruitment and indirect activation of dendritic cells (DCs). These experiments first allowed us to test whether our preliminary result showing reduced antibody production in IDO1 deficient mice in response to immunization with NP-KLH in alum is also found with a different, but similar adjuvant. Second, it gave us insight into the pathway that requires IDO1 for serum Ig responses.

C57BL/6 mice genetically deficient for IDO1 (IDO1 ko) and C57BL/6 control mice were immunized i.p. with 100μg NP-KLH emulsified in titermax (Fig. 1). Serum was collected at days 0, 6, 15, 22, and 29 and tested for the presence of anti-NP antibodies by ELISA. Anti-NP IgM titers were elevated by day 5 and remained steady for several weeks in both control and IDO ko C57BL/6 mice. In C57BL/6 control mice, titers of anti-NP IgG_1, $IgG2_{a/c}$, IgG_{2b}, and IgG_3 were also high starting on day 5 and then continued to increase over the next few weeks. In contrast, in IDO ko C57BL/6 mice, reduced levels of anti-NP IgG_1 and IgG_{2b} and undetectable $IgG_{2a/c}$ and IgG_3 were found at day 5. IDO ko mice did generate anti-NP IgG, as titers reached comparable levels to control C57BL/6 mice by day 10 for IgG_1 and IgG_{2b} and day 15 for $IgG_{2a/c}$ and IgG_3 (Fig. 1). These data suggest that IDO may play a role early in the response, but that additional factors are able to compensate for its loss later in the response.

Loss of IDO activity does not inhibit B cell responses to NP-KLH in CFA

To determine if an adjuvant that stimulates Toll-like receptors can overcome the defect in IDO ko mice, the model antigen NP-KLH was used in combination with complete Freund's adjuvant (CFA). CFA, derived from the microbacterium *m. tuberculosis*, stimulates Toll-like receptors (TLRs) to directly activate DCs and B cells, allowing us to test whether loss of IDO activity affects B cell activation directly.

IDO1 ko and C57BL/6 control mice were immunized i.p. with 100μg NP-KLH emulsified in CFA (Fig. 2). Serum was collected at days 0, 6, 15, 22, and 29 and tested for the presence of anti-NP antibodies by ELISA. The vast majority of anti-NP Ig in both control and IDO1 ko C57BL/6 mice was of the IgG_1 isotype. Titers of anti-NP IgG_1 were present at day 5 and then increased throughout the remainder of the experiment. Anti-NP $IgG2_{a/c}$, IgG_{2b}, and IgG_3 were also detectable at day 5, increased up to day 10-15, and then plateaued. Titers of anti-NP IgM were first detected at day 5 and peaked by day 10. No differences were detected between C57BL/6 mice with and without IDO1 (Fig. 2). Therefore, these data demonstrate that stimulation through TLRs is able to bypass the defect in IDO1 ko mice. This suggests that the defect in IDO1 ko mice is in the initial induction of serum antibody responses and can be overcome by TLR stimulation.

Loss of IDO activity does not inhibit B cell responses to NP-CGG in Alum

Due to the differences seen with NP-KLH in combination with the adjuvants alum and titermax

compared to CFA, we used a second NP conjugate, NP-chicken gamma globulin (CGG). NP-CGG, like NP-KLH, is a T-dependent antigen. B cells will still respond to the hapten NP; however T cell help will be generated in response to CGG, as opposed to KLH. In the future, these different hapten/carrier systems could be used to distinguish the role of IDO in T vs. B cells during primary and memory immune responses.

IDO1 ko and C57BL/6 control mice were immunized i.p. with 100μg NP-CGG in alum (Fig. 3). Serum was collected at days 0, 6, 15, 22, and 29 and tested for the presence of anti-NP antibodies by ELISA. Serum anti-NP IgM, IgG_1, $IgG_{2a/c}$, and IgG_{2b} responses were first detected at day 5, peaked at day 10, and then plateaued. Very little IgG_3 was present and didn't appear until day 10. Overall, titers of anti-NP were lower in response to NP-CGG in alum (Fig. 3) than they were to NP-KLH, in either alum (as shown in preliminary data) or titermax (Fig. 1). Surprisingly, and in contrast to immunization with NP-KLH in alum or titermax, no difference was seen between IDO1 ko and control C57BL/6 mice (Fig. 3). It is unclear whether the lack of difference is due to the difference in carrier used (KLH compared to CGG) or the fact that the overall antibody response to NP-CGG was lower than to NP-KLH.

Conclusions

As a group, adjuvants are thought to work by stimulating innate immune receptors on dendritic cells and monocytes, stimulating the production of chemokines and cytokines necessary for productive adaptive immune response. Just as different antigens can trigger altered immune profiles, different adjuvants have been used to stimulate different innate immune system players. The studies described in this update demonstrate that IDO1 deficient mice are able to mount productive antibody responses upon immunization with model antigens. The extent of the response varied depending upon the adjuvant used. IDO1 ko mice exhibited reduced antibody titers (particularly at early time points) in response to immunization using alum and titermax, two particulate adjuvants that lead to indirect activation of DCs and B cells through induction of chemokine production by macrophages and monocytes. In contrast, antibody titers in response to immunization using CFA, an adjuvant that stimulates TLRs to directly activate DCs and B cells, were indistinguishable between IDO1 ko and control C57BL/6 mice. Therefore, these studies suggest that IDO1 plays a role in the induction of serum antibody responses, but that IDO1 is dispensable when B cells receive a secondary stimulus through TLRs.

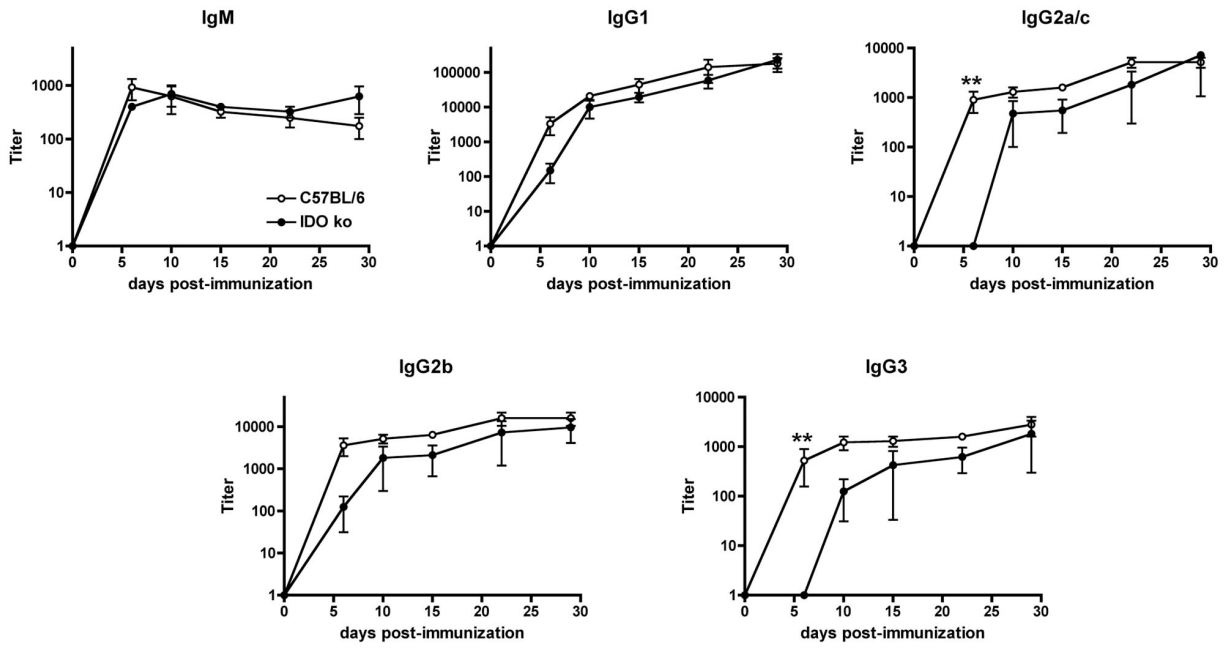

Figure 1. Loss of IDO activity inhibits early B cell responses to NP-KLH in Titermax. C57BL/6 mice and IDO ko mice were immunized i.p. with 100μg NP-KLH in Titermax on day 0. Serum samples were taken on days 0, 5, 15, 22, and 29 and analyzed for anti-NP IgM, IgG_1, $IgG_{2a/c}$, IgG_{2b}, and IgG_3 by ELISA. n=4 mice per group. **$p<0.05$

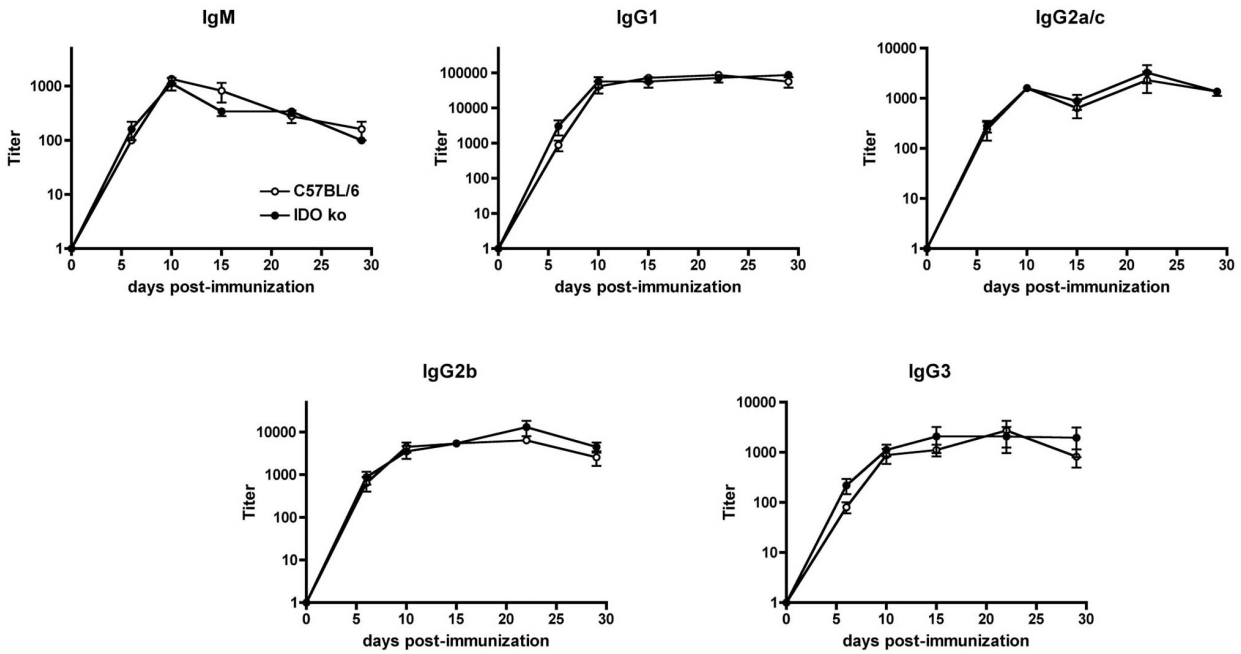

Figure 2. Loss of IDO activity does not inhibit B cell responses to NP-KLH in CFA. C57BL/6 mice and IDO ko mice were immunized i.p. with 100µg NP-KLH in CFA on day 0. Serum samples were taken on days 0, 5, 15, 22, and 29 and analyzed for anti-NP IgM, IgG_1, $IgG_{2a/c}$, IgG_{2b}, and IgG_3 by ELISA. n=5 mice per group.

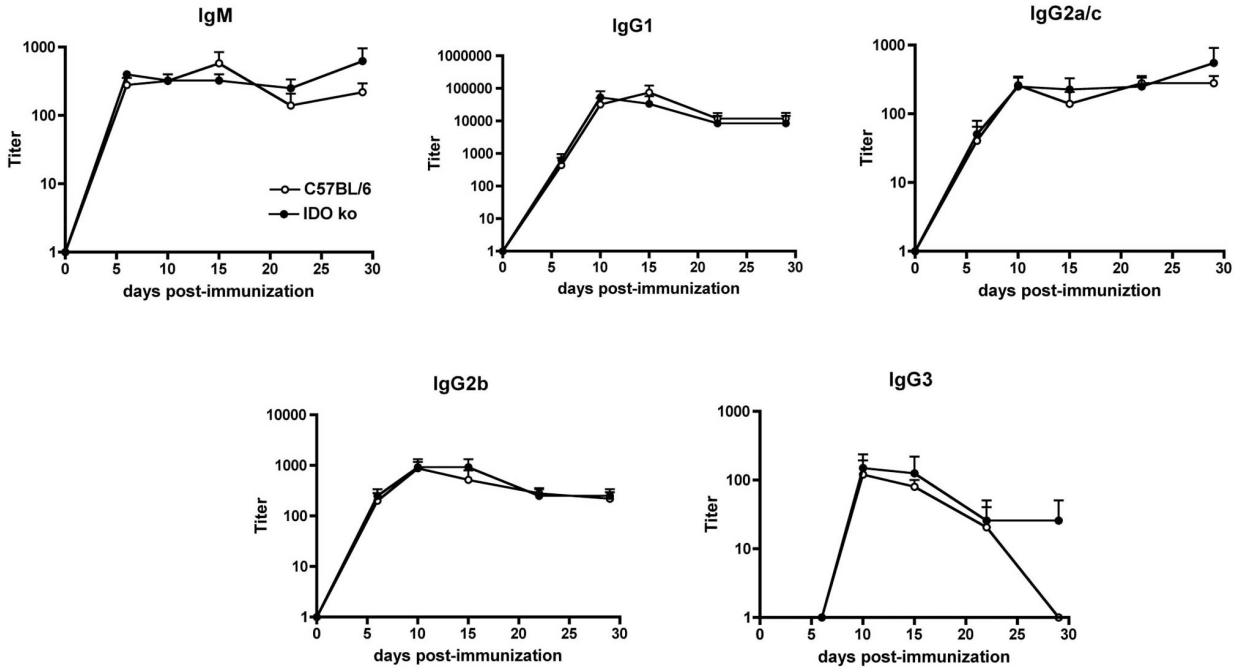

Figure 3. Loss of IDO activity does not inhibit B cell responses to NP-CGG in Alum. C57BL/6 mice and IDO ko mice were immunized i.p. with 100µg NP-CGG in alum on day 0. Serum samples were taken on days 0, 5, 15, 22, and 29 and analyzed for anti-NP IgM, IgG_1, $IgG_{2a/c}$, IgG_{2b}, and IgG_3 by ELISA. n=5 mice per group.