Lankenau Institute for Medical Research

Annual Progress Report: 2008 Formula Grant

Reporting Period

July 1, 2009 – December 31, 2009

Formula Grant Overview

The Lankenau Institute for Medical Research received $223,613 in formula funds for the grant award period January 1, 2009 through December 31, 2009. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Arsenic Drugs and Hydroxyethyl Disulfide in the Control of Cancer Cell Growth and Response to Therapy - The efficacy of arsenic trioxide (As_2O_3) as an anti cancer drug in leukemia and other cancer cells may be limited due to its detoxification by intracellular glutathione (GSH). Our preliminary results have demonstrated that hydroxyethyl disulfide (HEDS), a non-toxic oxidant, removes GSH and increases the response of glucose deprived cancer cells to radiation and chemotherapeutic agents. Increasing the susceptibility of the rest of the tumor cells containing glucose to HEDS is expected to improve the outcome of cancer therapy. In this project, we will determine the efficacy of combined treatment of HEDS and arsenic compounds (including but not limited to the FDA approved Trisenox) in increasing the response of human cancer cells to radiation and chemotherapeutic agents.

Duration of Project

1/1/2009 - 12/31/2009

Project Overview

Arsenic trioxide (Trisenox) is a Food and Drug Administration (FDA) approved drug for treatment of acute promyelocytic leukemia in the US. This compound has shown significant improvement in the outcome of leukemia patients. The efficacy of arsenic trioxide as an anti cancer drug for other types of cancer may be limited due to its detoxification by intracellular glutathione. Our preliminary results have demonstrated that hydroxyethyl disulfide (HEDS), a non-toxic oxidant, increases the response of glucose deprived cancer cells to radiation and certain chemotherapeutic agents by oxidizing glutathione (GSH). HEDS has no significant effect in cells with functioning oxidative pentose cycle, which uses glucose to detoxify HEDS. These results have raised the possibility to target therapy resistant hypoxic cancer cells since hypoxic cells are also glucose deprived in solid tumors. Our preliminary studies have demonstrated that HEDS treatment showed a significant (50% better) improvement in the response of breast tumor xenograft to etoposide. Although a significant percentage of cancer cells in solid tumor may be

glucose deprived, which can be targeted by HEDS, increasing the vulnerability of the rest of the tumor cells containing glucose to HEDS will improve tumor response to cancer therapy. We have previously demonstrated that phenylarsine oxide (PAO), another arsenic compound, inhibits the detoxification of HEDS by rodent cells. In this project, we will determine the combined use of non-toxic HEDS and arsenic compounds in controlling the growth and response of cancer cells to chemotherapeutic agents and radiation.

All of our studies will be carried out in human cancer cells with various concentrations of arsenic compounds and HEDS. A state-of-the-art high pressure liquid chromatography and electrochemical detection and other biochemical assays will be used to determine i) whether arsenic compounds are effective in inhibiting the detoxification of HEDS, ii) whether arsenic compounds are effective in increasing HEDS mediated oxidation of GSH and proteins, and iii) whether HEDS increases the cytotoxicity of arsenic compounds or arsenic compounds increase the cytotoxicity of HEDS in human cancer cells.

Principal Investigator

Iraimoudi S. Ayene, PhD
Associate Professor
Lankenau Institute for Medical Research
100 Lancaster Avenue
Wynnewood, PA 19096

Other Participating Researchers

Albert DeNittis, MD - employed by Lankenau Hospital
Jie Li, M.D., PhD - employed by Lankenau Institute for Medical Research

Expected Research Outcomes and Benefits

Chemotherapeutic agents and radiation are commonly used at tolerable doses to treat cancer. In particular, arsenic trioxide has been shown to be effective in the complete remission in some patients with relapsed or refractory acute promyelocytic leukemia. Most importantly, the patients did not exhibit severe marrow suppression. Due to the effectiveness of arsenic trioxide in leukemia cells, there is an upsurge of interest in understanding the mechanisms and efficacy of arsenic trioxide on other types of cancer cells. Most studies with other types of cancer cells have shown that arsenic trioxide is more effective in leukemia and myeloma cells than in lung, colon, ovarian, cervical, renal and breast cancer cells. A recent study has shown that human lung cancer cells A549 are highly resistant to arsenic trioxide. Glutathione plays a major role in the resistance of cancer cells to various therapeutic agents that include arsenic trioxide. We have recently demonstrated, for the first time, that hydroxyethyl disulfide (HEDS) in combination with phenylarsine oxide (PAO), an arsenic compound, increased the response of rodent cells by ten fold to γ radiation. These results also suggested that this approach could be used to increase the response of human cancer (leukemia and non leukemia) cells to radiation and chemotherapeutic agents. Our proposed studies will determine the efficacy of combined treatment of arsenic compounds and HEDS in the improvement of cancer cells response to radiation and

chemotherapeutic agents. To the best of our knowledge, our approach is the first ever to increase the response of cancer cells by targeting DNA repair protein and GSH using non-toxic disulfide (HEDS) in combination with one of the arsenic compounds. In conclusion, our approach is innovative since it consists of an experimental test of a novel hypothesis that inhibition of detoxification of HEDS (Specific Aim 1), inhibition of functions of glutathione and DNA repair proteins (Specific Aim 2) by combined treatment of HEDS and arsenic compounds can improve the response of cancer cells to radiation and chemotherapeutic agents (Specific Aim 3).

Summary of Research Completed

A. Specific Aims

The goal of this project is to determine the efficacy of combined treatment of arsenic trioxide and HEDS in increasing the sensitivity of human cancer cells with glucose to radiation and cisplatin. Our preliminary studies indicated that glucose deprived cells or cells impaired with OPPC activity are susceptible to HEDS mediated radiation sensitization. However, this approach does not enhance the response of cells with glucose to radiation since HEDS is detoxified by oxidative pentose cycle, which requires glucose. We have previously demonstrated that PAO, an arsenic compound, inhibits the detoxification of HEDS by 50% at 1μM PAO, and the combined treatment of HEDS and PAO sensitized Chinese hamster ovary (CHO) cells to radiation by 10 fold. Arsenic Trioxide (As_2O_3) in combination with HEDS is expected to improve the response of human cancer cells to cisplatin and radiation. We will test our novel and unique hypothesis in both sensitive and resistant human colon cancer cells.

In the previous annual progress report, we demonstrated the HEDS and arsenic trioxide mediated sensitization of p53 wild type HCT116 human cancer cells to cisplatin and radiation. In the second six months of the funding period, we have completed the studies proposed in specific aims 1 and 3 for p53 mutant HT29 human colon cancer cells. Additionally, we have also identified the mechanisms (specific aim 2) for HEDS and As_2O_3 mediated sensitization of both these cancer cells to radiation and cisplatin.

B. Studies and Results

Our major hypothesis is that cellular (PSH & GSH) redox modifier (HEDS) can be used to sensitize cancer cells to cisplatin, radiation and/or arsenic trioxide. This hypothesis is based on our previously published data that demonstrated HEDS and phenylarsine oxide mediated sensitization (cell death) of Chinese hamster ovary (CHO) cells to γ radiation.

PROJECTS CARRIED OUT FOR SPECIFIC AIM 1:

In specific aim 1, we proposed to determine whether arsenic trioxide is effective in inhibiting the detoxification of HEDS by human p53 wild type (HCT116) and p53 mutant (HT29) cancer cells. Since the submission of the last progress report, we have completed experiments to determine the effects of arsenic trioxide on the detoxification of HEDS in HT29 cells. HEDS is a disulfide dimer of the monomer sulfhydryl compound mercaptoethanol. Disulfides are known to oxidize protein and non protein thiols in cells. Mammalian cells convert HEDS, a thiol oxidant, into mercaptoethanol, a non toxic reductant. The detoxification of HEDS is carried out by most mammalian cells using oxidative pentose cycle. We have used radiation resistant p53 mutant

human colon cancer cells HT29 cells in vitro to determine the effect of different concentrations of arsenic trioxide on the detoxification of HEDS. Cells grown in a 60mm dish with normal *DMEM*, 10%FBS, 1% penicillin/streptomycin (P/S) and 20 mM HEPES were treated with different concentrations of arsenic compounds (As_2O_3). The arsenic compound treated cells were then incubated with HEDS. A stock solution of 0.1M HEDS was prepared fresh in glucose free DMEM growth medium. HEDS exposure was effected by replenishing cells with 1 (six well plate) and 1.5ml (60mm dishes) of fresh DMEM growth medium with glucose, 10% FBS, HEPES and desired concentrations of HEDS. Cells treated with and without HEDS were cooled on ice. To quantify the bioreduction/mercaptoethanol (ME) production (detoxification), 0.5 ml of extracellular medium was mixed with 0.5 ml of 100 mM sulfosalicyclic acid (SSA) lysis buffer in microfuge tubes and centrifuged at high speed in a Fisher 59A microfuge. These samples were used for the determination of mercaptoethanol produced from the detoxification of HEDS in these cells. Our results have demonstrated that arsenic trioxide inhibited the detoxification of HEDS in p53 mutant colon cancer cells (Figure 1).

PROJECTS CARRIED OUT FOR SPECIFIC AIM 2:

As part of specific aim 2, we proposed to determine whether arsenic trioxide is effective in increasing HEDS mediated oxidation of glutathione (GSH). The oxidation of protein thiols (PSH) and loss of function of DNA repair protein (Ku) will also be determined in HCT116 and HT29. In the second six months of the funding period, we determined the efficacy of arsenic trioxide and HEDS in the modification of GSH in HT29 cells. To quantify the intracellular non protein thiols GSH, the extracellular medium was removed after the incubation of cells with HEDS and the cells rinsed three times with PBS. The attached cells were lysed with 0.5 ml of ice cold SSA lysis buffer and 0.5 ml water. After 5 min on ice, the cells were scraped with a teflon spatula. The intracellular extract was individually transferred to microfuge tubes and centrifuged at high speed in a Fisher 59A microfuge. The supernatant of these samples was used for the quantification of GSH using Ellman's reagent. The results have demonstrated that arsenic trioxide in combination with HEDS decreased GSH in HT29 cells (Figure 2).

For quantification of protein thiols (PSH), cancer cells attached to the dishes were incubated with different concentrations of arsenic compound and HEDS. These cells were then washed three times with PBS and then treated with SSA. This treatment precipitates cellular macromolecules in place without loss of protein. The acid was removed and cells washed two more times with 5 ml SSA, which was removed by aspiration. Under these experimental conditions, only the NPSH were washed off the cells. Acid-fixed cells were then incubated with 1 ml of 100 mM phosphate buffer, pH 7.4, containing 1.5 mM dithiobis nitrobenzoic acid (DTNB) for 15 min at 37º and the absorbancy read at 412 nm. Protein thiols were quantified using an absorption coefficient for reduced DTNB of 13,600 at 412 nm. The results from these experiments have demonstrated that arsenic trioxide in combination with HEDS increased the oxidation of PSH (Figure 3).

The DNA binding efficiency of nuclear Ku was quantified by Ku DNA repair kits from Active Motif, USA. The nuclear proteins were prepared by membrane disruption using Active Motif hypotonic buffer, detergent and complete lysis buffer and centrifugation as per the manufacturer's instructions. The nuclear proteins were stored at –80ºC. Protein concentration was quantified by Biorad Reagent. Nuclear protein (1µg) mixed with AM6 binding buffer was incubated in an eight well strip provided by Active Motif for 1hr at RT, washed and incubated

with Ku70 antibody for 1hr. After incubation, the wells were washed, incubated with developing solution for 4min, mixed with stop solution and the O.D was read in a microplate reader at 450nm with a reference wavelength of 655nm. The results have demonstrated that arsenic trioxide together with HEDS inhibited the function of Ku protein in both HCT116 and HT29 cells (Figure 4).

PROJECTS CARRIED OUT FOR SPECIFIC AIM 3:

In specific aim 3, we proposed to determine whether arsenic trioxide in combination with HEDS improves the response of colon cancer cells to cisplatin and γ radiation. In the second six months of the funding period, we determined the efficacy of arsenic trioxide and HEDS in increasing the response of HT29 cancer cells to cisplatin or γ radiation. The results have demonstrated that arsenic trioxide in combination with HEDS improved the response of HT29 cells to cisplatin and radiation (Figures 5 and 6). The results have also demonstrated that HEDS improved the response of HT29 cells to arsenic trioxide suggesting that HEDS can regulate arsenic trioxide mediated cytotoxicity in cancer cells.

C. Significance

The results from human cancer cells HCT116 and HT29 demonstrated that arsenic trioxide in combination with HEDS can be used to sensitize colon cancer cells to cisplatin and radiation.

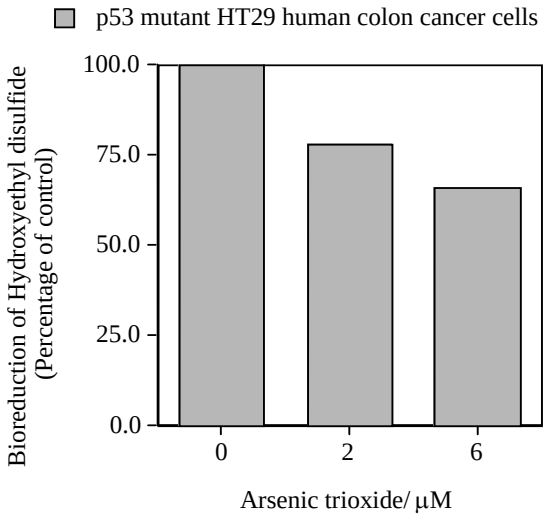

Figure 1. Quantification of detoxification of HEDS by p53 mutant HT29 human colon cancer cells by medium based assay that measures the conversion of HEDS into a non-toxic mercaptoethanol (ME). These results have demonstrated that arsenic trioxide inhibited the detoxification of HEDS up to 40% of the untreated control at concentrations as low as 6 μM.

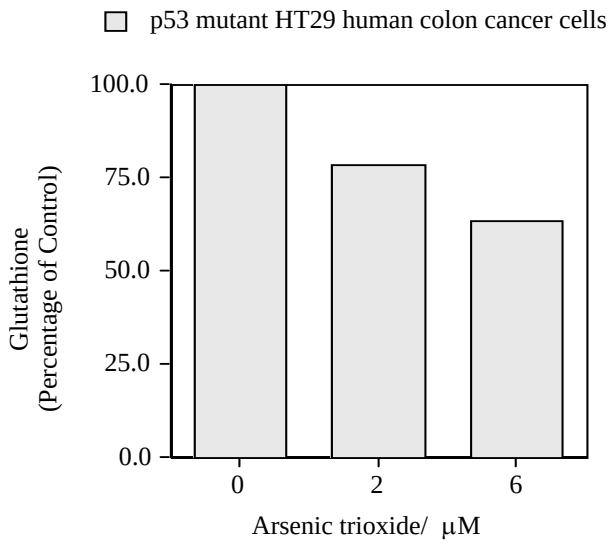

Figure 2. Quantification of intracellular dithiobis nitrobenzoic acid reactive non-protein thiol glutathione in p53 mutant HT29 human colon cancer cells treated with different concentrations of arsenic trioxide in combination with HEDS. The results have demonstrated that arsenic trioxide in combination with HEDS decreased non protein thiols in HT29 cells up to 40% at concentrations as low as 6μM

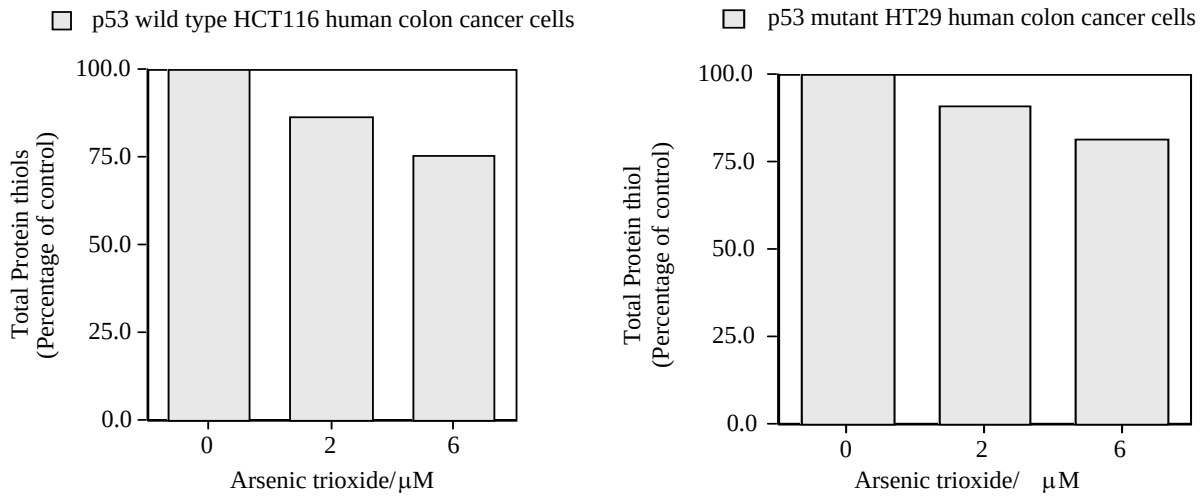

Figure 3. Quantification of intracellular protein thiols in p53 wild type HCT116 and p53 mutant HT29 human colon cancer cells treated with different concentrations of arsenic trioxide in combination with HEDS. The results have demonstrated that arsenic trioxide in combination with HEDS decreased non protein thiols in both these cells up to 30% at concentrations as low as 6μM.

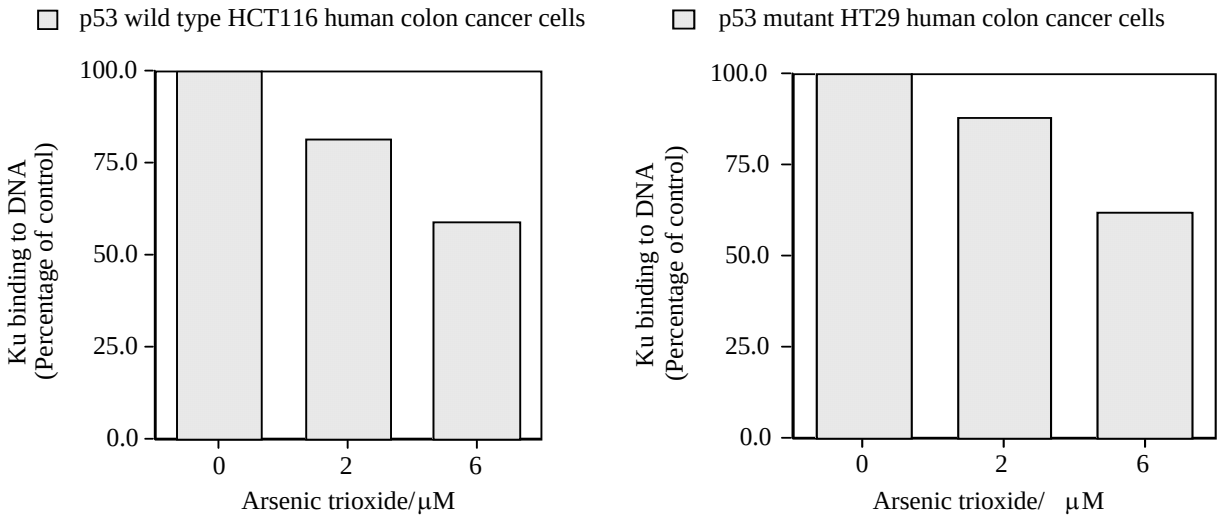

Figure 4. Quantification of Ku protein function in p53 wild type HCT116 and p53 mutant HT29 human colon cancer cells treated with different concentrations of arsenic trioxide in combination with HEDS. The results have demonstrated that arsenic trioxide in combination with HEDS decreased Ku protein function in both these cells up to 40% at concentrations as low as 6μM.

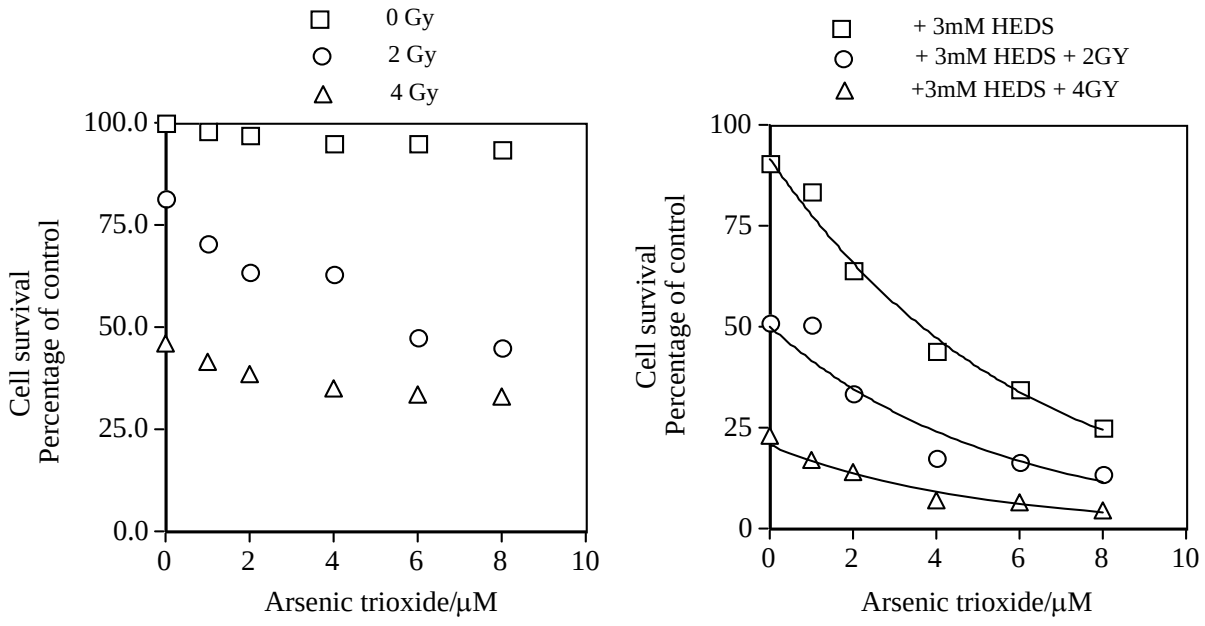

Figure 5. Effects of combined treatment of HEDS (3mM), arsenic trioxide (0-8μM) and radiation on the growth of HT29 human colon cancer cells. The results have demonstrated that HEDS improved the response of HT29 cells to combined treatment of arsenic trioxide and radiation.

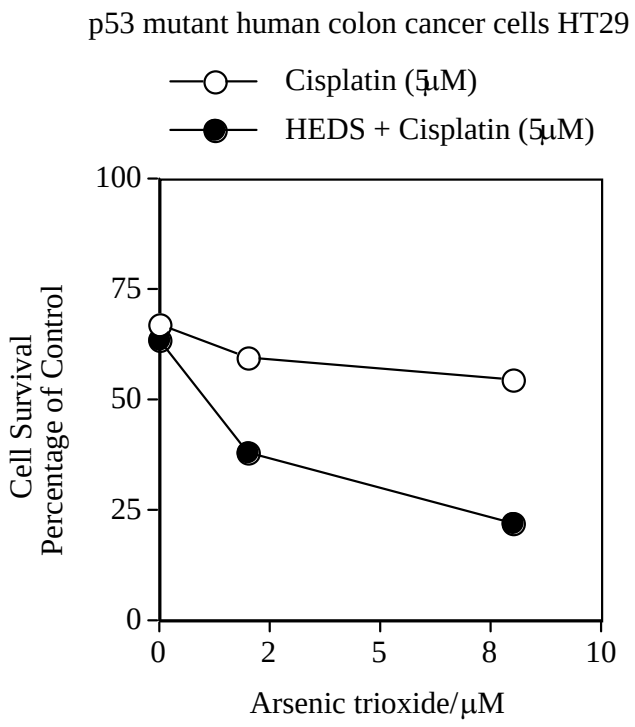

Figure 6: Effects of combined treatment of HEDS (2mM), cisplatin (5μM) and arsenic trioxide (0-8 μM) on the growth of HT29 human colon cancer cells. The results have demonstrated that HEDS improved the response of HT29 cells to combined treatment of arsenic trioxide and cisplatin.

<u>Research Project 2: Project Title and Purpose</u>

Role of IDO in B Cell-mediated Immunity and Autoimmunity - Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation and destruction of the joints. RA patients have an elevation in the level of activity of an immunoregulatory enzyme called IDO. This elevation in IDO 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. In this project, we will investigate the mechanism by which IDO inhibition alleviates arthritis development. The overall goal of this project is to define the mechanism by which IDO modulates the immune response leading to arthritis and provide new insights into strategies that can be used to manipulate this pathway to reduce or prevent debilitating disease.

Duration of Project

1/1/2009 - 12/31/2009

Project Overview

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease that affects 3 million Americans. It is characterized by chronic inflammation of the synovial joints, eventually leading to progressive and debilitating destruction of cartilage and bone. Current therapies focus on

controlling the symptoms of arthritis, not on the unknown underlying basis for its development. We propose to explore two immunomodulatory enzymes, IDO and IDO2, as disease modifiers of arthritis pathogenesis. Initially associated with protection of the fetus from the maternal immune system, IDO has also been shown to suppress effective immune responsiveness to a number of pathological conditions, including infection and tumorigenesis. However, the role of IDO in classic autoimmune disorders, such as RA, is not well characterized. The conceptualization that IDO acts solely to suppress effector T cell activation has led to the general assumption that inhibiting IDO will exacerbate autoimmune responses. However, recent indications in cancer models suggest a more complex biological role for IDO in actually molding the state of pathological inflammation commonly associated with and contributing to tumorigenesis. If IDO were to contribute similarly to other states of pathological inflammation, inhibiting IDO activity would have the unexpected consequence of ameliorating, rather than exacerbating symptoms. Along these lines, we have obtained preliminary evidence in the spontaneous K/BxN mouse model of RA that inhibiting IDO does indeed interfere with the manifestation of autoimmunity. IDO2, a novel IDO-related enzyme, is less well characterized, but has intriguing features that hint at IDO-related roles in dendritic cell function. In this project, we will use both pharmacological and genetic inhibition of IDO/IDO2 activity in the K/BxN model to determine the role that IDO/IDO2 plays in shaping the autoimmune response leading to arthritis. We suggest that targeting IDO/IDO2 has the exciting potential to lead to a new approach for the prevention and treatment of RA.

Aim 1. Determine which inflammatory features of arthritis induced in K/BxN mice can be ameliorated by targeting IDO/IDO2 activity.

Aim 2. Investigate whether IDO directly affects B cell activation or acts indirectly through modulatory effects on the microenvironment.

Principal Investigator

Laura Mandik-Nayak, PhD
Assistant Professor
Lankenau Institute for Medical Research
100 Lancaster Avenue
Wynnewood, PA 19096

Other Participating Researchers

None

Expected Research Outcomes and Benefits

Currently about 3 million people in the United States are living with RA, approximately 80% of those being women. While drugs to treat RA exist, better treatments to prevent and treat this disease are needed. In the context of cancer, the IDO/IDO2 enzymes are associated with pathological inflammation and immune escape and there is a substantial body of preclinical evidence that small molecule inhibitors of these enzymes 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/IDO2 activity may be therapeutically beneficial for suppressing the autoimmune symptoms associated with the spontaneous development of autoantibody-mediated arthritis. RA patients exhibit disease-associated elevation of IDO, leading us to hypothesize that the IDO/IDO2 enzymes may play an important contributory role to disease pathology in RA patients. In this project, we will employ genetic knockout mice and a set of specific small molecule inhibitors of IDO and/or IDO2 to probe the involvement of these enzymes in inflammatory processes linked to autoantibody-mediated autoimmunity. Our long-term goal is to determine whether IDO or IDO2 may influence the inflammatory characteristics of autoantibody-mediated autoimmunity, and if so, whether small molecule inhibitors of IDO or IDO2 now entering clinical trials may offer new options to prevent or treat these debilitating diseases.

Summary of Research Completed

This study supported by the Pennsylvania Department of Health is part of a larger ongoing study (currently supported by institutional funds) to determine the role of indoleamine 2,3-dioxygenase (IDO) in the development and progression of B cell mediated autoimmunity. During the 6 month period covered by this report, we have focused on experiments using the pharmacological inhibitors of IDO1 (L-1-methyl-tryptophan, L-1MT) and IDO2 (D-1MT), to determine if inhibition of IDO1, IDO2, or both is required for the amelioration of arthritis. Breeding is underway to generate arthritic mice that are genetically deficient for IDO1 or IDO2 to specifically test the role of each enzyme in the disease process. In vivo immunization experiments with model antigens, using both pharmacological inhibition of IDO with 1MT and IDO1 deficient mice, have also begun to define the role IDO normally plays in B cell activation and antibody production.

IDO2, not IDO1, drives arthritis in K/BxN mice

Our preliminary studies in K/BxN mice demonstrated that treatment with D/L-1MT resulted in a reduced autoantibody response and an attenuated course of arthritis. This treatment inhibits both IDO and IDO2, suggesting that IDO and/or IDO2 normally promote the development of inflammation in this model. To determine if inhibition of IDO1, IDO2, or both was responsible, small molecule inhibitors that target IDO1 or IDO2 (L-1MT and D-1MT, respectively) were used to pharmacologically block IDO activity. K/BxN mice were treated with D-1MT, L-1MT, or carrier alone starting at weaning *(Fig. 1)*. Mice were monitored for arthritis development by measuring joint inflammation by the change in ankle thickness and synovial proliferation and inflammatory cell infiltrates by histology. K/BxN mice that received carrier alone developed severe inflammation in their front and rear paws that began between 28 and 35 days of age. Administration of D-1MT delayed the onset and attenuated the severity of arthritis. In contrast, L-1MT did not inhibit arthritis, indicating that inhibition of IDO2, not IDO1, alleviates arthritis development in this model.

At the termination of the experiment (7wk of age), rear ankles were harvested and examined for histological evidence of arthritis by staining with hematoxylin and eosin *(Fig. 2)*. Carrier-treated mice showed classic signs of arthritis, with a greatly expanded synovium, panus formation, and

inflammatory cell infiltrates *(Fig. 2)*. In contrast, K/BxN mice treated with D-1MT, but not L-1MT, showed a reduction in the severity of arthritis with minimal synovial expansion and fewer infiltrating inflammatory cells. Together, these data suggest that IDO2, not IDO1, is responsible for driving arthritis.

D-1MT, not L-1MT inhibits autoreactive B cell response

Antibodies that recognize the glycolytic enzyme GPI are the key effector molecules in the disease process in K/BxN mice. Previously, we demonstrated that the reduced arthritis in D/L-1MT-treated mice was the result of a diminished GPI-specific B cell response. To determine if this was affected by inhibition of IDO1 or IDO2, K/BxN mice were treated with L-1MT, D-1MT, or carrier alone and followed for serum anti-GPI titers and the number of antibody secreting cells. Titers of anti-GPI Ig *(Fig. 3a)* were significantly lower in D-1MT compared to L-1MT or carrier treated mice. To determine if the reduced serum anti-GPI levels were due to D-1MT treatment affecting the magnitude of the anti-GPI B cell response, the number of antibody secreting cells (ASCs) was quantified in the lymph nodes draining the arthritic joints (dLNs) of L-1MT, D-1MT, or carrier-treated K/BxN mice *(Fig. 3b)*. GPI-reactive ASCs were present in large numbers in the dLNs of L-1MT and carrier-treated K/BxN mice. Numbers of anti-GPI ASCs were significantly reduced in the dLN of D-1MT-treated mice. Together, these data demonstrate that inhibition of IDO2, but not IDO1 activity, diminishes the pathogenic anti-GPI B cell response, resulting in reduced arthritis.

Neither L-1MT nor D-1MT alone is sufficient to alter cytokine response

IDO has been shown to be both an inhibitor of T cell activation and a modifier of the cytokine profile of the resulting T cell response. Our preliminary data showed that K/BxN mice treated with D/L-1MT had reduced levels of the inflammatory cytokines IL-6, MCP-1, and IL-10, while levels of IFNγ and TNFα remained unchanged. To determine if inhibition of IDO1 and/or IDO2 was responsible for this decrease in cytokines, K/BxN mice were treated with L-MT, D-1MT, or carrier alone and monitored for cytokine production. LNs draining the arthritic joints were harvested and stimulated overnight with PMA + ionomycin. Cytokine levels were measured in the culture supernatants by using the flow cytometry-based cytometric bead array assay *(Fig. 4)*. Carrier-treated mice had high levels of IFNγ, TNFα, IL-6, MCP-1, and IL-10. In contrast to the racemic D/L-1MT mix, neither L-1MT nor D-1MT alone affected inflammatory cytokine production *(Fig. 4)*. These data suggest that although inhibition of IDO2 alone is able to alleviate arthritis, inhibition of both IDO1 and IDO2 is required to decrease inflammatory cytokine levels.

IDO is also required in B cell responses to T cell-dependent model antigens

Our preliminary data demonstrate that IDO is required for the activation and/or differentiation of autoreactive B cells into autoantibody producing cells. These data suggest that IDO may play a more general role in the activation of B cells to non-self antigens. To begin to address the role of IDO on B cell activation, we inhibited IDO activity in mice immunized against the T cell-dependent model antigen, (4-hydroxy-3-nitrophenyl) acetyl - keyhole limpet hemocyanin (NP-KLH) and T cell-independent antigen NP-LPS. IDO activity was inhibited pharmacologically

using D/L-1MT and genetically, using IDO1$^{-/-}$ mice. 1MT-treated and carrier-treated C57BL/6 mice and untreated IDO1$^{-/-}$ mice were immunized with 100μg NP-KLH precipitated in alum or 50μg NP-LPS. Anti-NP Ig titers were measured in the serum over the next 4 weeks by ELISA. In response to both NP-KLH and NP-LPS, anti-NP IgG was first detected in carrier-treated C57BL/6 mice at day 5 and levels continued to increase over the next few weeks *(Fig. 5)*. IDO1$^{-/-}$ mice produced reduced levels of anti-NP Ig in response to NP-KLH. After an initial spike at day 5, 1MT-treated C57BL/6 mice also consistently produced lower levels of serum anti-NP IgG *(Fig. 5a)*. In contrast, both IDO1$^{-/-}$ and D/L-1MT treated C57BL/6 mice produced high levels of anti-NP Ig in response to NP-LPS *(Fig. 5b)*. These preliminary data suggest that IDO1 plays a role in the activation of B cells in response to T cell-dependent, but not T cell-independent antigens.

For T cell-dependent stimuli, the anti-NP B cell response can be followed *in situ* as well as in the serum. *In situ*, antibody secreting cell foci can be detected as early as day 2, and reach a peak at 5-8 days post-immunization. At this time, germinal centers begin to appear and remain for several weeks. To determine if 1MT affected the timing or location of antibody secreting cell foci or germinal centers, IDO1$^{-/-}$, carrier-treated, and D/L-1MT-treated C57BL/6 mice were immunized with NP-KLH. At 5 and 10 days after immunization, spleens were harvested, and frozen sections examined for the presence of antibody secreting cell foci and germinal centers. NP-specific B cells were identified by staining for Igλ-expression. Consistent with the reduced serum anti-NP response, both D/L-1MT-treated and IDO1$^{-/-}$ mice appeared to exhibit smaller numbers of antibody secreting cell foci at day 5 and germinal centers at day 10 than carrier-treated C57BL/6 mice *(Fig. 6)*.

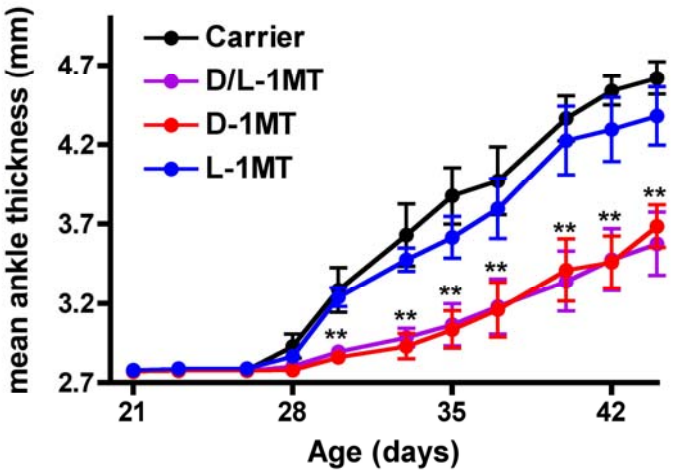

Figure 1. D, not L-1MT inhibits arthritis. K/BxN mice were treated p.o. with 400mg/kg D (red), L (blue), or D/L-1MT (purple) b.i.d. or carrier alone (black) starting at 21 days. Rear ankles were measured as an indication of arthritis and represented as the mean ankle thickness ± SEM. Representative experiment of 3 total, showing n=5 mice for each treatment. **p<0.01

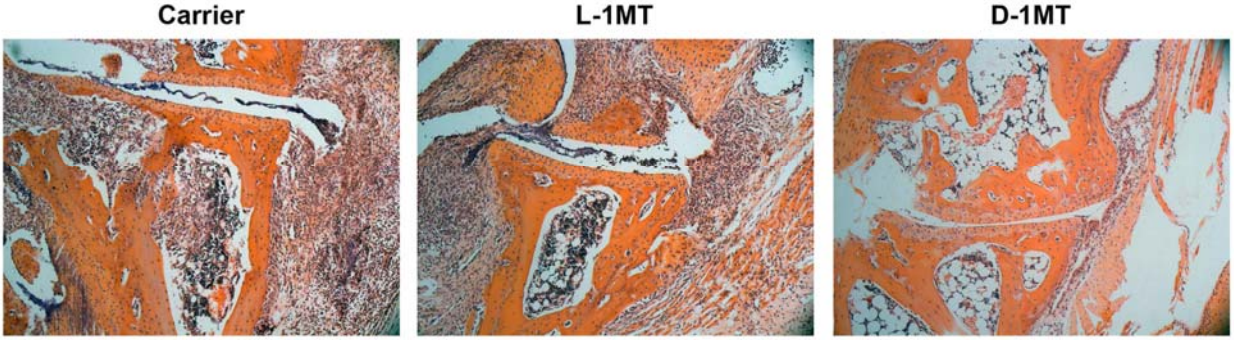

Figure 2. D, not L-1MT inhibits arthritis. Metatarsal joint from D-1MT, L-1MT, or carrier-treated K/BxN mouse at 42d of age stained with H&E. Representative sections from a total of n=5 mice for each treatment group. Original magnification 100X.

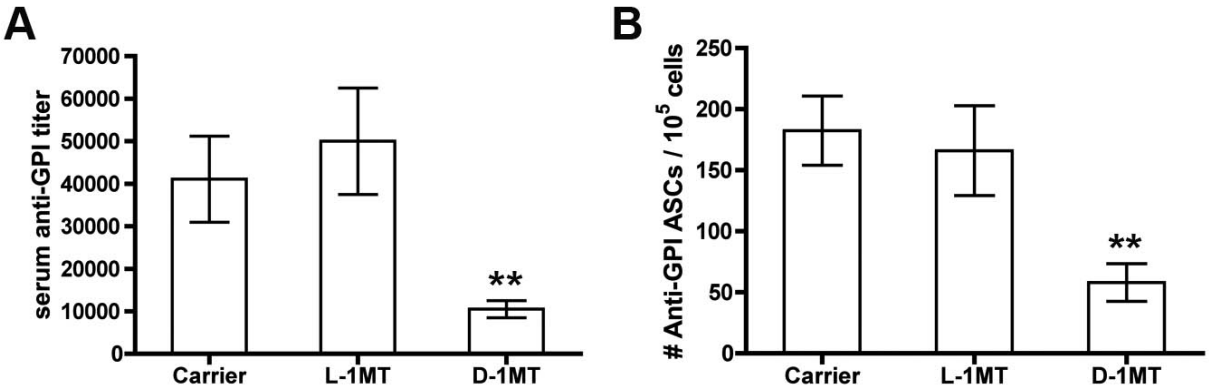

Figure 3. D, not L-1MT inhibits anti-GPI B cell response. K/BxN mice were treated with D-1MT, L-1MT, or carrier alone and analyzed at 6-8 wk of age. (A) The isotype of anti-GPI Ig was measured by ELISA and represented as the mean titer of Ig ± SEM. (B) Antibody Secreting Cells (ASCs) were measured using an ELISpot assay. The graph shows the mean number of ASCs ± SEM in each organ from a representative experiment of 3 total showing n=5 mice for each group. ** $p<0.05$

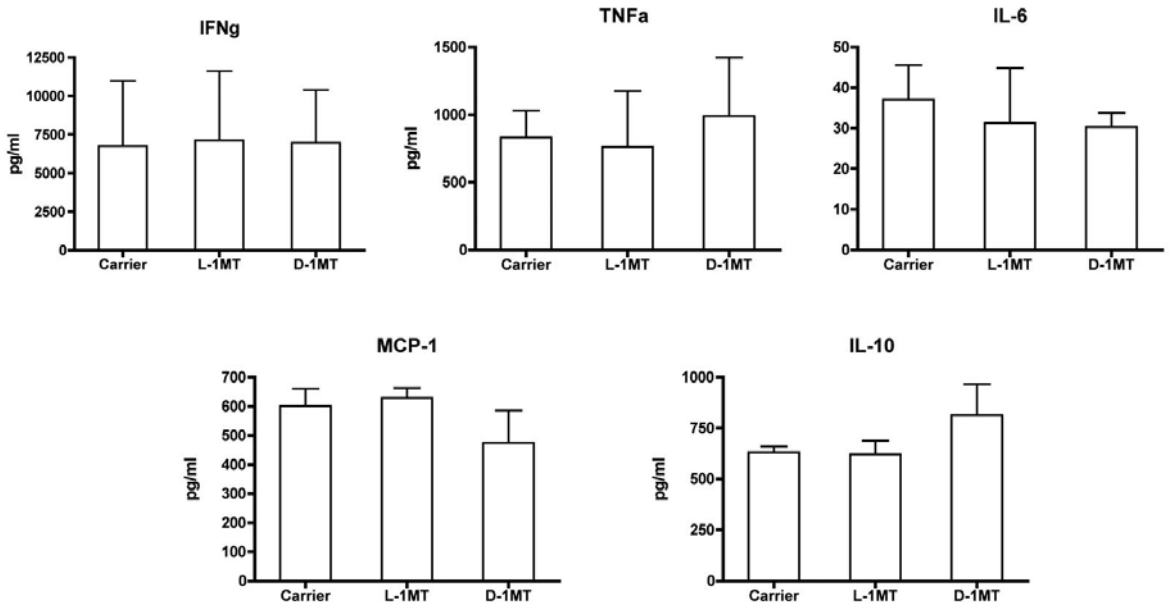

Figure 4. Neither D nor L-1MT treatment alone alters cytokine levels. K/BxN mice were treated with D-1MT, L-1MT, or carrier alone and analyzed at 7-8 wk of age. Cells from the joint dLNs were cultured overnight in PMA (50ng/ml) + Ionomycin (500ng/ml). Cytokines were measured in culture supernatants by cytometric bead array. Graphs show the mean concentration ± SEM from a representative experiment of two with n=5 mice per group.

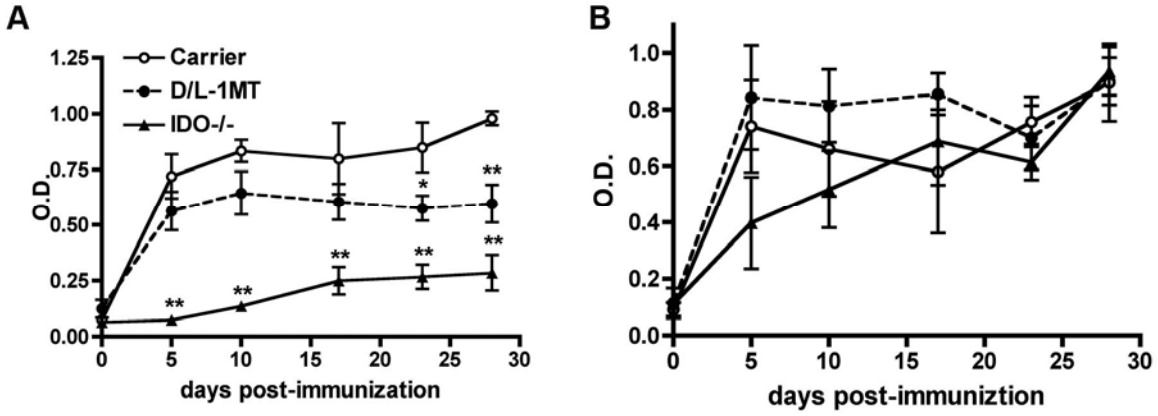

Figure 5. 1MT inhibits B cell response to T-dependent, but not T-independent Ag. Carrier- and D/L-1MT-treated C57BL/6 mice or untreated IDO1$^{-/-}$ mice were immunized with (A) 100mg NP-KLH in alum or 50μg NP-LPS on day 0. D/L-1MT (400mg/kg, p.o.) and carrier were given b.i.d. for the duration of the experiment. Serum samples were taken on days 0, 5. 20, 17, 24, and 28 and analyzed for anti-NP IgG1 by ELISA. Data are from an individual experiment with n=5 mice per group. *p<0.05, **p<0.01

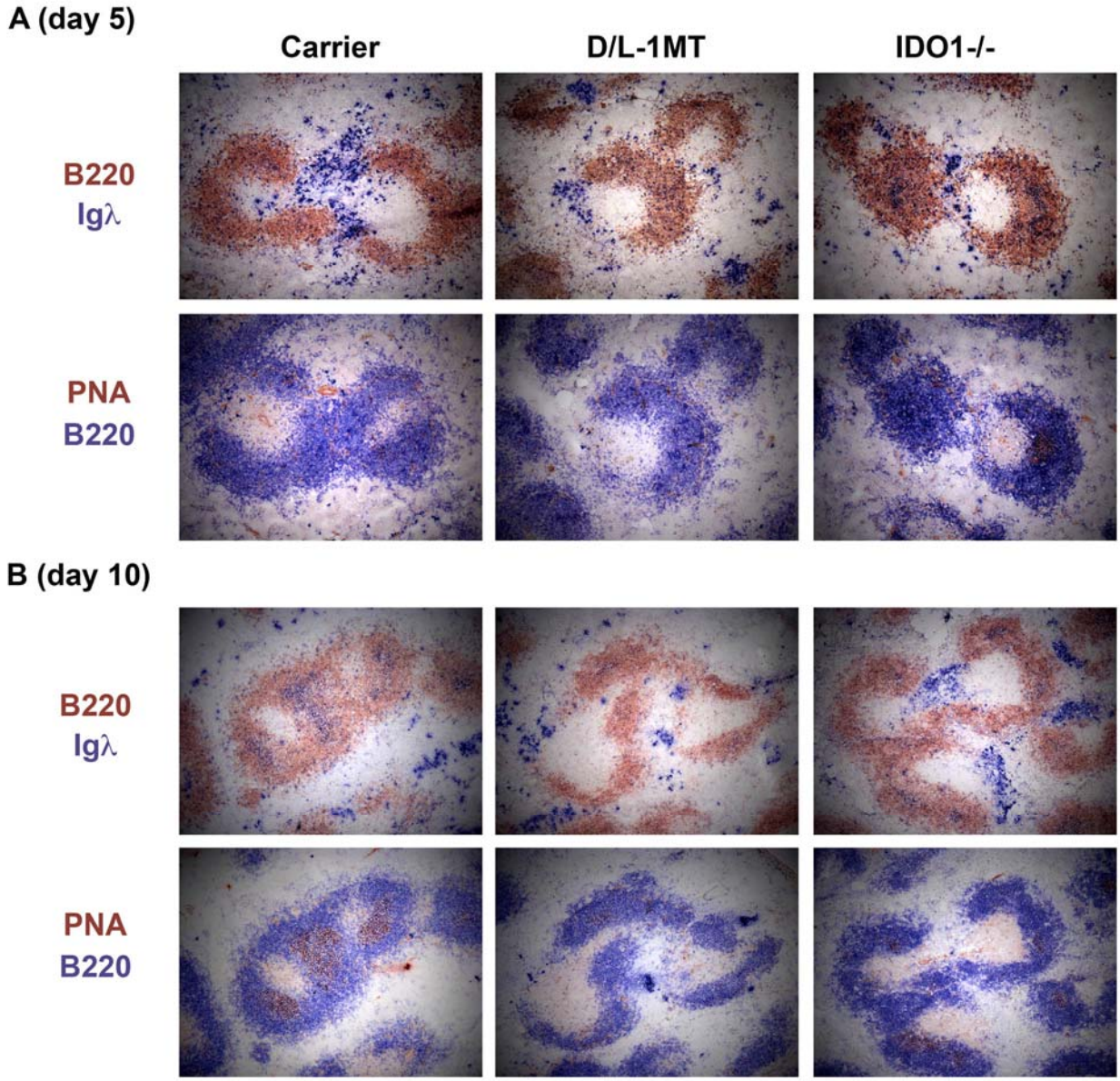

Figure 6. Reduced antibody secreting cells and germinal centers in D/L-1MT and IDO1$^{-/-}$ mice. Carrier- and D/L-1MT-treated C57BL/6 mice or untreated IDO1$^{-/-}$ mice were immunized with 100µg NP-KLH in alum on day 0. D/L-1MT (400mg/kg, p.o.) and carrier were given b.i.d. for the duration of the experiment. Spleens were harvested on days 5 and 10 post-immunization, frozen in OCT, sectioned, and stained with antibodies to B220 and (A) Igl to identify antibody secreting cells and (B) PNA to identify germinal centers. Shown are representative sections from n=5 mice for each group. Original magnification = 100x.