INVESTIGATION OF THE RELATIONSHIP BETWEEN INFLAMMATORY SIGNALING PATHWAYS AND INDOLEAMINE 2,3-DIOXYGENASE IN GLIOBLASTOMA MULTIFORME

An Undergraduate Research Scholars Thesis

by

ALEXANDER B. SALTZMAN

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

Approved by
Research Advisor: Raquel Sitcheran, Ph.D.

May 2013

Major: Biochemistry

TABLE OF CONTENTS

ABSTRACT

Investigation of the Relationship Between Inflammatory Signaling Pathways and Indoleamine 2,3-Dioxygenase in Glioblastoma Multiforme. (May 2013)

Alexander B. Saltzman
Department of Agriculture and Life Sciences
Texas A&M University

Research Advisor: Dr. Raquel Sitcheran, Ph.D.
Department of Molecular and Cellular Medicine

Pervasive tumors appear to evade immune detection through manipulation of the immune response, though precisely how this occurs is not well understood. A further understanding of these details may be achieved through the study of inflammation-driven tumors. While inflammatory cytokines increase blood flow to sites of inflammation for increased delivery of oxygen and nutrients, immune cells are also recruited and activated. Interferon-γ, an inflammatory cytokine, may suppress the actions of effector T cells through induction of indoleamine 2,3-dioxygenase-1 (IDO-1). IDO is involved in tryptophan metabolism and stimulates the recruitment and maturation of T regulatory lymphocytes while suppressing the activity and proliferation of T effector cells. IFN-γ appears to induce IDO-1 expression through NF-κB and JAK-STAT signaling pathways, though the precise signaling cascades are unclear. Here we investigate the relationship between IFN-γ and IDO in glioblastoma, an aggressive cancer of the brain with very poor prognosis. Reporter assays indicate that IFN-γ does increase transcriptional activation at the IDO-1 promoter, while protein analysis does not indicate a change of IDO-1 expression at the protein level. IFN-γ does appear to negatively affect transcriptional activation of the canonical NF-κB pathway, as seen by a loss in p65 phosphorylation, suggesting that IFN-γ signals independently, perhaps in opposition to, the canonical NF-κB transcriptional cascade.

ACKNOWLEDGMENTS

I would like to acknowledge the other researchers in the lab for their support. First, the P.I. and my research advisor Dr. Raquel Sitcheran gave me direction and guidence on suitable experiments to persue. I would also like to thank Dr. Dong Lee for his advice and availability whenever I had a question, as well as similar work had had done which he readily shared with me. Additionally, I would like to thank John Valenta for his assistance in experiments that I was performing for the first time, as well as assisting me in the interpretation of experimental results. Finally I want to recognize Evan Cherry who frequently shared medical information related to our research which gave me a more comprehensive understanding on the subject and its relevance.

NOMENCLATURE

IDO	Indoleamine 2,3-dioxygenase
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
IRAK1	Interleukin-1 receptor-associated kinase 1
AHR	Aryl hydrocarbon receptor
NF-κB	Nuclear factor kappa B
RHD	Rel homology domain
IκB	Inhibitor of κB
IKK	IκB kinase
NEMO	NF-κB essential modulator
IFN	Interferon
TNF-α	Tumor Necrosis Factor - alpha
TGF-β	Transforming Growth Factor - β
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin

CHAPTER I

INTRODUCTION

Glioblastoma Multiforme

Glioblastoma multiforme, a WHO grade IV astrocytoma, is an aggressive brain cancer with poor prognosis; the five year survival rate of those afflicted is below 5% [1]. The most recent advance in treatment, adjunct usage of the chemotherapeutic agent temozolomide with radiation in 2005 increased median survival time from 12 to 14.2 months [2]. More successful therapies are urgently needed, and a more complete understanding of the tumor environment is being actively pursued for this end.

Immunity in the Brain

The blood-brain barrier (BBB) limits the flow of immune cells into the brain; evidence of leukocyte influx after widespread inflammation is well defined (such as after ischemic stroke) but the capacity of lymphocytes to migrate into the brain during various disease states is less clear [3, 4]. The brain does have dedicated macrophages referred to as microglial cells who, not unlike macrophages in other tissues, transition from pro- to anti-inflammatory status during tissue repair and clearance of apoptotic cells [4]. T lymphocytes, including a significant population of T regulatory cells, have been reported in brain tissue of patients with glioblastoma [5], suggesting that tumor infiltrating lymphocytes exist and may serve to suppress inflammation and immune responses in tumors that evade immunosurveillance.

Nuclear factor - κB and Inflammation

Nuclear factor - kappa B (NF-κB) was first identified as a complex of transcription factors present but inactive in B cells until activated by inflammatory stimuli [6]. It has subsequently been identified in many other cell types with roles including normal and malignant development of immune cells and colorectal cancers [7,8]. Under normal circumstances, NF-κB activation after signaling from harmful external stimuuli allows cells to resist apoptosis and increase proliferation to repair damaged tissue. However, continual activation of NF-κB causes an extensive and aberrant response that can initiate and maintain cancer growth [9]. A more comprehensive understanding of NF-κB regulation and subsequent downstream effects has been continually investigated since its discovery.

NF-κB proteins and mediators can be divided into three general groups. p65/RelA, RelB, c-Rel, NF-κB1, and NF-κB2 are of the transcription factor family with a conserved Rel homology domain (RHD) that allows for DNA binding [10]. Importantly, NF-κB1 and 2 do not possess transactivation domains for induction of targeted gene expression. Secondly, inhibitors of κB (IκB) include IκBα, κBβ, IκBϵ, IκBζ, Bcl-3, p105, and p100[1]. Finally, the IκB kinases (IKK) are composed of IKKα, IKKβ, IKKϵ, and the scaffold protein IKKγ/NEMO. Though theoretically there are many possible combinations of the Rel transcription factor dimerizations, there are specific dimers that are commonly observed in nature. p65/p50 dimers make up the largest population and most studied, while RelB has been seen as a heterodimer with either p52 (or sequestered with the pre-processed p100) or p50. Two signaling pathways of NF-κB have been established, though other members of the NF-κB are also involved in signaling in less understood mechanisms and will not be explored here. The classical or canonical pathway involves liberation of p65/p50 heterodimers from inhibitory IκBα complexes in the cytoplasm. In response to external stimulus, IKKβ phosphorylates IκBα leading to polyubiquination and proteosomal degradation of the phosphorylated protein. No longer bound by IκBα, the p65/p50 dimer translocates to the nucleus via its where

[1]p105 and p100 are also NF-κB1 and 2, but the reference to NF-κB1 or 2 includes the processed forms of p105 and p100, p50 and p52, respectively.

it binds to promoter sites for gene transcription. One of the targeted gene promoters of the heterodimer is that encoding for IκBα, which in turn binds to nuclear p65/p50 with subsequent export from the nucleus in a negative feedback loop. The alternative, or noncannonical pathway is an IKKα dependent partial degradation of p100 (which sequesters RelB in the cytoplasm) to p52 after its polyubiquination. This p52/RelB heterodimer similarly translocates to the nucleus for gene transcription [11]. The two pathways are not mutually exclusive.

A simple model of two NF-κB pathways is displayed below [Figure 1].

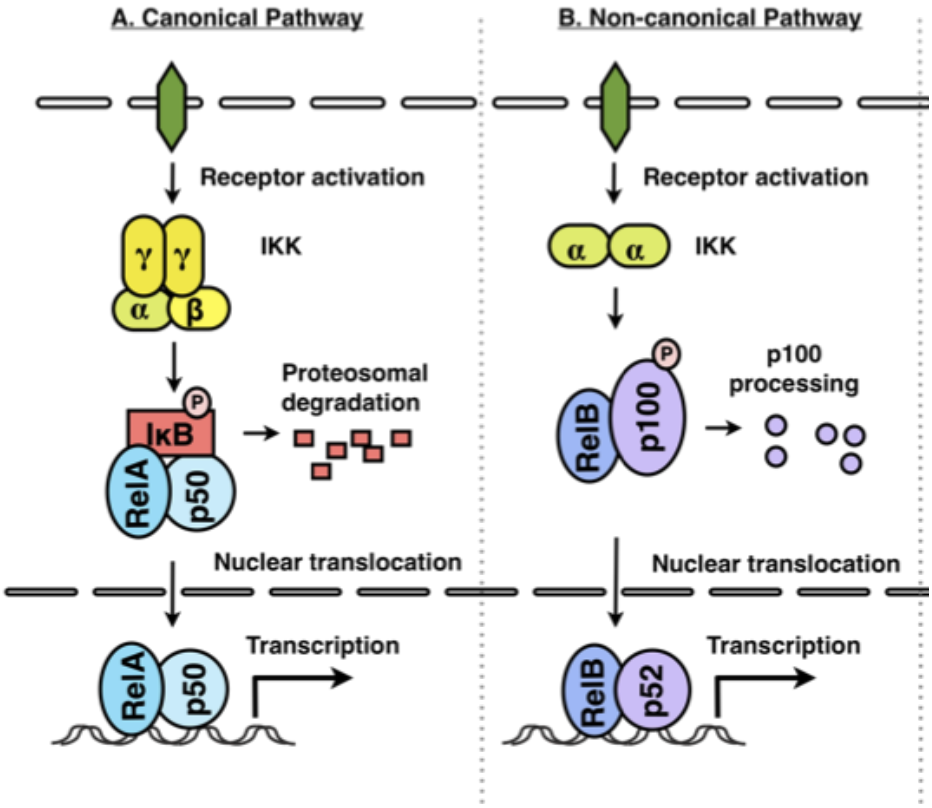

Fig. 1. A) Canonical signaling depends on IKKβ resulting in degradation of IB and activation of RelA/p50 complexes. B) Non-canonical NF-κB signaling involves IKKα-dependent processing of p100 to p52 and activation of RelB/p52 heterodimers. IKKγ = NEMO.

Indoleamine 2,3-Dioxygenase Mediated Regulation Immunity

Indoleamine 2,3-dioxygenase (IDO) is an enzyme for the rate limiting step in tryptophan metabolism, and its expression in some carcinomas is negatively correlated with patient survival [12]. The enzyme was first identified as an interferon-γ (IFN-γ) induced protein that suppressed the growth of a foreign pathogen, *Toxoplasma gondii*, within the host cell through depletion of free tryptophan[cite]. IDO was also found to exert control on allogenic fetus rejection through its enzymatic activity. This effect occurs through the resulting tryptophan metabolites, kynurenine. Kynurenine are endogenous ligands of the aryl hydrocarbon receptor (AHR), which plays an important role in immune suppression through growth arrest of cells such as cytotoxic and helper T lymphocytes, while also causing an expansion of the regulatory T lymphocytes. Previously mentioned IFN-γ, as well as transforming growth factor - β (TGF-β) have been shown to increase expression of IDO and stabilize protein levels through a recently characterized ITIM (immunoreceptor tyrosine-based inhibitory motif) [13]. IRAK1, which is involved in pro-inflammatory response via the cannonical NF-κB pathway, shifts the balance of NF-κB to its alternative signaling pathway. In plasmacytoid dendritic cells, the alternative NF-κB proteins (RelB-p52 dimers) but not canonical p65 have been found bound at the IDO promoter upon stimulation of TLR-7, suggesting the involvement of the alternative NF-κB pathway in a positive feedback loop for IDO expression [14].

While the alternative NF-κB pathway appears to be directly involved in IDO-1 expression, preliminary data suggests that the classical pathway is also involved as IDO-1 induction is lost after knockdown of p65[Lee and Sitcheran, unpublished data].

CHAPTER II

MATERIALS

All chemicals used were analytical grade or higher.

Cell Culture

U87 MG (ATCC Repository) glioblastoma cell line was cultured on 10 cm plates(Greiner CELLSTAR®) in Dulbecco's Modified Eagle Medium (DMEM, Gibco®) supplemented with 100 U/mL penicillin/streptomycin (Gibco®) and 10% by volume fetal bovine serum (FBS, Gibco®) within a humidified incubator at ambient oxygen, 37° Celsius, and 5% CO_2. At 80% confluence cells were dissociated with 0.25% trypsin-EDTA (Gibco®) for passage or experimentation.

Transient Transfection of Mammalian Cells

U87 MG cells were seeded in a 24 well plate at either 2×10^5 cells/well or 7.5×10^4 cells/well (for 24 and 96 well plates, respectively) in DMEM + 10% FBS. After 24 hr, cells were chemically transfected using a polyethyleneimine (PEI) : DNA ratio of 3:1. A total of 500 ng of DNA was used in each sample (except mock), with reporter plasmids at 100 ng/sample, constitutive lacZ plasmid at 10 ng/sample, any protein over-expression plasmids at 50 ng/sample, and an empty vector plasmid making up the remaining weight. After 15 minute incubation of the PEI:DNA solution at room temperature, cells were given their respective mixes along with fresh media.

Cytokine and Chemical Treatments

24 hr after cells had been transfected (or seeded for stably expressing cell lines), cells were treated with cytokines or chemicals of interest. IFN-γ (EMD Millipore®), TNF-α (Promega®), and TGF-β (Cell Signaling Technology®) were all used at a concentration of 10 ng/ml. TCDD (Supelco Analytical®) was used at a concentration of 30 nM.

Plasmid Constructs and Reporter Assays

Reporter Constructs

pcDNA3.1 pcmv-lacZ plasmid (Promega®) was used to normalize the luminescence via transfection efficiency across all samples subjected to transient transfection. pGL3 κB4-luc (luciferase driven by an artificial NF-κB binding element), pcmv-p65, pcmv-p50, and pcmv-Flag-p52 were gifts from Raquel Sitcheran, PhD. plenti6 pIDO1-luc and pLenti6-hRelB-(Delta)RI were gifts from Drs. Dong Lee and Raquel Sitcheran. U87 MG cells stably transformed with pcmv-luc, Δpcmv-luc, pIDO1-luc, and pIDO1-$\Delta\kappa$B-luc were also gifts from Drs. Dong Lee and Raquel Sitcheran.

Luciferase Assay

24 hr after treatment, cells were lysed with 50 μl of Mammalian Protein Extraction Reagent (M-PER®, Thermo Scientific®) : EDTA-free protease inhibitors (Halt™ Protease Inhibitor, Thermo Scientific®) at a ratio of 100:1. Plates were subsequently shaken on ice for 20 minutes. For experiments on a 24 well plate, 25 additional μl of M-PER was added to samples resistant to lysis after this time. Samples were subject to a freeze-thaw cycle to ensure complete lysis before analysis.

For experiments on 24 well plates, 10 μl of lysate was mixed with 50 μl of reconstituted, lyophilized D-Luciferin buffered solution (Pierce®) in a white 96 well plate. For experiments on 96 well plates, 20 μl of reconstituted D-Luciferin solution was added directly to each lysate. Reagents and samples were given a 10 minute incubation at room temperature before luminescence was measured on a Victor3 optical plate reader (Perkin Elmer®).

Beta-galactosidase Assay

As previously mentioned with regards to transient transfection experiments, a plasmid constitutively expressing β-galactosidase was included in all samples to normalize luminescence across samples to correct for variations in transfection efficiency. The level of β-gal was measured indirectly through the rate of appearance of chlorophenol red (through measuring absorbance at 550 nm, of which chlorophenol red has maximal absorbance) from its precursor chlorophenolred-β-D-galactopyranoside (CPRG). CPRG is galactose linked to chlorophenol red and has no significant absorbance at 550 nm at the concentration used in the assay. As β-galactosidase cleaves the β linkage between galactose and glucose that compose lactose, the endogenous ligand for the enzyme, CPRG is a suitable artificial substrate and in fact is more sensitive than other artificial substrates like ONPG [15]. 10 μL of cellular extract was mixed with 200 μL of pre-warmed CPRG solution(Z-buffer, composed of 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4 \bullet 7H_2O$, + 1.35 μL:mL β-mercaptoethanol + 2.5×10^{-5} M CPRG substrate) Absorbance readings were taken every minute for one hour at 37° Celsius, and the resulting slope of absorbance with respect to time served as a marker of the amount of β-gal, and thus overall efficiency in transfection.

Western Blotting

Bradford Assay and Sample Preparation

Cellular protein extracts were obtained as previously described with M-PER. Protein levels were quantified by Bradford assay, by which protein samples were compared to a set of standards of varying concentration of bovine serum albumin (Thermo Scientific®). Protein concentration was measured by the resulting absorbance at 595 nm after complexing with Quick-Start™ Bradford reagent (Bio-Rad®). A linear regression was generated from the data obtained from the standards, and the concentration of protein in the samples were assigned from their obtained absorbance and the calculated equation. After determining protein concentration, samples were prepared by diluting protein such to a final concentrations of 1μg/μL along with 25% v/v 4X sample buffer (Thermo Scientific®), 10% v/v dithiothreitol (for disruption of disulfide bonds), and lysis buffer to make up final volume. After preparation, samples were heated at 100° Celsius for 5 minutes before loading onto a denaturing polyacrylamide gel.

Polyacrylamide Gel Preparation and Electrophoresis

Polyacrylamide gels were self-cast with a lower separating gel composed of 8% polyacrylamide, 25% of total volume Tris/SDS buffer (pH 8.8), 0.5% v/v 10% ammonium persulfate, and 0.1% v/v tetramethylethylenediamine, along with an upper stacking gel composed of 4% polyacrylamide, 25% of total volume Tris/SDS buffer (pH 6.8), 0.5% v/v 10% ammonium persulfate, and 0.1% v/v tetramethylethylenediamine. Tris-glycine SDS was used as the running buffer for the gel. After samples were loaded, the gel was run at a constant 160 volts until loading dye had exited the bottom of the gel, approximately 1 hour.

Membrane Blotting and Protein Analysis

After running the gel, proteins were transfered to a nitrocellulose membrane using an Owl HEP-1 semi-dry transfer apparatus (Thermo Scientific®), incubated in Pierce® Fast Semi-dry Transfer Buffer at a constant 0.5 mAmps per membrane for 45 minutes. After transfer, membranes were incubated in 1:1 Odyssey® Blocking Buffer : dH_2O for 1 hour at room temperature, or overnight at 4° Celsius. After blocking, membranes were probed with primary antibodies specific to proteins of interest. Primary antibodies used were 1:1000 dilutions of anti-rabbit phospho-S536 p65 (CST-3033), RelB (CST-4922), and p52(CST-4822), 1:1000 dilutions of anti-mouse p65 (SCB-8008), p50 (SCB-53744), and 1:2500 dilution of anti-mouse β-actin (SCB-69879)[1]. Primary antibody dilutions were incubated with the membrane overnight at 4° Celsius. After primary antibody incubation, the membrane was subject to 3 washes of 1X TBST (1.37 M NaCl, 26.83 mM KCl, 247.65 mM Tris base, pH adjusted to 7.4, diluted 1:10 with dH_2O, and with addition of 0.1% v/v Tween-20 before use) at 10 minutes each (5 minutes when using antibodies specific for phosphorylated proteins). Following this, secondary infrared fluorescent antibodies were conjugated to the primary antibodies for visualization; 1:10,000 dilutions of IRDye 800CW goat anti-rabbbit and IRDye 680RD anti-mouse antibodies (LI-COR®) were incubated with the membrane in the dark for one hour. Before analysis, the membrane was again subject to 3 washes of 1X TBST at 10 minutes each (5 minutes when using antibodies specific for phosphorylated proteins) and then placed in cold PBS (1.37 M NaCl, 26.83 mM KCl, 101.4 mM Na_2HPO_4, 17.64 mM NaH_2PO_4, pH adjusted to 7.4 and diluted 1:10 with dH_2O before use). Membranes were then imaged on a LI-COR Infrared Imaging System (Odyssey®).

[1]CST = Cell Signaling Technology®, SCB = Santa Cruz Biotechnology®

CHAPTER III

RESULTS

Before investigating the possible role of NF-κB transcription factor pathways in the induction of IDO1 protein expression, luciferase assays were run with a synthetic κB4 sequence driving expression of luciferase; 4 repeats of cannonical NF-κB elements followed by the gene encoding for luciferase. U87 cells were transiently transfected with the κB4-luciferase plasmid, and samples were subjected to various other treatments to observe the resulting luciferase expression. Both RelA/p65 homodimers and RelA:p50 heterodimers bind to canonical κB elements, and TNFα is a well-documented inducer of canonical NF-κB signaling. As seen in Figure 2, both overexpression of p65 alone and with p50 drastically increased the luminescence resulting from increased expression of luciferase, as expected. While TNFα treatment did not induce luciferase expression significantly, the analysis took place 24 hours post-treatment; possibly exceeding the time of which the effects of TNFα on NF-κB induction is observed.

The reagents for luciferase expression analysis were changed from those previously used; the new reagents no longer included renilla expression for normalization. To control and normalize for transfection effeciency, a control plasmid containing cmv-lacZ was included in all samples and each was analyzed for the rate of production of CPRG. The assay was repeated with the κB4-luciferase plasmid [Figure 3A], which unexpectedly showed robust signal in the samples overexpressing RelB and p52, not the samples overexpressing p65 and p50. Western blot confirmed overexpression of RelB and p52, but p65 was not observed to be overexpressed due to the lack of increased phosphorylation of the protein relative to control[Figure 3B].

The assay was repeated to check the validity of the unexpected results. As seen in Figure 4A, overexpression of p65 and p50, but not with overexpression of noncanonical signaling

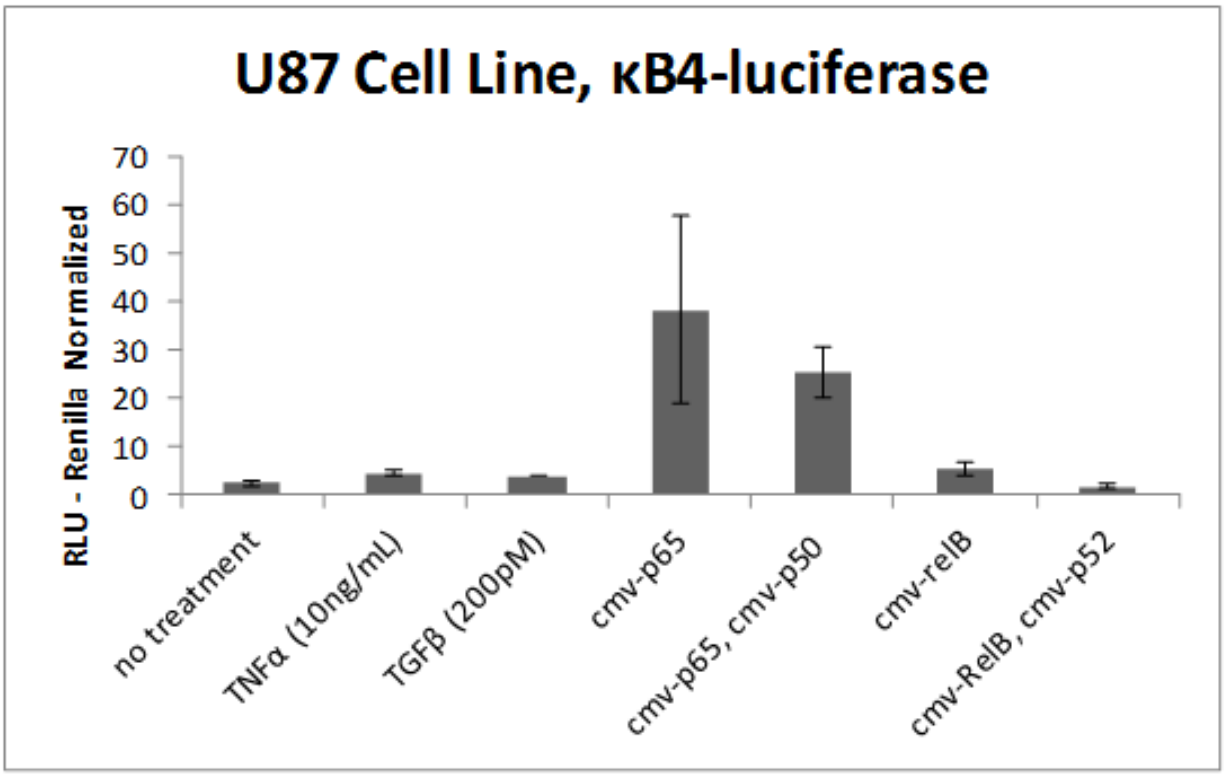

Fig. 2. Luminescence from luciferase expression directed from the κB4 promoter, normalized to Renella activity expressed from cmv-renilla plasmid transfected equally in all samples. TNFα or TGFβ was added to two of the samples 24 hours after tranfection, and all samples were analyzed 24 hours after that. Samples were performed in duplicate. Error bars indicate standard deviation.

proteins RelB and p50, resulted in the induction of luciferase expression. Overexpression was confirmed by western blot[Figure 4B].

The sequence encoding the promoter region of IDO1, and a shortened sequence excluding NF-κB and AhR/ARNT binding sites were cloned and inserted in a plasmid in front of the coding sequence of lucieferase [Lee and Sitcheran], represented graphically in Figure 5. The plasmids were transfected into U87 cells which were then subject to various treatments including combinations of TNFα, TGFβ, IFNγ, and TCDD - an exogenous ligand for AHR.

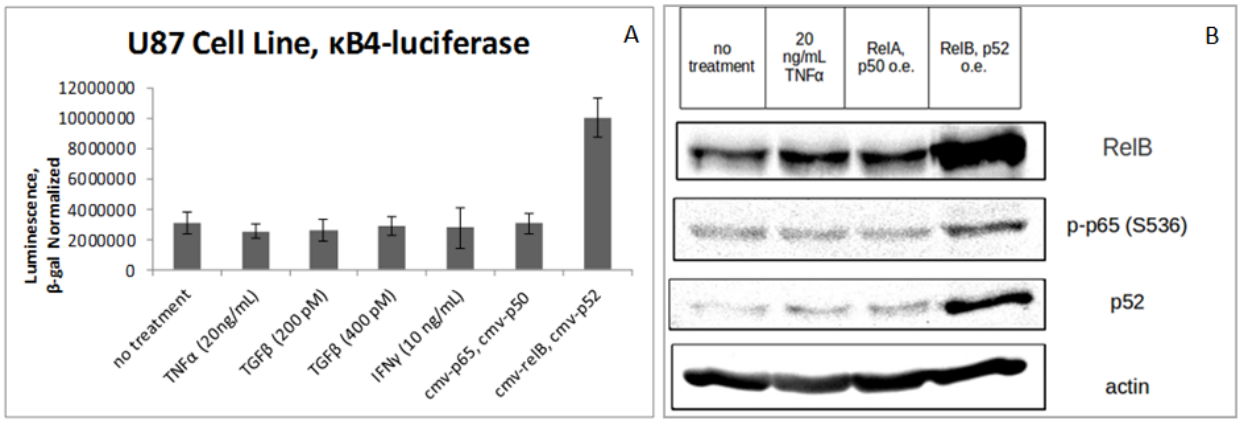

Fig. 3. A) Raw luminescence from luciferase expression directed from the κB4 promoter, normalizedto kinetic rate of β-gal expressed from cmv-lacZ plasmid transfected equally in all samples. TNFα, TGFβ, or IFNγ was added to designated samples 24 hours after tranfection, and all samples were analyzed 24 hours after that. Samples were performed in triplicate. Error bars indicate standard deviation. B) Western blot was performed from the same cell lysates used for the luciferase assay.

As seen in Figure 6, both the IDO1 promoter and that with deletion of the κB element were observed to have luminescence at or below that of the negative control.

A new plasmid construct was acquired [Lee and Sitcheran], designated here as pIDO1.2. U87 cells were transiently transfected with variations of this construct or the previously used κB4-luc reporter plasmid. As seen in Figure 7A, the κB4-luc reporter plasmid yielded results as seen previously with the induction of luciferase after overexpression of canonincal NF-κB proteins p65 and p50, but not noncanonical RelB and p50. In Figure 7B, all samples with the new construct had luminescence below that of the negative control.

The pIDO1-luc plasmid was again reconstructed [Lee and Sitcheran], designated here as pIDO1.3. U87 cells were transiently transfected with the new construct, along with the construct with the κB binding element removed and a negative control plasmid. As seen in Figure 8, treatment with IFNγ induced expression of luciferase in both the pIDO1.3-luc and pIDO1.3-$\Delta\kappa$B-luc plasmids relative to the untreated samples.

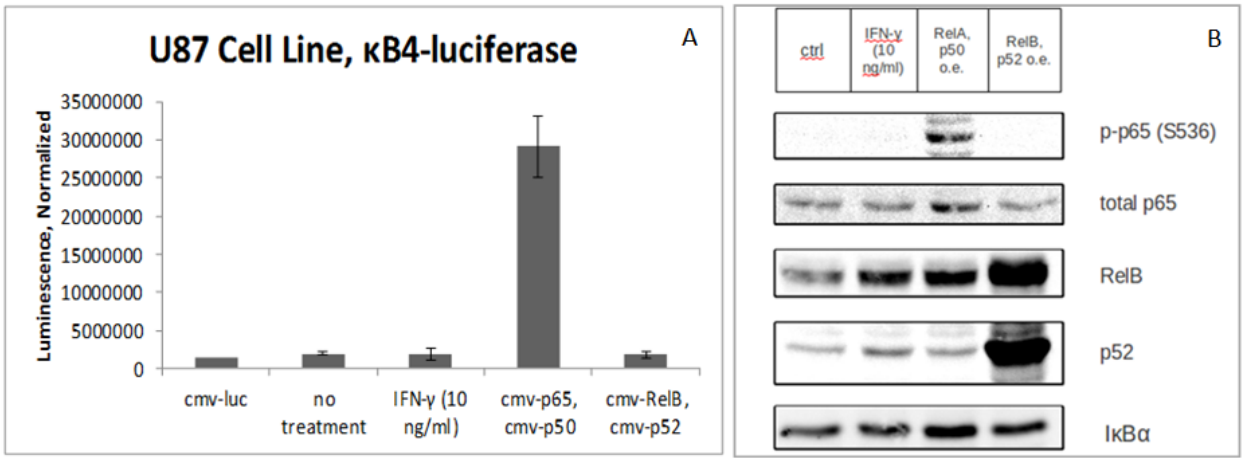

Fig. 4. A) Luminescence from luciferase expression directed from the κB4 promoter with data normalized to kinetic rate of β-gal expressed from cmv-lacZ plasmid transfected equally in all samples. IFNγ was added to the sample designated 24 hours after tranfection, and all samples were analyzed 24 hours after that. Samples were performed in triplicate. Error bars indicate standard deviation. B) Western blot was performed from the same cell lysates used for the luciferase assay.

Previous work has shown that cotreatment of IFNγ and TNFα increases transcription from the IDO1 promoter even further than IFNγ alone [Lee and Sitcheran, unpublished data]. However, these results were not reproduced when the experiment was repeated[Figure 9A]. Furthermore, IFN-γ did not show an increase in IDO-1 at the protein level, though did show a marked decrease in p65 phosphorylation at serine 536 - an indicator of transcriptional activity[Figure 9B].

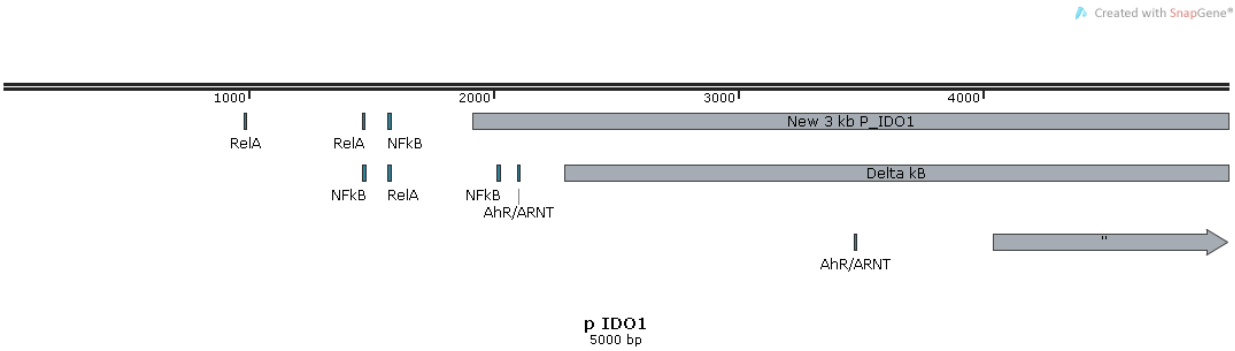

Fig. 5. Map of the IDO-1 promoter the regions used for luciferase reporter assays.

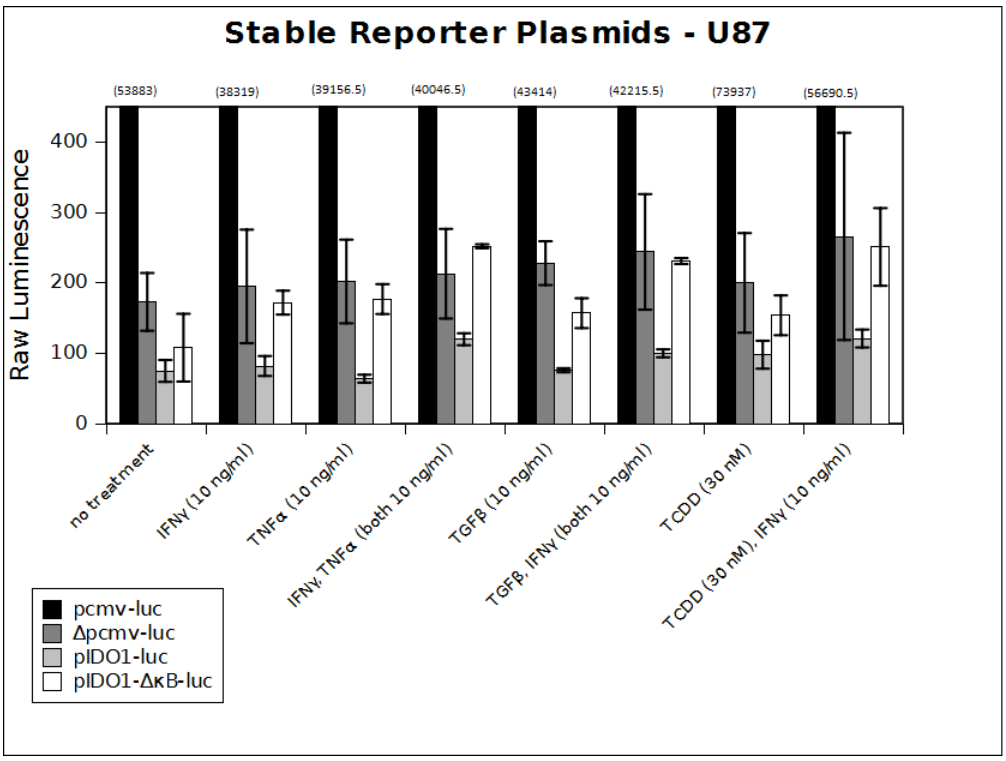

Fig. 6. Raw luminescence from luciferase expression directed from U87 cells stably tranfected with luciferase along with indicated promoters. TNFα, TGFβ, IFNγ and TCDD were added to designated samples 24 hours plating, and all samples were analyzed 24 hours after that. Samples were performed in triplicate. Error bars indicate standard deviation.

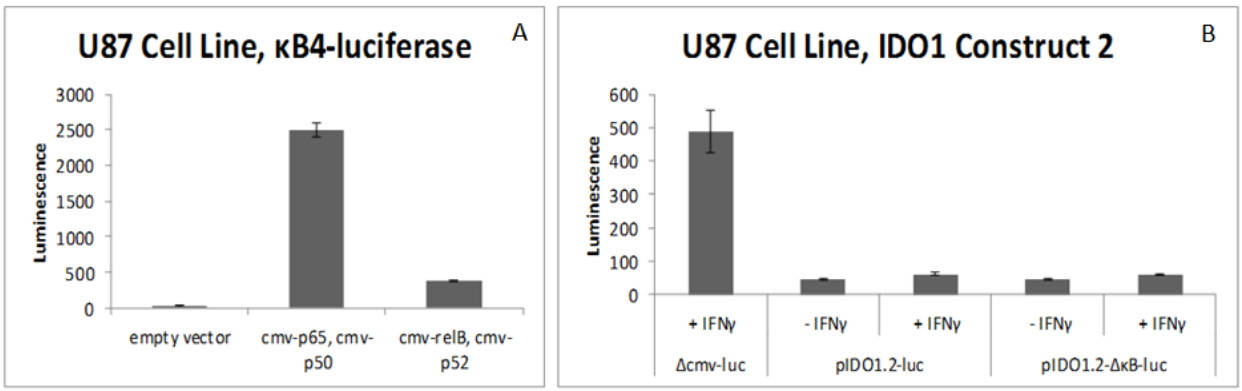

Fig. 7. Raw luminescence from luciferase expression directed from either the κB4 (A) or pIDO1.2 (B) promoter. IFNγ was added to designated samples 24 hours after tranfection, and all samples were analyzed 24 hours after that. Samples were performed in triplicate. Error bars indicate standard deviation.

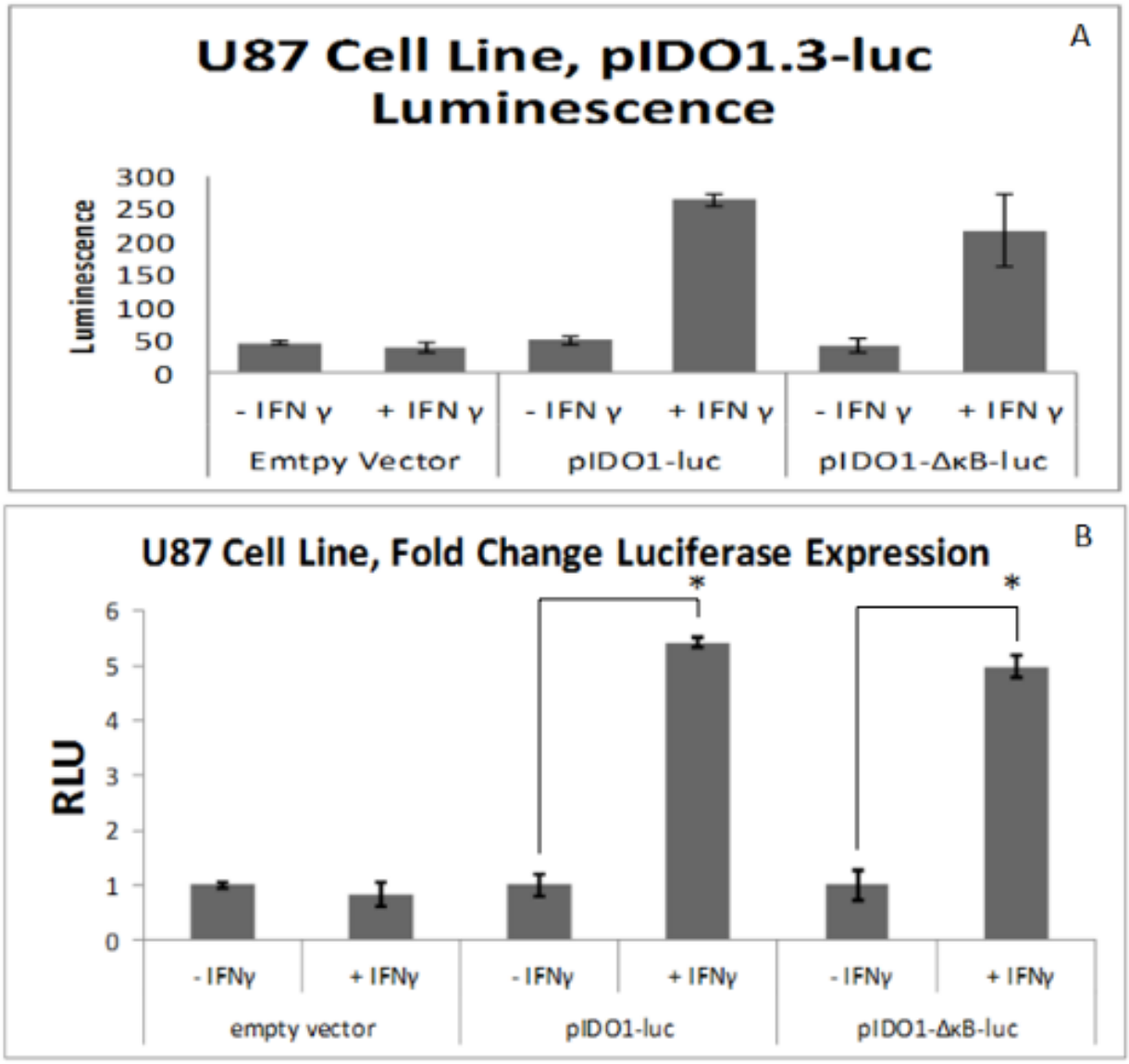

Fig. 8. Luminescence from luciferase expression directed from the pIDO1.3 promoter and its variant with deletion of the κB and AhR/ARNT binding elements. Raw luminescence is displayed in A, with data normalized to kinetic rate of β-gal expressed from cmv-lacZ plasmid transfected equally in all samples in B. IFNγ was added to designated samples 24 hours after tranfection, and all samples were analyzed 24 hours after that. Samples were performed in triplicate. * indicates $p < 0.01$, Student's 1-tailed t-test; error bars indicate SEM.

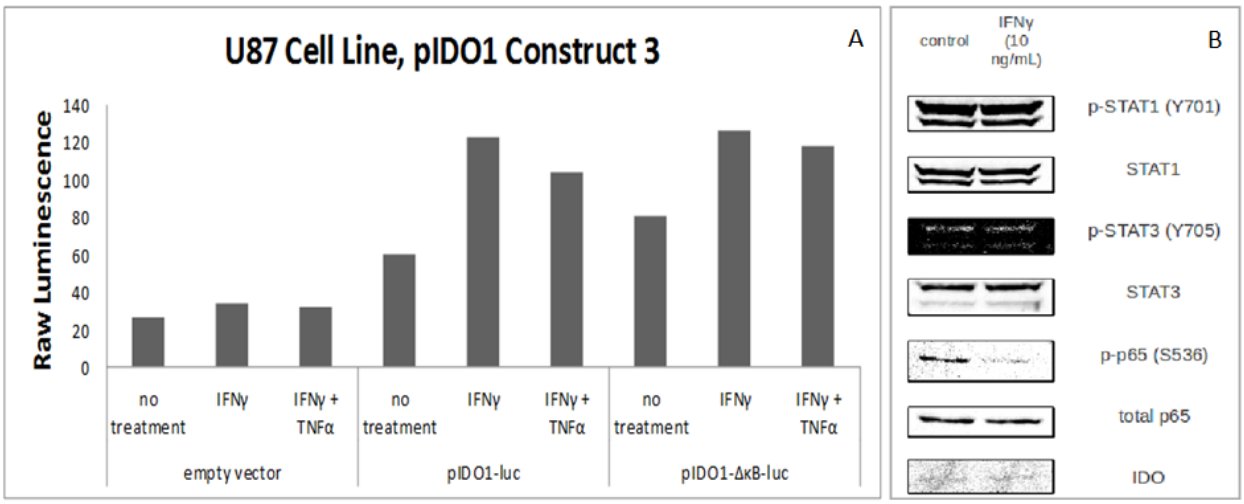

Fig. 9. A) Raw luminescence from luciferase expression directed from the pIDO1.3 promoter. IFNγ and TNFα was added to designated samples 24 hours after tranfection, and all samples were analyzed 24 hours after that. B) Western blot of U87 cells 24 hours after treatment with IFN-γ compared to untreated control.

CHAPTER IV
DISCUSSION

The result of the luciferase assay shown in figure 3A was suprising due to the lack of luciferase expression with cmv-p65 and cmv-50 transfection, yet with robust signal after overexpression of the noncanonical NF-κB proteins RelB and p52. However, a subsequent western blot from the samples show higher p65 phosphorylation on serine 536 (an indicator of transcriptional activation) in the RelB, p52 overexpressed sample and not the designated p65 overexpressed sample[Figure 3B]. This suggests that the cmv-p65 plasmid was mistakenly transfected along with cmv-relB and cmv-p52 instead of the correct sample.

While IFN-γ was shown to increase transcription from the IDO-1 promoter, these observations were not consistent nor observed at the protein level[Figures 8, 9A and Figure 9B]. This suggests that any effect that IFN-γ has on IDO-1 transcription is weak in this context. The decrease in phosphorylated p65 (S536) after treatment with IFN-γ suggests that the canonical NF-κB pathway is not involved in downstream targets in this cell line. This is supported in Figure 3A, in which IFN-γ treatment did not increase transcription from the synthetic κB4 promoter that responds to the canonical NF-κB protein (p65, p50) binding. However, While the reporter assays use part of the promoter sequence of IDO1 found in the human genome, they excluded the first 1900 or so bases that proved difficult to clone [Lee and Sitcheran, unpublished] which includes other NF-κB binding sites [refer to Figure5] and is unlike the native sequence that is within a chromatin environment. Thus other regulatory factors may be present and relevant, but not possible to observe through these assays alone.

The luminescence varied extensively between experiments; in Figure 4A the luminescence from the κB4 promoter of cmv-p65, cmv-p50 transfected cells was many times higher than in Figure 7B, even though conditions were kept the same; this variation suggests that small,

unobserved variations between experiments had an effect on results. The lack of luminescence in samples transfected with cmv-luc plasmid suggests a problem in in the plasmid, not the transfection, as luminescence was observed in other samples as at the κB4 promoter with overexpression of p65 and p50 [Figure 4].

It is possible that tryptophan-2,3-dioxygenase (TDO) is more importance than IDO-1 in glioblastoma, as previously shown that inhibition of TDO in human glioblastoma cells, but not IDO1/2, leads to a large decrease in kynurenine production while also reducing tumor volume in mice [16].

Future investigation of other members of the canonical NF-κB pathway; IκKβ, p50, and IκBα, as well as members of the noncanonical NF-κB pathway RelB, p52, and upstream IKKα in response to IFN-γ may further aid in our understanding of IFN-γ signaling cascades in glioblastoma. Previous work has shown the necessity of the canonical NF-κB pathway for IFN-γ induced expression of *ip-10* [17], so it is interesting that here a decrease in p65 phosphorylation was observed. Neither STAT1 nor STAT3 were observed to change in phosphorylation status with IFN-γ treatment, both of which are activated through tyrosine phosphorylation by Janus kinase (JAK) after cytokines, such as IFN-γ, bind to membrane receptors [18]. Analysis of effects from IFN-γ treatment over different timepoints may show differences in gene targets at different times, and help elucidate the directionality of IFNγ signaling and IDO1 expression as mediated through signaling pathways such as NF-κB.

REFERENCES

[1] CBTRUS, "Statistical Report: Primary Brain and Central Nervous System Tumors Diagnosed in the United States," tech. rep., CBTRUS, 2012.

[2] Johnson, D.R. and O'Neill, B.P. , "Glioblastoma survival in the United States before and during the temozolomide era," *Springer*, vol. 107, pp. 359–364, 2012.

[3] R. Daneman and M. Rescigno, "The Gut Immune Barrier and the Blood-Brain Barrier: Are They So Different?," *Immunity*, vol. 31, pp. 722–735, 2009.

[4] C. Colton, "Heterogeneity of Microglial Activation in the Innate Immune Response in the Brain," *Journal of Neuroimmune Pharmacology*, vol. 4, pp. 399–418, 2009.

[5] A. Andaloussi and M. Lesniak, "An increase in CD4+CD25+FOXP3+ regulatory T cells in tumor-infiltrating lymphocytes of human glioblastoma multiforme," *Neuro-Oncology*, vol. 8, no. 3, pp. 234–243, July 2006.

[6] Baltimore, David, "Discovering NF-[kappa]B," in *NF-[kappa]B: a network hub of controlling immunity, inflammation, and cancer*, Cold Springs Harbor Laboratory Press, 2009.

[7] S. Vallabhapurapu and M. Karin, "Regulation and Function of NF-[kappa]B Transcription Factors in the Immune System," *Annual Review of Immunology*, vol. 27, no. 1, pp. 693–733, 2009. PMID: 19302050.

[8] A. Schottelius and H. Dinter, "Cytokines, NF-[kappa]B, Microenvironment, Intestinal Inflammation and Cancer," in *The Link Between Inflammation and Cancer : Wounds that do not heal*, Springer Science, 2006.

[9] A. Betof and M. Dewhirst, "Establishing the tumor microenvironment," in *Tumor Microenvironment*, John Wiley & Sons, 2011.

[10] Oeckinghaus, A. and Ghosh, S. , "The NF-[kappa]B family of transcription factors and its regulation," in *NF-[kappa]B: a network hub of controlling immunity, inflammation, and cancer*, Cold Springs Harbor Laboratory Press, 2009.

[11] Wertz, Ingrid and Dixit, Vishva, "Signaling to NF-[kappa]B: Regulation by Ubiquinitination," in *NF-[kappa]B: a network hub of controlling immunity, inflammation, and cancer*, Cold Springs Harbor Laboratory Press, 2010.

[12] S. Lob, A. Konigsrainer, H.-G. Rammensee, G. Opelz, and P. Terness, "Inhibitors of indoleamine-2,3-dioxygenase for cancer therapy: can we see the wood for the trees?," *Nature Review Cancer*, vol. 9, pp. 445–452, 2009.

[13] M. Pallotta, C. Orabona, C. Volpi, C. Vacca, M. Belladonna, R. Bianchi, G. Servillo, M. Brunacci C, Calvitti, S. Bicciato, E. Mazza, L. Boon, F. M. Grassi, F and, F. Fallarino, P. Puccetti, and G. U, "Indoleamine 2,3-dioxygenase is a signaling protein in long-term tolerance by dendritic cells," *Nature Immunology*, vol. 12, pp. 870–880, 2011.

[14] O. Manches, M. Fernandez, J. Plumas, L. Chapergot, and N. Bhardwaj, "Activation of the noncanonical NF-[kappa]B pathway by HIV controls a dendritic cell immunoregulatory phenotype," *Proceedings of the National Academy of Sciences*, vol. 109, pp. 14122–14127, 2012.

[15] Eustice, D.C., Feldman, P.A., Colberg-Poley A.M., Buckery R.M., and Neubauer, R.H, "A sensitive method for the detection of beta-galactosidase in transfected mammalian cells.," *Biotechniques*, vol. 11, pp. 739–40, 742–3, 1991.

[16] C. Opitz, U. Litzenburger, F. Sahm, M. Ott, I. Tritschler, S. Trump, T. Schumacher, L. Jestaedt, D. Schrenk, M. Weller, M. Jugold, G. Guillemin, C. Miller, C. Lutz,

B. Radlwimmer, I. Lehmann, A. von Deimling, W. Wick, and M. Platten, "An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor," *Nature*, vol. 478, pp. 187–203, 2011.

[17] Shutz, D and Sandhya Rani, MR and Fuller, JD and Ransohoff, RM and Stark, GR, "Roles of IKK-[beta], IRF1, and p65 in the Activation of Chemokine Genes by Interferon-[gamma]," *Journal of Interferon & Cytokine Research*, vol. 29, pp. 817–824, 2009.

[18] Aaronson, DS and Horvath, CM, "A Road Map for Those Who Don't Know JAK-STAT," *Science*, vol. 296, pp. 1653–1655, 2002.