

**MECHANISMS OF SELECTIVE IMMUNOSUPPRESSIVE EFFECTS OF  
INDOLEAMINE 2, 3-DIOXYGENASE AND BORRELIDIN**

**by**

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## **Abstract**

Indoleamine 2, 3-dioxygenase (IDO), a tryptophan degrading enzyme, is a potent immunomodulatory factor that has been considered as a promising candidate in down-regulating alloimmune responses. IDO expression generates a tryptophan-deficient environment that selectively induces apoptosis in immune cells but not in primary skin cells. However, the mechanism(s) underlying these selective effects of IDO is not elucidated. In this doctoral research project, we hypothesize that different sensitivity of immune cells versus skin cells to IDO-induced tryptophan-deficient environment is due to the differential activity of general control non-derepressible-2 (GCN2) kinase stress-responsive pathway and its inhibitor, protein IMPACT homolog, in these cells. Considering the selective effect of tryptophan-deficiency on immune cells, we also proposed that borrelidin, a small molecule agent that mimics the effects of amino acid deprivation, may provide potential therapy for acute lymphoblastic leukemia (ALL) via its selective apoptotic effects. To this end, we investigated whether borrelidin selectively inhibits the proliferation of malignant ALL cell lines and studied the mechanism by which this drug acts.

Three specific objectives were accomplished in this study. We first showed that in the presence of IDO, higher activation of GCN2 in immune cells leads to apoptosis and is due to the very low or undetectable expression of IMPACT in these cells. Our gain- and loss-of-function findings suggest that high expression of IMPACT in non-immune cells acts as a protective mechanism against IDO-induced apoptosis. In the next phase, in order to stably express IDO in grafts to prolong their survival, a pure population of IDO-expressing bystander fibroblasts was generated using two simple yet effective lentiviral-based approaches. Finally, borrelidin was used as a novel strategy in treating ALL. We showed that borrelidin treatment potently and

selectively inhibits proliferation, induces apoptosis and mediates G<sub>1</sub> arrest in ALL cell lines and that borrelidin treatment in these cells is correlated with activation of the GCN2 pathway.

The findings presented in this thesis collectively demonstrate the mechanism of IDO action and pave the way towards application of IDO as an immunosuppressive factor in development of long-lasting non-rejectable allografts. Our findings also reveal the potential application of borrelidin in treating ALL.

## Preface

The work presented in this thesis has already been published or submitted for publication as co-authored works. This is to confirm that Darya Habibi is the first author of all publications included in this thesis as shown below:

Chapter 2: Habibi D, Jalili RB, Forouzandeh F, Ong CJ, Ghahary A. High Expression of IMPACT Protein Promotes Resistance to Indoleamine 2, 3 Dioxygenase-Induced Cell Death. J Cell Physiol 225: 196 -205, 2010

Chapter 3: Rezakhanlou AM\*, Habibi D\*, Lai A, Jalili RB, Ong CJ, Ghahary A. Highly Efficient Stable Expression of Indoleamine 2, 3 Dioxygenase Gene in Primary Fibroblasts. Biol Proced Online 12:107-112, 2010 \* Co-first Authors

Chapter 4: Habibi D, Ogloff N, Jalili RB, Yost A, Weng AP, Ghahary A, Ong CJ. Borrelidin, a Small Molecule Nitrile-Containing Macrolide Inhibitor of Threonyl-tRNA Synthetase, is a Potent Inducer of Apoptosis in Acute Lymphoblastic Leukemia. Invest New Drugs, DOI 10.1007/s10637-011-9700-y, 2011

Dr. Aziz Ghahary and Dr. Christopher Ong were the principal investigators of the research project. The main idea of using IDO as an immunosuppressive factor and studying its mechanism of effects was initially introduced by Dr. Ghahary. The idea of using borrelidin in mimicking the effects of amino acid deficiency and in treatment of acute lymphoblastic leukemia is credited to Dr. Ong. In addition, Dr. Ghahary and Dr. Ong supervised all experimental designs and analysis and critically reviewed all the manuscripts included in this thesis. The financial support for this thesis was provided through Dr. Ghahary and Dr. Ong's research grants. Darya Habibi had the prime role in the design, performing the research, data analysis, and preparation of the manuscript for all the work described in this thesis with the exception of:

Chapter 3: Darya Habibi and Alireza Moeen Rezakhanlou equally contributed to the data presented in this manuscript. Alireza Moeen Rezakhanlou performed the experiments in Fig. 3.2 shown in this manuscript.

Other co-authors were involved in providing ideas, assisting in experiments, and reviewing the manuscripts.

The work described in this thesis has been conducted with the approval of the University of British Columbia Biosafety and Ethics Committees under the certificate numbers B09-0298, and H05-70537.

## Table of Contents

Abstract.....	ii
Preface.....	iv
Table of Contents.....	vi
List of Figures.....	viii
Acknowledgements.....	x
Dedication.....	xi
CHAPTER 1. Introduction.....	1
1.1. Overview.....	1
1.2. Amino Acid Metabolism and Immune System.....	2
Immune System and Its Regulation .....	2
Amino Acid Catabolism as an Immunosuppressive Mechanism .....	3
IDO Structure, Expression and Regulation.....	5
Role of IDO in Pregnancy and Mechanisms of IDO-Mediated Tolerance Induction .....	7
The Immunomodulatory Role of IDO .....	8
Selective Apoptotic Effects of IDO .....	9
IDO and Tumor Immunity.....	11
Effects of IDO on Tumor Cells.....	12
IDO Inhibitors.....	13
GCN2 Pathway as a Potential Mechanism for IDO Function .....	14
Protein IMPACT Homolog, a GCN2 Kinase Inhibitor .....	16
Applications of IDO in Preventing Allograft Rejection .....	17
Strategies for Induction of IDO Expression in Primary Cells .....	17
1.3. Amino Acid Deprivation and Treatment of Cancers of Immune System.....	19
Acute Lymphoblastic Leukemia .....	19
Amino Acid Deprivation in Treatment of ALL.....	20
Borrelidin Structure and Function .....	21
1.4. Hypotheses and Specific Objectives.....	22

CHAPTER 2 .....	29
2.1. Introduction.....	29
2.2. Materials and Methods.....	31
2.3. Results.....	40
2.4. Discussion .....	54
CHAPTER 3 .....	59
3.1. Introduction.....	59
3.2. Materials and Methods.....	61
3.3. Results and Discussion .....	65
CHAPTER 4 .....	74
4.1. Introduction.....	74
4.2. Materials and Methods.....	77
4.3. Results.....	80
4.4. Discussion .....	89
CHAPTER 5. Conclusion and Suggestions for Future Work.....	92
5.1. General Discussion and Conclusion .....	92
5.2. Suggestions for Future Work .....	101
Bibliography .....	103

## List of Figures

Figure 1.1. Mechanisms of IDO-induced immunosuppression. ....	8
Figure 1.2. Mechanism of IDO-induced GCN2 kinase pathway activation. ....	15
Figure 1.3. The proposed mechanism for selective suppressive and apoptotic effects of IDO on immune cells versus primary skin cells. ....	24
Figure 2.1. IDO expression in fibroblasts in response to IFN- $\gamma$ treatment. ....	41
Figure 2.2. Selective GCN2 kinase pathway activation in skin cells versus Jurkat cells co-cultured with IDO-expressing fibroblasts. ....	43
Figure 2.3. Differential expression pattern of IMPACT protein in skin cells versus Jurkat cells exposed to IDO-expressing fibroblasts. ....	45
Figure 2.4. Lower expression of IMPACT protein in primary human T cells compared to that of primary human fibroblasts. ....	46
Figure 2.5. Increased IMPACT expression level in fibroblasts cultured in tryptophan-deficient media. ....	47
Figure 2.6. Effects of IDO expression on the level of CHOP expression and proliferation rates of IMPACT knocked-down fibroblasts. ....	50
Figure 2.7. Effects of IDO expression on the death rates of primary IMPACT knocked fibroblasts. ....	52
Figure 2.8. Effects of tryptophan deficiency or IDO exposure on the proliferation of IMPACT-expressing Jurkat cells. ....	54
Figure 3.1. Construction of a lentiviral-based vector for delivering the IDO gene. ....	67
Figure 3.2. Transduction of mouse fibroblasts with IDO/pLC-E -expressing lentiviral vector. ..	71



Figure 3.3. Transduction of primary mouse fibroblasts with IDO/FUGWBW-expressing lentiviral vector. ....	72
Figure 4.1. Selective effects of borrelidin treatment on the proliferation and growth of ALL cell lines. ....	81
Figure 4.2. Increased apoptosis in borrelidin-treated Jurkat cells and CEM cells.....	83
Figure 4.3. Induced G1 arrest in borrelidin-treated Jurkat and CEM cells.....	85
Figure 4.4. Activation of the GCN2 kinase pathway and CHOP induction in borrelidin-treated Jurkat cells. ....	87
Figure 4.5. Activation of GCN2 kinase pathway and CHOP induction in borrelidin-treated CEM cells. ....	88

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## **Dedication**

This work is dedicated to my parents, for their love.

## **CHAPTER 1. Introduction**

### **1.1. Overview**

Rejection of allogeneic grafted tissue due to the host immunological response is a major barrier in all types of transplantation (1). Many types of injuries occur in graft and host tissues post transplantation that can induce inflammatory responses such as cytokine production, infiltration of leukocytes and lymphocytes, and altered gene expression in the stressed tissues (2). Different cell types participate in the process of graft rejection; however, only T lymphocytes appear to be absolutely required for acute rejection (occurring within the first few weeks post transplantation) (3, 4). Therefore, the most effective immunosuppressive drugs are those that inhibit T cell proliferation such as cyclosporine and tacrolimus. The success of transplantation mostly depends upon long-term use of immunosuppressive drugs that have many side effects such as lack of specificity on T cell proliferation and systemic immunosuppressive effects on the patient's immune system (1). Therefore, current research is focused on finding new strategies to induce host tolerance by mimicking the naturally occurring mechanisms of self-tolerance (5).

Our group has successfully used a novel approach whereby an immunosuppressive factor is locally expressed in the allograft, creating a microenvironment in which infiltrating immune cells are unable to proliferate and destroy the grafts (6-9). We have also shown that this immunosuppressive factor, that leads to degradation of an essential amino acid, has differential apoptotic effects on immune cells versus skin cells (10, 11). However, the mechanism of this selective apoptotic effects has not been elucidated. Thus, in my doctoral research project we

aimed to study the downstream mechanism(s) of this immunosuppressive factor and generate a gene delivery system in order to achieve a high stable expression of this factor in primary bystander fibroblasts. Moreover, we also aimed to find a novel small molecule drug that can mimic the effects of this immunosuppressive factor and study its potential application in treatment of the diseases of the immune system, including acute lymphoblastic leukemia (ALL).

## **1.2. Amino Acid Metabolism and Immune System**

### **Immune System and Its Regulation**

Vertebrates have a complex immune system due to the evolution of ancient immunity as well as the presence of the lymphoid structures. Invertebrate immune mediators such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) as well as its receptor are still well conserved in higher vertebrates (12). Innate immunity is the first line of defense against common pathogens, comprises the mechanisms that defend the host in a non-specific manner, and is thought to have developed around 500 million years ago. The strategy of the innate immune system is to identify conserved patterns of pathogens using its generic receptors and initiate an inflammatory response against the invading pathogen (13-16). However, many pathogens are not recognized by the innate immunity. As a result, adaptive immunity evolved in order to provide an increased level of protection (17). The adaptive immune system is comprised of highly specialized mechanisms to recognize antigens and includes a "memory" in order to make more efficient future responses against a specific antigen. Adaptive immunity, however, has not replaced innate immunity. In fact, innate immunity has a key role in initiating and directing adaptive immune responses (13, 16).

An important caveat in the adaptive immune system is the possibility of autoimmunity: considering self-structures as pathogens, which can eventually lead to destroying the organism. As such, immune regulation has evolved in higher organisms in order to control the response of the adaptive immune system to self-antigens as well as to reduce exaggerated inflammation and innate immunity (18, 19). Different specialized cell types are responsible for immunoregulation which ultimately lead to immune tolerance. These cells are supported by anti-inflammatory cytokines including transforming growth factor  $\beta$  (TGF- $\beta$ ) and interleukin-10 (IL-10). Although the immunoregulatory pathway has the ability to change over evolutionary time due to a high level of plasticity; in mammals, immune tolerance still relies on mechanisms based on ancestral metabolic pathways (19, 20).

### **Amino Acid Catabolism as an Immunosuppressive Mechanism**

The balance between responsiveness to the infectious agents and tolerance to non-harmful antigens is constantly adjusted by the immune system. Mammalian hosts take advantage of amino acid catabolism in order to control pathogen invasion and also regulate their own immune response (21). Tryptophan catabolism along with the kynurenine metabolic pathway has been considered as one of the mechanisms that is involved in mediating tolerance (22). Tryptophan is the least abundant of the essential dietary amino acids in mammals (23, 24). A small amount of the dietary tryptophan is used to synthesize the neurotransmitter serotonin as well as melatonin. The majority of tryptophan is metabolized through the kynurenine pathway (24), leading to the biosynthesis of nicotinamide adenine dinucleotide (NAD). The first and rate-limiting step of the kynurenine metabolic pathway is the conversion of tryptophan to kynurenine that occurs during the oxidative cleavage of the pyrrole ring of tryptophan. Two enzymes

catalyze this step, hepatic enzyme tryptophan 2, 3-dioxygenase (TDO) (25), and indoleamine 2, 3-dioxygenase (IDO) an enzyme that is inducible in a series of tissues and cell types (23). Recently, a novel enzyme with homology to IDO and the same enzymatic activity has been discovered and termed indoleamine 2, 3-dioxygenase-2 protein (IDO2) (26). At the amino acid level, IDO2 shares 43% identity to IDO1 and is encoded by a gene adjacent to IDO1 on chromosome 8 both in mouse and human (26). In mice, IDO1 and IDO2 are expressed in different cell types which suggests that they are non-redundant genes (27). Tryptophan breakdown has been shown to be necessary in maintenance of immune tolerance (28). Different findings on mammalian pregnancy (29-31), chronic infections (32-34), tumor resistance (35, 36), and autoimmune diseases (37) support the importance of the immunoregulatory function of IDO.

The other pathway that is considered to play a role in mediating tolerance is the arginine catabolic pathway. Arginine is a semi-essential dibasic amino acid naturally ingested in our diet (38, 39). It is suggested that arginine is one of the amino acids that is essential for T cell activation and proliferation (40). Arginase 1 is an enzyme that metabolizes arginine to ornithine and urea (38). Arginase 1 is induced in myeloid cells by T helper 2 (Th2) cytokines and inflammatory agents (39, 41). Arginase-expressing myeloid cells deplete arginine from the surrounding environment which can in turn exert suppressive effects of T cell proliferation (40).

Therefore, by depriving the environment of the relevant amino acids, or by generating downstream metabolites, IDO and arginase control T cell activation and the immune system (21). It should be mentioned that IDO and arginase are not the only amino acids that can have regulatory effects of T cells. New evidence show that L-phenylalanine, L-cystine / cysteine, L-glutamine, and L-histidine may also be involved in immunoregulation (21).

## **IDO Structure, Expression and Regulation**

The strong immunomodulatory enzyme IDO (EC 1.13.11.42) was first discovered in rabbit intestine by Osamu Hayaishi in 1967 (42). Human IDO is a 45 kDa cytosolic monomeric hemoprotein of 403 aa long (43). The overall structure of IDO shows two distinct alpha-helical domains, one small and one large, with the heme prosthetic group between them (44).

Different cells and tissues express IDO in a constitutive or inducible manner. IDO expression is reported in mucosal surfaces of the gut, lung, lymph nodes, spleen, uterus during pregnancy, in epididymis and thymus during their normal function (45-47). IDO expression is inducible in different cell types, such as endothelial cells, fibroblasts, certain myeloid-lineage cells (monocyte-derived macrophages and dendritic cells) and some tumor-cell lines (48), upon exposure to IFN- $\gamma$  (46, 49-52). Post-translational modifications to IDO proteins are required for enzyme function; therefore, expression of IDO protein may not always correlate with IDO enzyme activity (53).

In mice and humans, the IDO protein (apoenzyme) is encoded by a single gene, named *Indo*, with 10 exons spanning ~ 15 kbp of DNA that is located on the short arm of chromosome 8 (8p12-8p11) (28, 54, 55). Inflammatory mediators, most prominently interferon- $\gamma$  (IFN- $\gamma$ ) (56), tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), or toll-like receptor (TLR) ligation (e.g. through lipopolysaccharide) induce IDO gene transcription (28, 57, 58). IDO gene promoters contain several sequence elements that respond to type I (IFN- $\alpha/\beta$ ) and, type II (IFN- $\gamma$ ) interferons (46, 59-61). Within the immune system, certain types of antigen-presenting cells (APCs), such as dendritic cells (DCs), express IDO mainly in response to proinflammatory stimuli or when exposed to signals from activated T cells (62). IDO expression is also reported to be induced by the anti-inflammatory cytokine interleukin 10 (IL-10) (63). Intracellular signaling that follows



the ligation of IDO inducers and results in the expression of IDO occurs along the JAK-STAT pathway and nuclear factor  $\kappa$ B (NF $\kappa$ B) (64-66). IDO can be expressed as an enzymatically inactive protein. Therefore, both transcriptional and posttranslational modifications are necessary for IDO competence (67). Data show that the control of IDO transcription might be a cell-type specific and complex phenomenon due to the differences in the other signaling pathways and cytokines that might regulate IDO expression in each specific cell type (68, 69).

The tumor suppressor gene *Bin1* (Bridging integrator 1), located on chromosome 2q14, encodes for a member of the BAR family of adapter proteins and has a role in controlling the expression of the *Indo* gene (70, 71). *Bin1* expression is attenuated in some human cancers such as prostate, colon, breast and lung cancers (72). Methylation of the *Bin1* gene promoter CpG islands results in *Bin1* inactivation and is associated with prostate and breast cancer, however no methylation is detected in *Bin1* CpG islands in normal tissues (73). It has been shown that attenuation of *Bin1* results in increased basal and IFN-induced activity of the *Indo* promoter (74). In *Bin1*<sup>-/-</sup> tumours, use of an IDO inhibitor (1-methyl-tryptophan, 1-MT) inhibits the growth of tumours in syngeneic hosts, which shows that induction of IDO as a result of *Bin1* loss is an important mediator of immune escape (75). Together, it is suggested that *Bin1* suppresses the development and/or progression of epithelial cancers of the lung, liver, colon, prostate, and breast.

A variety of physiopathological processes could be affected by IDO activity. A high level of tryptophan degradation is observed in diseases concomitant with cellular immune activation or dysfunction. Involvement of the kynurenine pathway has been shown in several neurodegenerative diseases (76). Neurological or psychiatric symptoms can occur due to free serum tryptophan deprivation and induction of neuroactive kynurenine metabolites in diseases

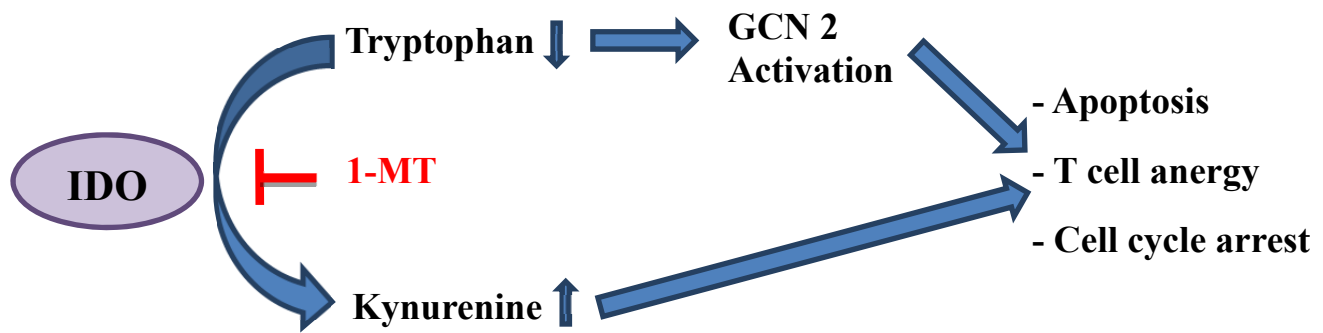
such as epilepsy, Parkinson's, Huntington's, and Alzheimer's disease, and in mental disorders such as depression and schizophrenia (77, 78).

### **Role of IDO in Pregnancy and Mechanisms of IDO-Mediated Tolerance Induction**

The very existence of mammals requires the presence of immunosuppressive processes that prevent semi-allogeneic fetal rejection in utero. This is considered one of nature's most formidable examples of tolerance induction. Munn and Mellor showed that placental cells synthesizing IDO are involved in suppressing maternal T cell responses against the fetus and therefore, at least in part, promote immune tolerance, resulting in immunologic acceptance of the fetus in a mouse model (31). Treatment of pregnant mice with an inhibitor of IDO, 1-MT, blocks tryptophan catabolism and results in the immune-mediated rejection of fetal allografts. These investigators further showed that peripheral tolerance is induced by IDO-mediated tryptophan catabolism (30, 31). There are two proposed mechanisms to explain the effects of IDO induction on T cells (Figure 1.1):

- 1) Toxic tryptophan metabolites generated by IDO, kynurenines, increase the susceptibility of T cells to Fas-independent apoptosis (79). Tryptophan-deprived conditions suppress T cell proliferation and make them susceptible to FasL-dependent apoptosis (76, 80, 81).

- 2) IDO activity creates a tryptophan-deficient microenvironment in which T cell cycle arrest occurs in a late G1 phase (31, 51, 82). Recently, it has been suggested that general control non-derepressible-2 (GCN2) stress kinase mediates this IDO-responsive signaling system in T cells (83).



**Figure 1.1. Mechanisms of IDO-induced immunosuppression.**

IDO is the first and rate-limiting enzyme in the metabolism of tryptophan. IDO enzymatic activity leads to a local decrease in the amount of tryptophan as well as local induction in the levels of kynurenine metabolites. Decreased levels of tryptophan results in the induction of uncharged transfer RNA (tRNA) in neighboring T-cells. This activates the stress responsive kinase GCN2 that in turn leads to apoptosis, cell cycle arrest and anergy induction in responding T cells. Kynurenine metabolites can also cause cell cycle arrest and apoptosis. 1-methyl tryptophan (1-MT) acts as a specific inhibitor of IDO function.

### **The Immunomodulatory Role of IDO**

Previous findings suggest that IDO was primarily used as an innate host defense mechanism against infectious pathogens, and gained its immunoregulatory role only later during evolution (43). IDO expression is also considered to be a counter-regulatory mechanism to suppress excessive immune activation (62). DCs are potent APCs that are able to increase (immunity) or inhibit (tolerance) T cell responses (84, 85). DCs present antigens from different cells and tissues. Depending on the circumstances, T cell responses occur that have the potential to destroy or protect infected or healthy cells (45). It has been shown that in the case of tolerance induction, IDO regulation in DCs plays a crucial role in changing the balance between tolerance and immunity (76). Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), known to be constitutively expressed on certain regulatory T cells (Tregs), has an important role in the induction of immune tolerance. Binding of costimulatory B7 molecules on DCs by CD28 results in stimulatory DCs, whereas CTLA-4 blocks the CD28-B7 costimulatory signaling and results in

tolerogenic DCs (86-88). CTLA-4 immunoglobulin (CTLA-4 Ig) causes IFN- $\gamma$  upregulation that mediates the induction of IDO expression in DCs in an autocrine or paracrine fashion and provides the conditions for long-term survival of allografts (89). When co-cultured with CTLA-4-expressing T cells, DCs that express B220, CD8 $\alpha$ , CD19, and relatively high levels of CD11c upregulate IDO expression (90, 91). Therefore, IDO induction through a CTLA-4 dependent mechanism has been implicated as a possible immunosuppressive effector mechanism of regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells (Tregs) (88). There are also reports indicating that naive T cells may convert to foxp3<sup>+</sup> Tregs in the presence of kynurenines (92). IDO-expressing DCs mediate peripheral T cell tolerance through multiple mechanisms such as induction of T cell anergy, Treg-cell activity and enhancement of activated T cell apoptosis.

The immunomodulatory effects of IDO may contribute to various conditions such as autoimmunity, HIV infection, tumor immunology, allergy and transplantation (62, 93-97). IDO might prevent exaggerated immune responses by reducing immune effector mechanisms or even causing a state of immunosuppression upon its sustained activity (23, 35). However, IDO knockout mice (ido<sup>-/-</sup> mice), or mice treated for more than 28 days with IDO inhibitors, do not develop autoimmune disorders (48, 98, 99). Moreover, DC and Treg development in ido<sup>-/-</sup> mice seem unaffected (100). As such, IDO does not seem to be required for maintaining self-tolerance. Therefore, IDO effects are selective and focused on the specific forms of acquired peripheral tolerance.

### **Selective Apoptotic Effects of IDO**

In a co-culture system, IDO expression by genetically modified fibroblasts has been shown to induce apoptosis in bystander immune cells. However, the levels of IDO-mediated

apoptosis in different types of immune cells have been shown to be different. The number of apoptotic bystander immune cells in the presence of IDO have been shown to be highest in Jurkat cells (82.7%), followed by THP-1 cells (35.8%), CD4<sup>+</sup> lymphocytes (30%), PBMCs (18%), and a B-cell enriched (16.4%) population (10). IDO-mediated apoptosis in immune cells have been confirmed by propidium iodide (PI) staining and TUNEL assay. Adding tryptophan or 1-MT partially reverses these apoptotic effects of IDO and significantly decreases the IDO-mediated apoptosis in immune cells (10). In addition, it has been shown that despite the suppressive effects of IDO on CD4<sup>+</sup> and CD8<sup>+</sup> T cells, CD4<sup>+</sup> T cells are more resistant to the apoptotic effects of IDO (101).

IDO expression has been shown to have selective apoptotic and suppressive effects on immune cells, but not on non-immune cells such as primary skin cells (10, 11, 102-104). When cultured in the presence of IDO, the number of apoptotic bystander primary skin cells has been shown to be significantly lower than immune cells. Only 3.5% of primary human fibroblasts and 2.6% keratinocytes were PI-positive in the presence of IDO. These results showed that primary skin cells and endothelial cells are resistant to the tryptophan-deprived environment caused by IDO (10). In addition, it has been shown that IDO expression in genetically modified fibroblasts and keratinocytes, infected with an IDO adenoviral vector, does not affect the proliferation rate of these cells (10).

This differential sensitivity to IDO expression is especially of interest in the field of transplantation immunology in order to develop non-rejectable allografts. It is suggested that in such grafts, local expression of IDO inhibits the infiltration of immune cells but has less apoptotic effects on the primary cells of the allograft.

## **IDO and Tumor Immunity**

Immune surveillance mechanisms usually eliminate developing tumor cells. However, some tumor cells escape these surveillance mechanisms and survive to form mature tumors. These tumors establish immune privilege long before any clinical manifestation. Solid tumors begin as clusters of cells at one location; therefore, local immune privilege at the cluster site is sufficient and these tumor cells do not need systemic tolerance. Local immune privilege will reduce systemic immunity to tumor antigens and weaken immunological barriers against tumor metastasis (105). It has been shown that APCs from the tumor microenvironment have a defect in T cell stimulation suggesting that these APCs are either immature or suppressive (106). These APCs then migrate to tumor draining lymph nodes and present the tumor antigens to T cells in a suppressive manner (107).

IDO expression is one of the several known suppressor mechanisms in tumor draining lymph nodes. It has been shown that a population of IDO-expressing DCs in tumor-draining lymph nodes acts as a potent immunosuppressive mechanism in tumor-bearing mice. These tolerogenic DCs are able to reduce the number of antigen-specific T cells or induce the number of Tregs (36, 108). IDO is also expressed by different primary human tumors and human tumor cell lines constitutively or upon exposure to IFN- $\gamma$  and IDO expression by solid tumors correlates with a poor clinical prognosis (46, 48, 74). IDO expression in biopsy samples taken from patients with colorectal cancer as well as patients with hepatocellular carcinoma revealed that increased IDO expression in these samples correlated with metastasis (109, 110). It is suggested that local IDO expression by malignant cells could have a role in local immunosuppression at the site of the tumor either by inhibiting effector T cells or by activating Tregs (62, 92). IDO inhibitors are therefore suggested to be used in combination with current chemotherapeutic agents to thwart the

immune-editing process. Currently, phase I clinical trials are underway to test the efficacy of the IDO inhibitor, 1-MT, as a cancer vaccine adjuvant therapy (53).

### **Effects of IDO on Tumor Cells**

Studies have shown that an IDO-induced tryptophan-deprived environment as well as the cytotoxic metabolites of tryptophan degradation can affect tumor cell proliferation. It has been shown that upon treatment of human cancer cells with IFN- $\gamma$ , IDO expression is increased which leads to tryptophan degradation and inhibition of proliferation (111). This suggests that IDO induction can act as a mechanism by which IFN- $\gamma$  inhibits the growth of cancer cells (111). In a study on patients with hepatocellular carcinoma, it has been also shown that IDO expression correlates positively with progression-free survival (112). In cancer patients, increased expression of IDO in endothelial cells of tumors correlated with long-term patient survival. Moreover, the number of IDO-positive microvessels and the number of proliferating tumor cells correlated inversely in primary and metastatic renal cell carcinoma (113).

Studies have shown that patients receiving recombinant IFN- $\gamma$  as well as chemotherapeutic drugs show significantly longer survival in comparison with patients receiving only chemotherapy (114, 115). Moreover, many adjuvants that are used for cancer therapy including Toll-like receptor 9 (TLR9) and CpG-rich oligonucleotides, potentially induce IDO expression in cells (116, 117). Therefore, IDO can act as a double-edged sword and has also the ability to inhibit tumor growth (113).

## IDO Inhibitors

In order to pharmacologically and pathophysiologically characterize IDO, and to validate IDO as a clinically useful target, development of potent and selective IDO inhibitors are required. Therefore, multiple studies have been undertaken and have provided diverse classes of IDO inhibitors (118, 119).

**Competitive Inhibitors:** The first weak and non-selective IDO inhibitor amino acid L-2,5 di-hydro-phenylalanine was discovered in 1978 (120). Cady and Sono showed that analogues of L-Trp with the replacement of the indolic nitrogen with N-methyl substitution at the indolic ring (1-MT) is able to inhibit the activity of IDO (121). This inhibitor has both L- and D-isomers. 1-MT has favourable characteristics including oral availability, low protein binding and low clearance (74). In 2005, Muller et al. screened available libraries for indole derivatives and identified methyl-thiohydantoin-tryptophan (MTH-Trp), as a potent IDO inhibitor (74). Using the same screening method, Muller et al. disclosed brassinin, a natural product, as a moderately active compound that competitively inhibits IDO (122). D-1-MT has been recently used in clinical trials as anticancer therapy (123).

**Non-competitive Inhibitors:**  $\beta$ -Carboline derivatives were the first non-competitive IDO inhibitors (124, 125). Other compounds of this class of non-competitive enzymatic IDO inhibitors are phenyl-imidazole and brassilexin (125).

**Other inhibitors:** Another natural and potent IDO inhibitor compound is a marine natural product named Annulin C (126). Exiguamine A is another natural product isolated from marine sponge with potent IDO inhibitory characteristics (127).

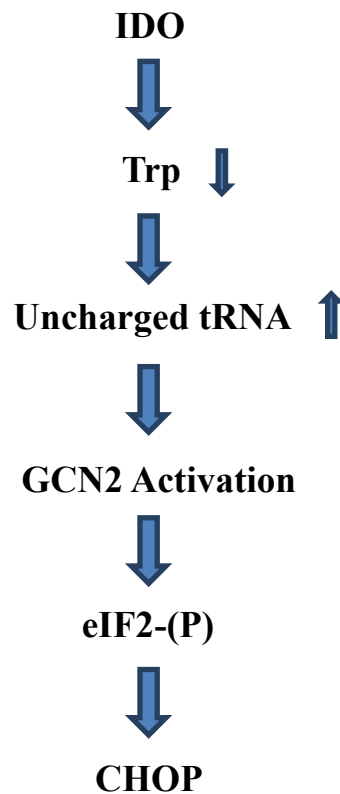
Recent screenings have led to identification of naphthoquinone derivatives, such as vitamin K3, as new IDO inhibitors. These compounds are shown to be uncompetitive inhibitors



of IDO (128). N-methyl-N'-9-phenanthrenyl-imidodicarbonimic diamide is also a novel non indolic structure with an unknown mode of inhibition against IDO and is known to be a potent IDO inhibitor (129).

### **GCN2 Pathway as a Potential Mechanism for IDO Function**

IDO depletes tryptophan in the microenvironment; therefore, it might affect pathways known to respond to amino acid metabolism. One of these amino acid sensitive pathways acts through eukaryotic initiation factor 2 $\alpha$  (eIF2 $\alpha$ ) kinase GCN2. Activation of the GCN2 kinase pathway can result in apoptosis, cell cycle arrest, differentiation, or compensatory adaption depending on the initiating stress and cell type. The GCN2 kinase pathway has been recently suggested as an intracellular signaling pathway in T cells that mediates key biological effects of IDO. It has been shown that IDO expression in APCs activates GCN2 kinase pathway in responding T cells. In addition, GCN2-knockout T cells are not susceptible to IDO suppressive effects (62, 83). Amino acid deprivation (tryptophan in case of IDO) can cause a rise in the levels of uncharged transfer RNA (tRNA) in cells (Figure 1.2). This activates the regulatory domain of the GCN2 kinase, triggers its kinase activity, and initiates downstream signaling resulting in eIF2 $\alpha$  phosphorylation (130). Phosphorylation of eIF2 $\alpha$  leads to repression of global protein synthesis but causes enhanced translation of selected messenger RNAs (mRNAs), such as activating transcription factor 4 (ATF4) mRNAs (131-134). Induced levels of ATF4 causes an induction in the levels of other transcriptional regulators such as ATF3 and CHOP (CCAAT/enhancer-binding protein homologous protein)/GADD153 (growth arrest and DNA-damage-inducible protein 153); as a result, a program of gene expression important for cellular remediation and apoptosis will be induced (135-137).



**Figure 1.2. Mechanism of IDO-induced GCN2 kinase pathway activation.**

IDO-induced tryptophan (Trp) deficiency causes a rise in the levels of uncharged tRNA, which activates the GCN2 kinase and initiates downstream signaling. This leads to phosphorylation of eIF2 $\alpha$  and induction in the levels of CHOP protein.

CHOP (also known as GADD 153) is a DNA damage-inducible nuclear leucine zipper protein that has role in apoptosis and differentiation. CHOP gene is a downstream target gene in the GCN2 kinase pathway, is a well-accepted marker for GCN2 activation (132). CHOP expression can be induced by different types of stress but its induction through amino acid deprivation has been shown to be particularly mediated by GCN2 (83, 132).

GCN2 knockout mice are viable, however, they show muscle loss, abnormal protein synthesis in liver, and increased morbidity when facing amino acid starvation (138). In mice with diets poor in essential amino acids, phosphorylation of eIF2 $\alpha$  is observed in the anterior piriform cortex. Although wild-type animals avoid such diets, GCN2 knockout mice fail to reject diets poor in essential amino acids and show increased morbidity due to failure to coordinate protein synthesis (139, 140).

### **Protein IMPACT Homolog, a GCN2 Kinase Inhibitor**

Human IMPACT homologue (imprinted and ancient) is an evolutionary conserved gene located on chromosome 18q11.2-12.1 and a recently described GCN2 kinase regulator (141). IMPACT mRNA has been shown to be highly expressed in tissues that are able to express IDO such as placenta, testis, and kidney, and has a very low expression in thymus (141). IMPACT protein is also expressed in brain (142). IMPACT abundance inversely correlates with the levels of phosphorylated eIF2 $\alpha$  in different areas of the brain (143). IMPACT protein binds to GCN1, an activator of GCN2, and inhibits GCN2 kinase activation (143). IMPACT protein overexpression in mouse embryonic fibroblasts inhibits GCN2 activation under amino acid starvation conditions, hence abolishing the expression of downstream target genes in GCN2 pathway including ATF4 and CHOP (142, 143).

## **Applications of IDO in Preventing Allograft Rejection**

Due to the potent immunoregulatory activity of IDO and its counter-regulatory effects to T cell stimulation, it has been suggested that IDO can actively down-regulate allogeneic immune responses in transplantation and promote tolerance at the graft site independently of immunosuppressive drugs. Ideally, IDO immunosuppressive effects act locally and in an antigen-specific manner at the graft site. IDO-mediated immune regulation appears to have considerable potential as an innovative immunotherapeutic approach to moderate clinically undesirable T cell responses (e.g induction of specific transplantation tolerance) (23). It has been shown that transplantation of IDO-overexpressing cells and organs leads to prolongation of the survival of pancreatic islet cells (7, 9, 144, 145), lung (146), cornea (147), skin substitutes (6, 8), cardiac and liver allografts (148, 149). Additionally, adenoviral-mediated IDO gene transfer into the donor heart attenuated acute allograft cardiac rejection (148).

In a rat model, IDO expression in primary human fibroblasts embedded within collagen protected xenogeneic fibroblasts and accelerated wound healing by promoting neovascularization (6). In addition, engraftment of IDO-expressing skin substitutes significantly suppressed T cell infiltration and improved healing progression in a rat model (8). It has also been shown that local expression of IDO by collagen-populated fibroblasts within which allogeneic islets were embedded, normalized the amount of blood glucose and prolonged the survival of the allograft in diabetic immunocompetent mice (9).

## **Strategies for Induction of IDO Expression in Primary Cells**

Different methods exist in order to induce IDO expression in primary cells. IFN- $\gamma$  and adenoviral vectors have previously been used in order to express IDO in target cells (7, 10, 11,

102, 145). However, both these methods have the ability to only transiently express IDO protein and can also result in inflammation or immunogenicity. Due to the potential application of IDO expression in different clinical settings, it is necessary to have a sustained efficient IDO activity in target cells and organs in order to be used in treatment strategies.

Gene manipulation of primary cells is known to be challenging due to unsatisfactory expression of the exogenous gene (150, 151). Lentiviral vectors have the ability to permanently and efficiently express different exogenous genes in target cells. Lentiviral vectors are a class of the Retroviridae family (150, 151). Retroviruses are RNA viruses that use a DNA intermediate for replication. These viruses are made of two single-stranded RNA molecules as well as replication enzymes present in a viral protein core. The viral envelope, consisting of cell membrane and viral-encoded envelope glycoproteins, surrounds this structure. During the replication cycle of a generic retrovirus, the viral envelope glycoprotein interacts with the cellular receptor and infects the target cell. This leads to the release of the virus core inside the cytoplasm of the target cell. In the cytoplasm, retroviral RNA will then be reverse transcribed into double-stranded DNA. This DNA is transported into the nucleus and gets permanently integrated inside the host genome, replicated during DNA synthesis and passed on to the daughter cells. Viral RNA then gets translocated into the cytoplasm and translates into viral enzymes and proteins. These proteins assemble with the viral RNA in order to generate new virion cores. These cores gain the envelope proteins during budding from the cell membrane. These progeny virions release from the host cell and are able to infect other cells. Retroviruses contain three genes including *gag*, *pol*, and *env*. *Gag* encodes the viral core proteins, while *pol* encodes for the viral replication enzymes and *env* encodes the viral envelope glycoprotein (150, 151).

Lentiviral vectors have the unique ability to transduce both dividing and non-dividing cells and are useful in order to permanently introduce exogenous genes of interest inside the target cells. Therefore, lentiviral vectors are important as gene delivery vehicles in gene therapy. These vectors also have the advantage of efficiently delivering genes of interest into hard-to-transfect cells such as primary cells. Due to their importance in gene delivery, multiple efforts have been made to generate efficient self-inactivating lentiviral vectors in order to inhibit the vector to one round of infection. As such, different generations of the lentiviral vectors have been engineered. Improvements that aim to delete some sequences minimize the chance of homologous recombination. These efforts include: deletion of the envelope gene and replacing it with VSVG envelope, replacing some LTR sequences with CMV promoter, deletions of genes encoding the accessory factors, separating the necessary genes on three or four different plasmids, and deletion in the 3' LTR of the transfer plasmid (151).

### **1.3. Amino Acid Deprivation and Treatment of Cancers of Immune System**

#### **Acute Lymphoblastic Leukemia**

Acute lymphoblastic leukemia (ALL) is a clonal proliferation and expansion of immature lymphoid cells and results in the accumulation of leukemic blasts in the bone marrow and different extramedullary sites (152-154). ALL affects both children and adults, and nearly 5000 ALL cases are annually diagnosed in the United States (155). ALL is the most common pediatric cancer, representing one-third of all childhood cancers worldwide. Few cases of ALL are associated with inherited genetic syndromes; however, the cause of ALL is still largely unknown (154). ALL clinical presentation is mostly nonspecific. ALL symptoms include easy bruising or

bleeding, dizziness, infections, fatigue, and lack of energy, weight loss, fever, joint pains, and night sweat can occur (152).

Significant improvements in early therapy for childhood ALL has led to an overall cure rate of about 80%. Contrary to the successes obtained with pediatric patients, treatment outcomes for adult patients has been far less rewarding, with only 40% of patients being long-term survivors (152, 153, 156). Adult patients with ALL have a very poor prognosis and few effective therapeutic options. The poor outcome in adult ALL is due to the increased rate of high-risk leukemia with greater drug resistance, unwillingness to accept temporary toxic effects, poorer tolerance of and compliance with treatment, and less effective treatment regimens, as compared with childhood ALL (157).

In spite of current treatment protocols which lead to improvements in therapy, resistance to treatments usually develop and complications in patients are frequent. Therefore, novel therapies that increase the efficacy of the treatments and that prolong ALL patient survival are needed.

### **Amino Acid Deprivation in Treatment of ALL**

ALL therapy is one of the most complex therapies in anticancer programs and includes using 6–12 drugs (152, 158). L-asparaginase (ASNase) has been the keystone of pediatric and adult ALL therapies since the 1970s (154). L-asparaginase catalyzes the hydrolysis of L-asparagine (Asn) to aspartic acid and ammonia and therefore depletes asparagine from the serum (158). Malignant T cells need high levels of nutrients in order to maintain their increased proliferation rate. Asn depletion leads to an increase in intracellular levels of uncharged tRNA, nutritional deprivation and inhibition of protein biosynthesis, which then leads to inhibition of

RNA and DNA synthesis, causing subsequent apoptotic cell death of the leukemic cells (155, 158). Despite the efficacy of L-ASNase in treatment of ALL, therapy is discontinued since allergic or anaphylactic reactions can develop after repeated administration of this bacterially derived enzyme. Adult patients have a higher chance of developing antibody positivity and clinical reaction to ASNase therapy as well as more toxic reactions to ASNase treatments (155). Generally, allergic reactions are the reason for the discontinuation of the drug (154). Moreover, the presence of asparaginase antibodies can cause drug inactivation. ASNase is administered in patients either intravenously or subcutaneously/ intramuscularly. It has been shown that GCN2 stress-responsive kinase is required to sense and respond to stress conditions generated by asparagine deprivation in mice treated with L-ASNase (159, 160).

As noted earlier, development of novel small molecule inhibitor drugs is vital to the therapeutic success especially in adult ALL. ASNase use in ALL therapy suggests the application of amino acid deprivation strategies in treatment of ALL. In addition, amino acid limitation strategies have been used in order to treat several tumors including hepatocellular carcinomas and melanomas (161, 162).

### **Borrelidin Structure and Function**

Borrelidin (C<sub>28</sub>H<sub>43</sub>NO<sub>6</sub>) is a novel nitrile-containing macrolide antibiotic isolated from the *Streptomyces* species. Borrelidin was first structurally determined in 1967 and then was subsequently refined using NMR analysis and X-ray crystallography (163, 164). Borrelidin was originally identified as an anti-bacterial, anti-viral, and anti-malarial agent. This drug also has strong antiangiogenic activity, inhibits the formation of new capillary tubes, and increases the collapse of formed capillary tubes through induction of apoptosis. It has been shown that



borrelidin-induced apoptosis in endothelial cells is through activation of caspase-3 and -8 (165). In animal models, borrelidin has also been shown to have anti-metastatic effects. More recently, borrelidin was found to also be a selective and potent inhibitor of bacterial and eukaryal threonyl-tRNA synthetase (ThrRS), an enzyme which functions to couple the essential amino acid threonine with its cognate tRNA. Aminoacyl-tRNAs are the products of the first step in protein biosynthesis, and borrelidin-mediated inhibition of aminoacyl-tRNA synthesis, causes an induction in the cytoplasmic levels of uncharged tRNA and eventually leads to inhibition of protein synthesis (165-167).

#### **1.4. Hypotheses and Specific Objectives**

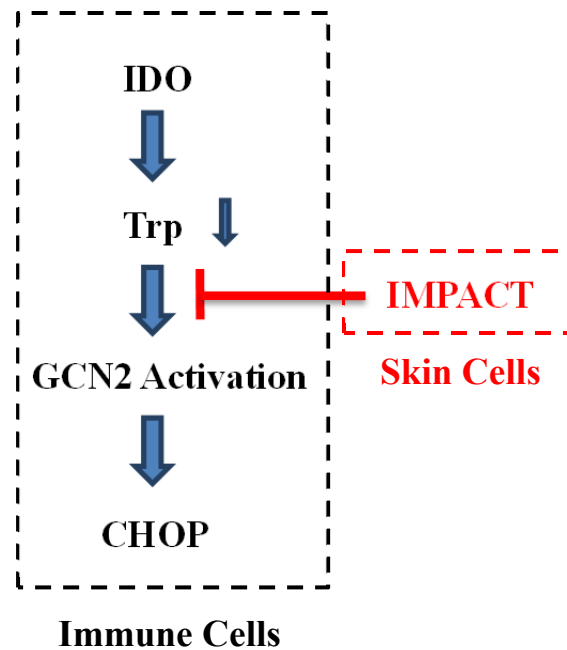
The present thesis was evolved from our previous studies which showed that IDO expression in dermal fibroblasts causes a substantial suppression in proliferation of bystander immune cells (10). We also showed that IDO selectively induces apoptosis in immune cells and that primary skin cells are resistant to the IDO-mediated tryptophan-deficient environment (11). However, the mechanism(s) of the selective effects of IDO on immune cells was not elucidated.

Our research group also used the local immunosuppressive effects of IDO expression to successfully develop a non-rejectable skin substitute as well as a non-rejectable islet allograft. We showed that local IDO expression in dermal fibroblasts, transduced with an IDO-expressing adenoviral vector, of skin substitute or in the bystander fibroblasts embedding the islet graft, suppresses the infiltrated T cells; this prevents the recipient's immune responses to the graft without affecting the function of the graft or the general immune system of the graft recipient (6-9). However, a critical aspect of an immunological intervention using IDO is the requirement to

achieve effective long-term expression of this gene. Previously, our research group has used IFN- $\gamma$  or adenoviral vectors in order to induce IDO expression (7, 10, 11, 104, 145). Nevertheless, the immunogenicity and transient gene expression of adenoviral vectors may hinder its clinical use in transplantation. Likewise, IFN- $\gamma$ -induced IDO expression is transient and IFN- $\gamma$  is known to be a proinflammatory agent which limits its clinical application. Therefore, it is necessary to sustain the efficacy of IDO activity in the local environment of the transplanted organ. In order to study the possible mechanism(s) that can explain the selective IDO-induced apoptosis in immune cells and in order to develop a novel method to prolong high expression of functional IDO protein in dermal fibroblasts to be used in future transplant models, we tested Hypothesis #1 and the specific objectives described below.

## Hypothesis 1

We hypothesize that different sensitivity of immune cells versus skin cells to IDO-induced tryptophan-deficient environment is due to the differential activity of the GCN2 kinase stress-responsive pathway and its inhibitor, the IMPACT protein in these cells (Figure 1.3).



**Figure 1.3. The proposed mechanism for selective suppressive and apoptotic effects of IDO on immune cells versus primary skin cells.**

We propose that high expression of protein IMPACT homologue in skin cells inhibits the IDO-induced GCN2 kinase activation which results in resistance of these cells to the effects of IDO. On the other hand, we suggest that lack or low levels of IMPACT expression in immune cells, results in IDO-induced activation of GCN2 kinase pathway and induction of apoptosis in these cells.

### **Objective 1.1**

To address our hypothesis, in Chapter 2, we studied the activation of the GCN2 pathway and the expression level of IMPACT protein in IDO-sensitive versus IDO-resistant cells. We also aimed to study the regulation of the GCN2 kinase pathway and IMPACT expression in these cells in the presence or absence of IDO. IDO-expressing or control human fibroblasts were co-cultured with bystander Jurkat cells, human T-cells, fibroblasts or keratinocytes. Activation of GCN2 kinase pathway and levels of IMPACT expression in bystander cells were studied at both the mRNA and protein level by RT-PCR and western blot analysis, respectively. Our findings revealed that activation of GCN2 pathway was significantly higher in immune cells exposed to an IDO-expressing environment compared to that of skin cells. In contrast, at both the mRNA and protein levels, IMPACT was highly and constitutively expressed in skin cells while its expression was very low in stimulated T-cells and undetectable in Jurkat cells.

### **Objective 1.2**

In order to examine the effects of IDO expression on IMPACT downregulated fibroblasts and IMPACT over-expressing Jurkat cells, we performed a series of loss of function and gain of function experiments in primary fibroblasts and Jurkat cells. Using siRNA against IMPACT, we downregulated the expression of IMPACT protein in fibroblasts. IMPACT-expressing lentiviral vectors were constructed and used to stably transduce Jurkat cells. These cells were then co-cultured with IDO-expressing or control human fibroblasts. Methyl thiazolyl tetrazolium (MTT), viability, and tritiated thymidine incorporation assays were performed. Our result (described in Chapter 2), further confirmed that IDO expression causes significant suppressive as well as apoptotic effects on IMPACT downregulated fibroblasts. Proliferation of IMPACT-expressing Jurkat cells was significantly rescued in a tryptophan-deficient environment.

### **Objective 1.3**

After demonstrating the mechanism of selective apoptotic effects of IDO on immune cells and its effects on immunosuppression, there was a need for a stable source of IDO expression in primary cells in order to overcome the current hurdles and to be used in our future transplant models. To address this, we aimed to perform lentiviral-mediated expression of IDO in primary fibroblasts in order to test its efficacy as an efficient delivery for effective stable expression of the IDO gene. For this, we used two approaches in order to construct two different IDO-expressing lentiviral vectors that either express mCherry as a fluorescence marker or blasticidin S resistance gene as a selection marker. The rationale for generating two different types of vectors is that in our transplant settings, based on the study type, we either need to be able to detect and track the transgene expression or have a very pure population of cells in a short period of time. Our results (described in Chapter 3), show that polybrene was able to increase the transduction efficiency of IDO-expressing lentiviral vector containing mCherry 10-fold. Moreover, using fluorescence activated cell sorting (FACS), we were able to obtain a greater than 95% pure population of IDO-expressing cells. In addition, using blasticidin S-containing lentiviral vector as a simple and yet effective strategy helped us to obtain a pure population of IDO-expressing primary fibroblasts without the need to use FACS.

Use of IDO and amino acid deprivation strategies in treatment of immune diseases was raised from the observation that during pregnancy, local IDO expression by trophoblastic cells of placenta prevents rejection of the allogeneic fetus. Moreover, as noted earlier, ASNase, which also leads to amino acid deprivation, has been used in treatment of ALL for the last few decades which suggests that amino acid limitation strategies can be used in ALL treatment. IDO and

ASNase have been shown to mediate their effects through induction in the levels of uncharged-tRNAs and activation of the GCN2 kinase stress-responsive pathway. Although IDO is a potent local immunosuppressive factor, it needs to be expressed intracellularly in order to function and mediate its effects. On the other hand, allergic hypersensitivity reactions can occur in patients after repeated administrations of ASNase which lead to discontinuation of this enzyme. Selective suppressive as well as apoptotic effects of amino acid deficiency on immune cells open up new avenues for treatment of diseases of the immune system including ALL. As such, we propose that borrelidin – a small molecule inhibitor of threonyl-tRNA synthetase that mimics the effects of amino acid deprivation - may provide potential therapy for ALL via its selective apoptotic effects. Therefore, to test the efficacy of borrelidin in suppressing the proliferation of malignant ALL cell lines, to study its mechanism of effects, and to find potential novel therapeutic options in treating ALL, we tested Hypothesis #2 and the specific objectives stated below.

## **Hypothesis 2**

We hypothesized that borrelidin treatment would selectively inhibit the proliferation of malignant ALL cell lines and induce apoptosis in these cells in association with the activation of GCN2 stress kinase pathway.

### **Objective 2.1**

In order to study the potential role of borrelidin in targeting ALL cell lines, Jurkat and CEM cells, cells were treated with different concentrations of borrelidin. Proliferation assay was performed and the growth rate of these cells was also studied. Propidium iodide staining and flow cytometry were performed to study the apoptotic effects of borrelidin on these cells as well as to study the effects of borrelidin on cell cycle progression. Our results (described in Chapter

4), show that borrelidin was able to increase apoptosis in ALL cell lines and also induced G1 arrest in these cells.

### **Objective 2.2**

To study the involvement of GCN2 kinase pathway as one of the downstream mechanisms by which borrelidin affects lymphoblastic cells, cells were treated with different concentrations of borrelidin. The levels of different proteins involved in the GCN2 kinase pathway were evaluated by western blot analysis. Our results (described in Chapter 4), showed that activation of the GCN2 kinase pathway was significantly higher in ALL cell lines treated with borrelidin compared to that of the control non-treated cells.

## CHAPTER 2

### Differential Expression of IMPACT Protein Promotes Resistance to Indoleamine 2, 3-Dioxygenase-Induced Cell Death <sup>1</sup>

#### 2.1. Introduction

Indoleamine 2, 3-dioxygenase (IDO) is a cytosolic monomeric hemoprotein that has been shown to have potent immunomodulatory activity (42, 168). IDO catalyzes the first and rate limiting step of the conversion of tryptophan, the least abundant of the essential dietary amino acids in mammals, to kynurenine as the main tryptophan metabolite (23, 24). IDO expression is inducible in different cell types, such as fibroblasts, endothelial cells, certain myeloid-lineage cells (monocyte-derived macrophages and dendritic cells) and some tumor-cell lines (48), upon exposure to interferon gamma (IFN- $\gamma$ ) which is a potent inducer of IDO (46, 49-52). It has been shown that placental cells synthesizing IDO are involved in the immunologic acceptance of the fetus in a mouse model (31). It has been suggested that IDO-mediated immunosuppression is due to the depletion of tryptophan and/ or accumulation of toxic tryptophan metabolites, kynurenines (36, 92). In a series of studies conducted by our research group, we have shown that induction of IDO expression in dermal fibroblasts by different approaches including IFN- $\gamma$  treatment causes a remarkable suppression in proliferation of bystander immune cells co-cultured with IDO-expressing fibroblasts (10, 102-104). We have also shown that when co-cultured with IDO-expressing fibroblasts, the apoptotic death rates of different types of immune cells such as bystander Jurkat cells, THP-1 cells, PBMC, CD8<sup>+</sup> and CD4<sup>+</sup> primary lymphocytes increases

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<sup>1</sup> A version of this chapter has been published. With kind permission from Wiley: J Cell Physiol. High Expression of IMPACT Protein Promotes Resistance to Indoleamine 2, 3 Dioxygenase-Induced Cell Death, 2010, 225: 196 -205, Habibi D, Jalili RB, Forouzandeh F, Ong CJ, Ghahary A.



significantly (10). We also demonstrated that the apoptotic effects of IDO are selective and primary dermal fibroblasts, keratinocytes, and endothelial cells are resistant to the IDO-mediated tryptophan-deficient environment (10, 101, 169).

Recent studies suggest that the stress-responsive kinase general control nonderepressible 2 (GCN2) signaling pathway enables T cells to sense and respond to stress conditions generated by IDO (83). Amino acid deprivation, tryptophan in the case of IDO, can cause a rise in the amount of uncharged transfer RNA (tRNA) in cells that activates the regulatory domain of GCN2, therefore triggers its kinase activity and initiates the downstream signaling. Activation of the GCN2 kinase pathway can lead to apoptosis, cell cycle arrest, differentiation, or compensatory adaption depending on the initiating stress and cell type (132, 170, 171). The CCAAT/enhancer-binding protein homologous protein (CHOP) gene (also known as GADD 153) is a downstream target gene in the GCN2 pathway and is a well-accepted marker for GCN2 activation (132). CHOP is a DNA damage-inducible nuclear leucine zipper protein that has a role in apoptosis and differentiation. Protein IMPACT homolog (imprinted and ancient) is a recently identified GCN2 kinase regulator. IMPACT homolog is an evolutionary conserved gene located on chromosome 18q11.2–12.1 (141, 172). It has been shown that by binding to GCN1, an activator of GCN2, the IMPACT protein inhibits the activation of GCN2, hence abolishing the expression of its downstream target genes ATF4 and CHOP (143).

Due to the immunoregulatory effects of IDO, this enzyme has been considered as a promising candidate in down-regulating allogeneic immune responses in different types of transplantation (144, 146-148). In a series of studies conducted by our group, we have shown that: 1) IDO can be used successfully as a local immunosuppressive factor to develop a non-rejectable skin substitute (6); 2) local IDO expression by syngeneic fibroblasts can suppress

allogeneic immune response to pancreatic islets (7, 145); and 3) the level of CHOP protein increases in immune cells, but not in skin cells, when co-cultured with IDO-expressing fibroblasts (11). These findings set the stage for the current study through which we examine a possible mechanism that can explain the selective IDO-mediated apoptosis in immune cells but not in skin cells. To address this issue, we therefore asked whether: a) the expression level of IMPACT protein is different in IDO sensitive versus IDO resistant cells; b) the IMPACT protein expression is differentially regulated in immune and skin cells exposed to IDO-expressing cells; c) the stress environment created by IDO expression can affect the viability and proliferation of IMPACT knocked-down fibroblasts; and d) overexpression of IMPACT protein in stably transduced Jurkat cells can rescue the proliferation of these cells in a tryptophan-deficient environment.

## **2.2. Materials and Methods**

### **Fibroblasts and Keratinocytes Culture**

Neonatal foreskin was used as source of fibroblasts and keratinocytes and the procedure was done based on the approval of Ethics committee of the University of British Columbia. Cultures of human foreskin fibroblasts were established as described previously by Ghahary et al (173). In brief, punch biopsy samples were prepared from human foreskin. The tissue was collected in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS; GIBCO, Grand Island, NY), minced into small pieces of less than 0.5 mm, washed with sterile medium six times, and distributed into 60×15 mm Petri culture dishes (Corning Inc., Corning, NY), four pieces per dish. A sterile glass cover-slip was attached to the dish with a drop of sterile

silicone grease to immobilize the tissue fragment. DMEM plus antibiotics (penicillin G sodium 100 U/ml, streptomycin sulfate 100 µg/ ml, and amphotericin B 0.25 µg/ml; GIBCO) with 10% FBS was added to each dish and incubated at 37°C in a water-jacked humidified incubator in a 5% CO<sub>2</sub> atmosphere. The medium was replaced twice weekly. After 4 weeks of incubation, cells were released from dishes by brief (5 min) treatment with 0.1% trypsin (Life Technologies Inc., Gaithersburg, MD) and 0.02% ethylenediaminetetraacetic acid (EDTA) (Sigma, St. Louis, MO) in phosphate-buffered saline solution (PBS; pH 7.4) and transferred to 75 cm<sup>2</sup> culture flasks (Corning Inc.). Thereafter, once visual confluence was reached, the cells were subcultured 1:6 by trypsinization. Fibroblasts from passages 3 to 6 were used for this study. To culture human foreskin keratinocytes, keratinocyte serum-free medium (KSFM, GIBCO) supplemented with bovine pituitary extract (25 µg/ml) and epidermal growth factor (0.5 ng/ml) were used. Primary cultured keratinocytes at passages 3–5 were used for this study.

### **Isolation of Human Peripheral Blood Mononuclear Cells (PBMCs)**

Total PBMCs were isolated from whole blood of volunteers by using density gradient sedimentation on Histopaque-1077 (Sigma) according to the manufacturer's protocol. Briefly, whole blood was layered on an equal volume of Histopaque and centrifuged at 2,000 rpm for 20 min at 20°C and stopped without any brake. PBMC were isolated and added to RPMI 1640 (Hyclone, UT) plus 10% FBS and pelleted by centrifugation at 2,000 rpm for 10 min and were further washed twice in PBS plus 1% FBS.

### **Isolation of T Cells from PBMCs**

Upon isolation of PBMCs, CD3<sup>+</sup> T cells were isolated by using the EasySep negative selection human T cell enrichment kit (StemCell Technologies, Vancouver, BC, Canada) using the manufacturer's recommendations. Cell suspension at a concentration of  $5 \times 10^7$  cell/ml was prepared using PBS containing 2% heat-inactivated fetal bovine serum (FBS; GIBCO). The EasySep negative selection T cell enrichment cocktail was added to a 12 × 75-mm polystyrene tube, at 50 µl/ml cell suspension. The mixture was incubated for 10 min at room temperature. Then, EasySep magnetic nanoparticles (StemCell Technologies) were added at 50 µl/ml cell suspension and incubated at room temperature for another 10 min. After mixing, the sample tube was placed without cap into the magnet (EasySep Magnet 18000; StemCell Technologies) for 5 min and wanted cells were subsequently poured off into a new tube. After being sorted from blood, T cells were propagated in RPMI 1640 (Hyclone, UT) supplemented with 10% FBS, 0.1 U penicillin/ml, and 0.1 mg streptomycin/ml at 37 ° C in a humidified 5% CO<sub>2</sub> atmosphere. T cells were stimulated with anti-CD3 (1 µg/ml; BD) using a procedure previously described (101).

### **Jurkat Cell Culture**

The Jurkat cells (ATCC) were maintained in RPMI 1640 (Hyclone, UT) containing 10% FBS and 2 mM glutamine. All cells were cultured at 37° C in a 5% CO<sub>2</sub> atmosphere. All media were supplemented with antibiotic-antimycotic preparations (100 U/ml penicillin, 100 mg/ml streptomycin, 0.25 mg/ml amphotericin B (Invitrogen, Carlsbad, CA)).

### **IFN- $\gamma$ Treatment of Fibroblasts**

In order to induce IDO in fibroblasts, cells were seeded onto 30  $\mu$ m Millicell Sterilized Culture Plate Inserts (Millipore, Bedford, MA), and treated with 0 or 1000 U/ml IFN- $\gamma$  (Sigma) for a period of 48 hours. The expression of IDO was confirmed by the detection of IDO protein expression using western blot as well as measuring the level of kynurenine in conditioned medium.

### **Co-Culture Systems of IDO-Expressing Fibroblasts and Bystander Cells**

Using 30  $\mu$ m Millicell Sterilized Culture Plate Inserts (Millipore, Bedford, MA), we set up a co-culture system in which IDO-expressing fibroblasts were grown on the upper chamber of a six-well plate. Keratinocytes, fibroblasts, Jurkat cells or T cells were cultured as bystander cells on the lower chamber. Therefore, there was no direct contact between IDO-expressing fibroblasts and bystander cells. IFN- $\gamma$ -treated fibroblasts were washed three times with PBS before co-culturing them with other cells, to remove any excess IFN- $\gamma$ .

### **Kynurenine Measurement in the Conditioned Medium**

The biological activity of IDO was evaluated by measuring the levels of tryptophan degradation product, L-kynurenine, present in conditioned medium. The amount of L-kynurenine was measured by the previously established method (174). Briefly, proteins in conditioned medium were precipitated by trichloroacetic acid. After centrifugation, 0.5 ml of supernatant was incubated with equal volume of Ehrlich's reagent for 10 min at room temperature. Absorption of resultant solution was measured at 490 nm by spectrophotometer. The values of kynurenine in

conditioned medium were calculated according to a standard curve with defined kynurenine concentration (0–20 µg/ml).

### **Western Blot Analysis**

Bystander cells were harvested 72 hours after the co-culture and washed twice with PBS. Cells were then lysed in lysis buffer (50 mM Tris–HCl, pH 7.4; 10 mM EDTA; 5 mM EGTA; 0.5% Igepal; 1% Triton X-100, and protease inhibitor cocktail (Sigma). Cell lysates were centrifuged at 14,000 g for 10 min. The protein concentration of supernatant was measured by BCA protein assay kit (Pierce, Rockfield, IL). Equal amounts of cell lysates were separated by SDS-PAGE. Proteins were then transferred to a PVDF membrane (Millipore Corp., Bedford, MA). After blocking with 5% skim milk, the blots were probed with the following antibodies: polyclonal anti-IDO antibody produced in rabbits (1:5,000, Washington Biotechnology Inc., Baltimore, MD), anti phospho-GCN2 produced in rabbits (Thr898, 1:1000 dilution, Cell Signaling Technology INC., Beverly, MA), anti GCN2 produced in rabbit (1:1000 dilution, Cell Signaling), anti CHOP produced in mouse (1:1000 dilution, Cell Signaling), anti cleaved PARP (Asp214) produced in rabbits (1:1,000, Cell Signaling) and anti-IMPACT produced in mouse (1:500 dilution, Abcam, Cambridge, MA). Horseradish peroxidase conjugated goat anti-rabbit IgG and horseradish peroxidase conjugated goat anti-mouse IgG served as a secondary antibodies for the enhanced chemiluminescence detection system (ECL; Amersham Biosciences, UK). Blots were then stripped and reprobed for  $\beta$ -actin (1:25,000 dilution, Sigma) and used as a control of protein loading.

### **IMPACT Reverse Transcriptase-PCR Analysis**

Total RNA was isolated using a RNeasy kit (Qiagen, Maryland). cDNA was synthesized using the Thermo-Script reverse transcriptase (RT)-PCR System (Invitrogen). The primers used were as follows: IMPACT: sense 5'-ACCAGAGGCAGAATGAGGAA- 3', antisense 5'-CCAAGGAGCATTCAACTGGT-3'; glyceraldehyde- 3-phosphate dehydrogenase: sense 5'-CGAGATCCCTCCAAAATCAA - 3' antisense 5'-TGTGGTCATGAGTCCTTCCA- 3'. Amplified PCR products were separated by 1% agarose gel electrophoresis and visualized with ethidium bromide staining. DNA bands were visualized under UV light.

### **Small Interfering RNA (siRNA)**

In order to knockdown IMPACT expression in fibroblasts, ON-TARGET plus SMART pool siRNAs (Dharmacon, Lafayette, CO) directed against IMPACT, was used. Oligofectamine (Invitrogen Life Technologies, Inc.) was used to transfect and increase the siRNA uptake into the fibroblasts according to the manufacture's instructions. In brief, fibroblasts were transfected with various concentrations of siRNA (1, 5, 10 and 20 nM) after pre-incubation for 20 min with 4 mg/ml oligofectamine in serum-free OPTI-MEM (Invitrogen Life Technologies). Four hours after the start of the incubation, DMEM/15% FBS was added at a final concentration of 5% FBS. Cells were harvested or used for co-culture experiments 48 hours post-transfection. A non-silencing siRNA (Qiagen, Maryland) was used as a control.

### **Methyl Thiazolyl Tetrazolium Proliferation Assay**

A colorimetric methyl thiazolyl tetrazolium (MTT) [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay was used to evaluate the effects of IDO on proliferation of

IMPACT siRNA transfected fibroblasts. After being in co-culture with either IDO-expressing or non IDO-expressing fibroblasts for 72 hours, MTT (Sigma) solution (5 mg/ml) was added to bystander non-transfected or siRNA transfected fibroblasts and incubated at 37°C for 5 hours. The formazin crystals were solubilized in 500 µl of dimethyl sulfoxide at the end of incubation, and the optical density of the solutions was measured at 570 nm.

### **Live/Dead Viability Assay**

A fluorescence-based live/dead viability kit (Invitrogen) was used to analyze the effects of IDO on the death rates of fibroblasts transfected with IMPACT siRNA according to the manufacturer's protocol. In brief, after being in co-culture with either IDO-expressing or non IDO-expressing fibroblasts for 72 hours, bystander non-transfected or siRNA transfected fibroblasts cultures were washed with PBS. The live/dead working solution containing Ethidium homodimer-1 (EthD-1, 4 µM) and calcein AM (2 µM) in PBS was prepared and added to each well of bystander fibroblasts. Samples were incubated at room temperature for 30 min (protected from light). Images were obtained using a Zeiss Axiovert 200 M fluorescence microscope (Carl Zeiss, Inc. Cologne/Oberkochen, Germany) and Northern Eclipse image analysis software. Death rates were determined using plate reader according to the manufacturer's protocol. Specificity of the IDO effect was determined by addition of 1-methyl-tryptophan (1-MT), an IDO inhibitor (Aldrich Chemical Co., Milwaukee, WI), to IDO-expressing fibroblasts co-culture at the final concentration of 800 µmol/L (145).



## **Plasmid Constructions**

A lentiviral construct for expressing the IMPACT gene was generated using the FUGWBW vector modified from the lentiviral backbone FUGW where the GFP gene was replaced by the Gateway cassette and a blasticidin S resistance gene expression cassette was inserted downstream of the Gateway cassette (175, 176). The human IMPACT gene (BC034016, MGC-22261, ATCC) was generated by PCR using a full-length cDNA encoding the gene as template and the forward primer (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGCTGAGGGGGACGCAGGGAG-3') and reverse primer (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAATGTTTCATTCCTCTTCTTGTCTTTTC-3'). The amplified PCR product was first inserted into an entry vector (pDON201), and then Gateway (Invitrogen) cloned into a lentiviral FUGWBW expression vector. The plasmid was amplified in competent DH10-B bacteria and purified using the Qiagen Plasmid DNA Maxi-prep kit (Qiagen). Sequence of the IMPACT/FUGWBW construct was confirmed by DNA sequencing analysis. Empty FUGWBW vector was used as the control.

## **Lentiviral Vector Production and Cell Transduction**

Replication-defective lentiviral vectors were generated as previously described (177, 178) by transient transfection of 293T human kidney cells with either IMPACT/FUGWBW vector or empty vector, R8.9 (packaging plasmid) and VSV-G (envelope plasmid) using ProFectin (Mammalian Transfection System Calcium Phosphate; Promega). Supernatant containing the vector stocks was harvested and stored at -80°C until use. For transduction, Jurkat cells were seeded on flat bottom 6-well cell culture plates (Corning Incorporated, Corning, NY, USA) and incubated with the same titer of IMPACT lentiviral vector or the control empty vector for 24

hours. Blasticidin selection (5 µg/ml) was subsequently performed in order to obtain a stable population of IMPACT-expressing or control Jurkat cells.

### **Tritiated Thymidine Incorporation Assay**

Proliferation was measured by [<sup>3</sup>H] thymidine incorporation assay. Jurkat cells were seeded in 96 well plates either in regular or tryptophan-deficient media or in the presence or absence of IDO and were incubated for 24 hours. Cells were then pulsed by [<sup>3</sup>H] thymidine (1 µCi/well, PerkinElmer, MA) and incubated for 16 hours. Cells were lysed by freezing and thawing at -80° C and harvested on a Harvester 96 Mach III M (TOMTEC, Milano, Italy). The amount of incorporated thymidine was measured using a Wallac-1450-MicroBeta Jet β-scintillation machine (American Laboratory Trading, Inc. CT).

### **Statistical Analysis**

All data are reported as mean ± SD. Statistical significance was calculated using two-tailed unpaired Student's *t*-test or one-way analysis of variance (ANOVA) with post-hoc test using Bonferroni correction in case of multiple comparisons. *p*-values < 0.05 were considered to be statistically significant in this study.

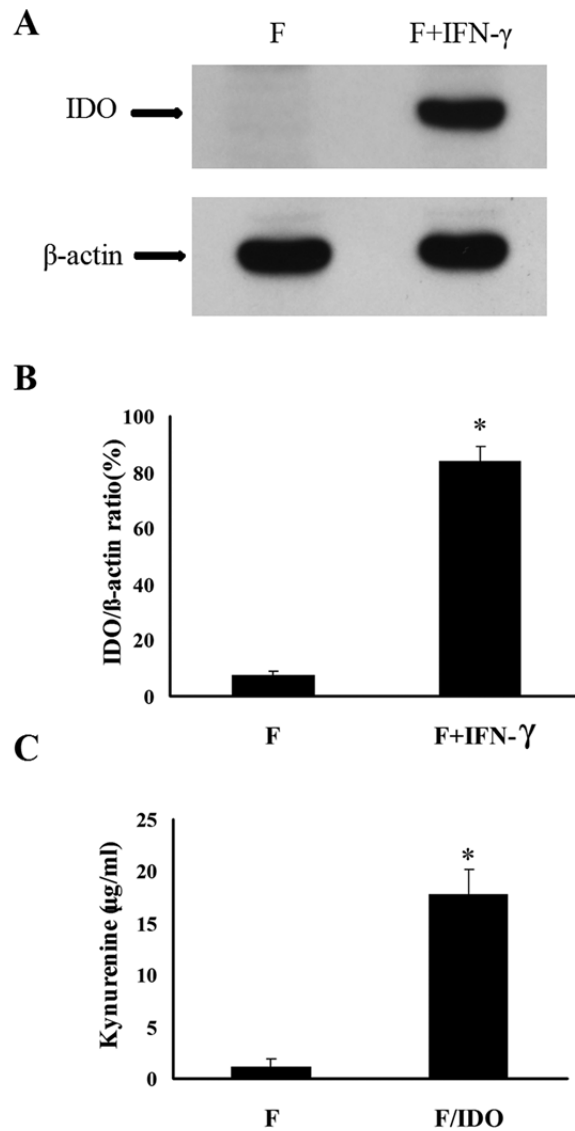
## **2.3. Results**

### **Expression of Functional IDO Protein in IFN- $\gamma$ -Treated Human Primary Fibroblasts**

In order to induce the expression of IDO in fibroblasts, primary human fibroblasts were treated with IFN- $\gamma$  (1000 U/ml), which is a potent inducer of IDO expression (10, 102-104). After 48 hours of incubation, cells were harvested and conditioned medium was collected. IDO protein expression in treated cells was validated by western blot analysis (Figure 2.1A). Meanwhile, IDO expression was not detectable in the control non-treated fibroblasts. The quantitative analysis shown in Figure 2.1B indicated a significant expression (more than 7-fold) in IDO protein level in treated fibroblasts. In order to show that the expressed IDO is functional, the level of kynurenine was measured in the conditioned media of both groups of fibroblasts. The average of three separate experiments showed a significant increase in kynurenine level ( $1.2 \pm 0.7$ ,  $17.7 \pm 2.4$   $\mu\text{g/ml}$  for non-treated and treated fibroblasts respectively;  $P < 0.0001$  relative to values of control group,  $n=3$ ; Figure 2.1C).

### **Activation of GCN2 Kinase Pathway in Jurkat Cells Co-Cultured with IDO-Expressing Fibroblasts**

It is suggested that activation of GCN2 kinase pathway by uncharged tRNA acts as a downstream mechanism to sense the suppressive effects of IDO. Therefore, we asked whether the activation of GCN2 pathway in response to IDO expression is selective in primary skin cells versus immune cells. IDO-expressing (IDO) or control non-treated fibroblasts (NT) were co-cultured with either Jurkat cells, keratinocytes or another strain of fibroblasts obtained from a different donor for three days. Seventy two hours after being in co-culture, conditioned media was collected for kynurenine assay and bystander Jurkat cells, keratinocytes, and fibroblast were

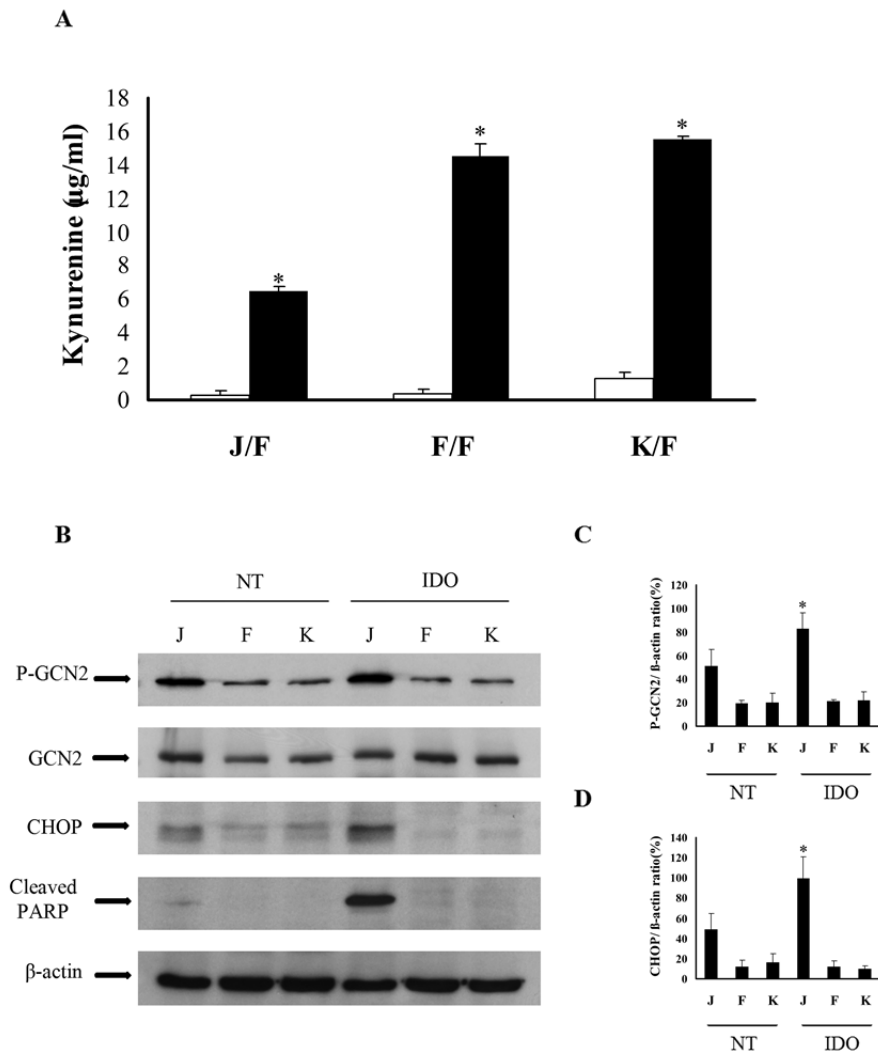


**Figure 2.1. IDO expression in fibroblasts in response to IFN- $\gamma$  treatment.**

Primary human fibroblasts were treated with IFN- $\gamma$  (1,000 U/ml) for 48 hours. A) After 48 hours, cells were harvested and IDO protein expression in non-treated (F) and treated (F+IFN- $\gamma$ ) fibroblasts was analyzed by western blot. Blots were then stripped and reprobed for  $\beta$ -actin as equal loading control. A representative experiment is shown. B) The mean ratio of densities of IDO band to that of  $\beta$ -actin band in treated cells (F+IFN- $\gamma$ ) or control (F) fibroblasts. C) The level of kynurenine was measured in the conditioned media of non IDO-expressing (F) and IDO-expressing (F/IDO) fibroblasts after 48 hours of treatment. Statistical significance was calculated using two-tailed unpaired Student's *t*-test. Significant (*p* value < 0.0001) differences between the two groups are indicated by asterisks (\*), *n*=3. IDO, indoleamine 2,3-dioxygenase.

harvested and evaluated for the expression of phosphorylated GCN2, total GCN2, CHOP (as a marker for GCN2 kinase pathway activation), and cleaved PARP (as a marker for apoptosis). Results of kynurenine assay shown in Figure 2.2A indicated that the level of kynurenine is significantly increased in the conditioned media of bystander Jurkat cells, fibroblasts, and keratinocytes in the IDO-exposed group ( $6.5 \pm 0.3$ ,  $14.5 \pm 0.7$  and  $15.5 \pm 0.2$ , respectively) when compared with that of bystander cells co-cultured with control fibroblasts ( $0.2 \pm 0.3$ ,  $0.3 \pm 0.2$ ,  $1.3 \pm 0.3$   $\mu\text{g/ml}$ , respectively;  $P < 0.001$ ,  $n=3$ ; Figure 2.2A). These results confirmed the functional IDO expression in the co-cultures.

Western blot analysis (Figure 2.2B) revealed that in the presence of IDO, GCN2 kinase is selectively activated in Jurkat cells compared with that of primary skin cells, fibroblasts and keratinocytes. Jurkat cells co-cultured with IDO-expressing fibroblasts showed significantly higher amount of phosphorylated GCN2 and CHOP compared to that of Jurkat cells co-cultured with non-treated fibroblasts. Moreover, the level of CHOP expression showed a positive correlation with that of phosphorylated GCN2 (Figure 2.2B). Expression of CHOP was either very low or undetectable in fibroblasts and keratinocytes groups co-cultured with either IDO-expressing or control fibroblasts. Western blot analysis also demonstrated a significant higher expression of cleaved PARP in Jurkat cells co-cultured with IDO-expressing fibroblasts (Figure 2.2B). Quantitative analysis shown in Figure 2.2C and 2.2D indicated a significant increase in the levels of GCN2 phosphorylation and CHOP protein respectively in Jurkat cells co-cultured with IDO-expressing cells relative to those of fibroblasts and keratinocytes.



**Figure 2.2. Selective GCN2 kinase pathway activation in skin cells versus Jurkat cells co-cultured with IDO-expressing fibroblasts.**

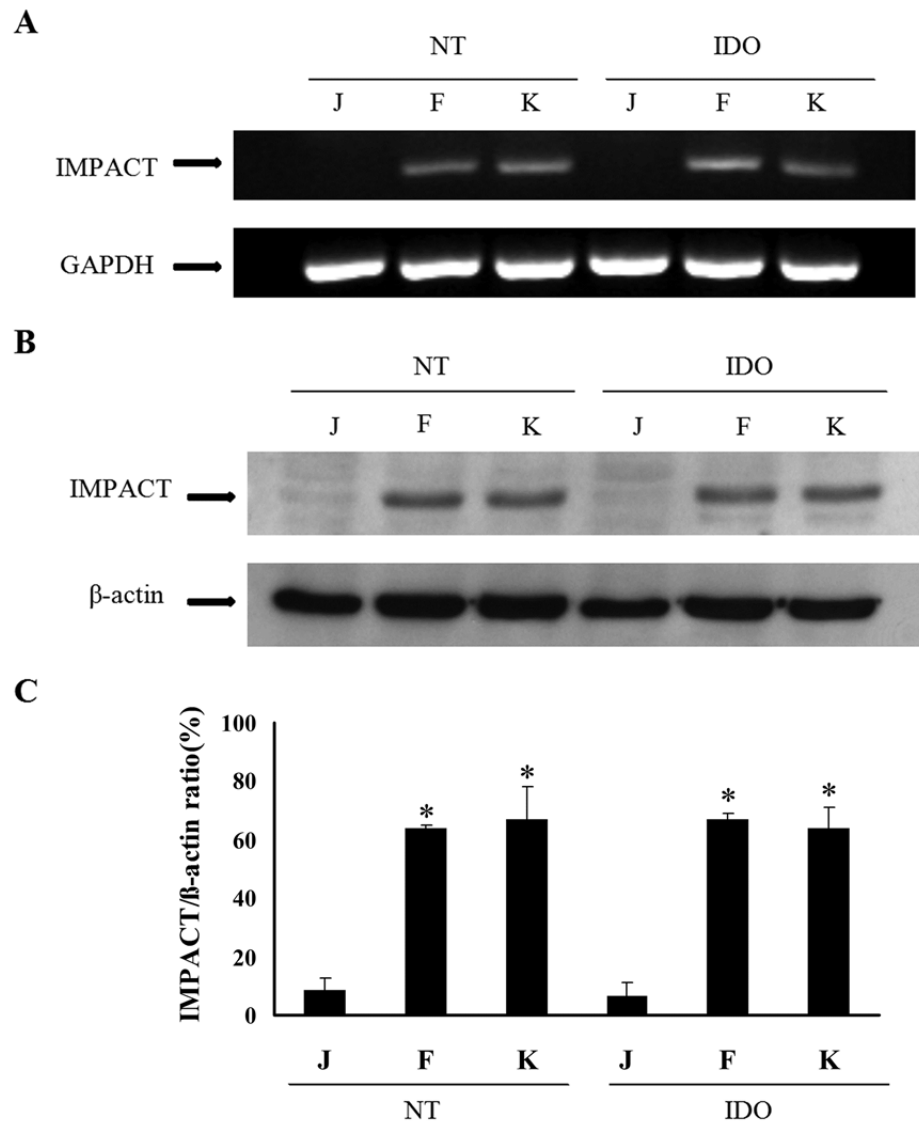
IDO-expressing (IDO) or control fibroblasts (NT) were co-cultured with bystander Jurkat cells (J), keratinocytes (K), or a different strain of fibroblasts (F) for 72 hours in a two-chamber culture plates. A) Kynurenine levels in the conditioned media were measured in each indicated group co-cultured with non IDO-expressing (open bars) or IDO-expressing fibroblasts (solid bars). Lower level of kynurenine in Jurkat cells co-cultured with IDO-expressing fibroblasts is due to the lower amount of tryptophan in the RPMI media. ANOVA with Bonferroni correction post-hoc test was used to calculate statistical significance. Significant (p value < 0.0001) differences have been indicated by asterisks (\*), n=3. B) After co-culture for 72 hours, bystander cells were harvested and cell lysates prepared and analyzed for the expression of phospho-GCN2 (upper row), total GCN2 (second row), CHOP (third row) and cleaved PARP (fourth row) using western blot. Blots were stripped and reprobed for β-actin as equal loading control. A representative experiment is shown. C-D) The mean ratio of densities of phospho-GCN2 and CHOP protein bands to that of β-actin band in indicated cells co-cultured with either IDO-expressing (IDO), or control (NT) fibroblasts. ANOVA with Bonferroni correction post-hoc test was used to calculate statistical significance. The significant (p value < 0.05) differences in phospho-GCN2 and CHOP level between the IDO exposed and the control Jurkat cells are indicated by asterisks (\*), n=3. P-GCN2: phosphorylated GCN2.

### **Differential Expression of Protein IMPACT Homolog in Primary Skin Versus Jurkat Cells**

Previous reports indicated that IMPACT protein binds to GCN1 and acts as an inhibitor of GCN2 kinase and thereby abolishes the expression of its downstream target CHOP under amino acid starvation conditions (143). We hypothesize that the selective activation of GCN2 kinase pathway in Jurkat cells versus skin cells co-cultured with IDO-expressing fibroblasts is due to differential expression of IMPACT protein. In order to examine this hypothesis, Jurkat cells and non-immune skin cells were co-cultured with IDO-expressing and control fibroblasts for three days. The bystander cells were then harvested and evaluated for the expression of IMPACT at the level of mRNA and protein production by RT-PCR and western blot analysis respectively. The results confirmed that at both mRNA (Figure 2.3A) and protein level (Figure 2.3B), IMPACT is expressed constitutively at high levels in skin cells but undetectable in Jurkat cells co-cultured with either IDO or non-IDO-expressing fibroblast cells. Moreover, the level of IMPACT expression was independent of IDO-induced low tryptophan environment. The quantitative analysis shown in Figure 2.3C indicated a significantly higher (more than 10-fold) constitutive expression of IMPACT protein in skin cell than Jurkat cells.

### **Low Expression of IMPACT Protein in Primary Human T Cells in Compare with Human Fibroblasts**

In order to evaluate the expression of IMPACT protein in primary human T cells and compare to that of primary fibroblasts, T cells were isolated from PBMCs. Isolated T cells were then stimulated with anti-CD3, co-cultured with IDO-expressing or control fibroblasts and harvested after three days. As shown in western blot results in Figure 2.4A, IMPACT protein expressed constitutively at a very low level in primary human T cells compared to that of

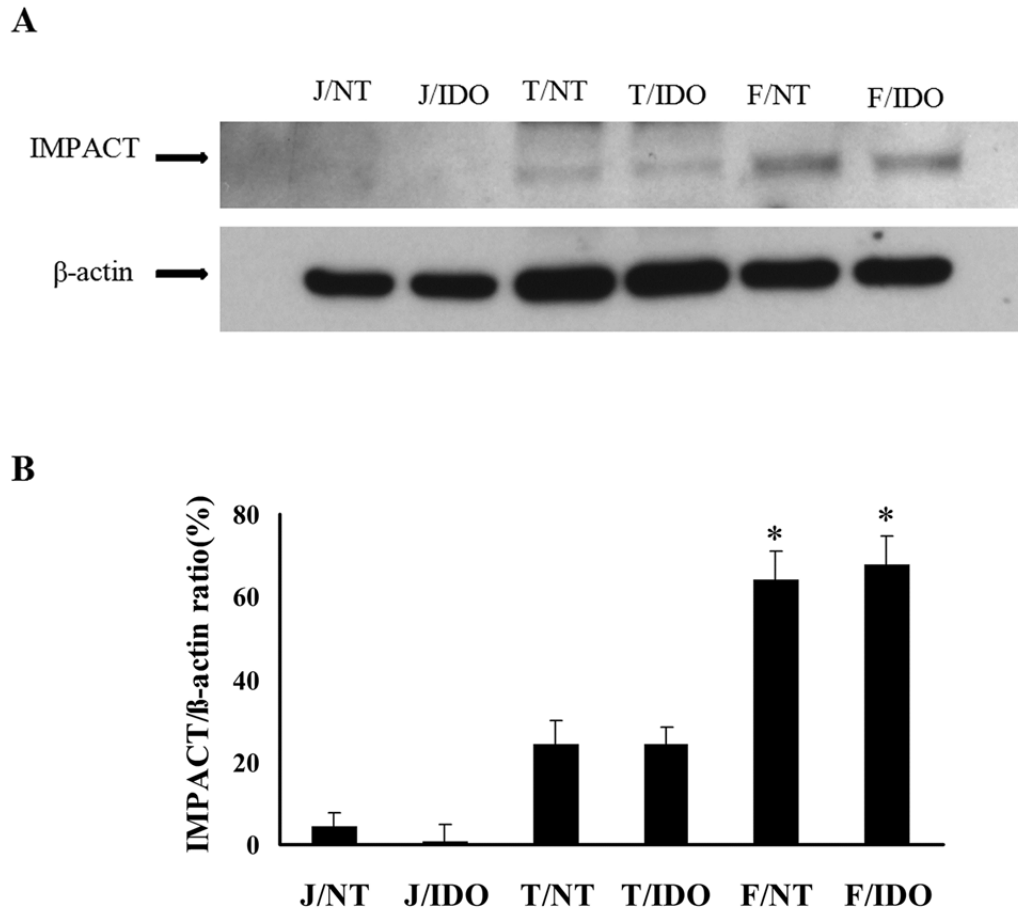


**Figure 2.3. Differential expression pattern of IMPACT protein in skin cells versus Jurkat cells exposed to IDO-expressing fibroblasts.**

IDO-expressing (IDO) or control fibroblasts (NT) were co-cultured with Jurkat cells (J), keratinocytes (K), or a different strain of fibroblasts (F) for 72 hours in a two chamber co-culture system. Bystander cells were harvested and analyzed for the expression of IMPACT mRNA and protein. A) IMPACT RT-PCR. GAPDH was used as the internal control. B) Western blot analysis of IMPACT. Blots were stripped and reprobed for  $\beta$ -actin as equal loading control. A representative experiment is shown. C) The mean ratio of densities of IMPACT protein band to that of  $\beta$ -actin band in indicated cells co-cultured with either IDO-expressing (IDO) or control (NT) fibroblasts. ANOVA with Bonferroni correction post-hoc test was used to calculate statistical significance. Significant (p value <0.0001) differences in IMPACT level between skin cells and Jurkat cells are indicated by asterisks (\*), n=3.



fibroblasts and this level of expression was independent of IDO-induced low tryptophan environment. The quantitative analysis shown in Figure 2.4B indicated a significantly higher expression (3-fold) of IMPACT in fibroblasts compared to that of T cells.

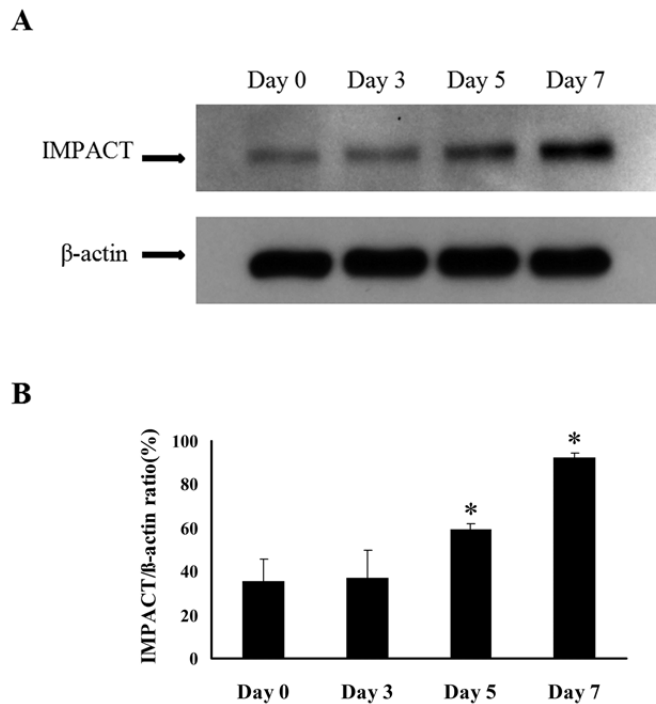


**Figure 2.4. Lower expression of IMPACT protein in primary human T cells compared to that of primary human fibroblasts.**

IDO-expressing (IDO) or control fibroblasts (NT) were co-cultured with purified stimulated human T cells (T), Jurkat cells (J) or a different strain of fibroblasts (F) for 72 hours in a two chamber co-culture system. Bystander cell lysates were prepared and analyzed for the expression of IMPACT protein by western blot. A) Result of western blot analysis for IMPACT. Blots were stripped and reprobed for  $\beta$ -actin as equal loading control. A representative experiment is shown. B) The mean ratio of densities of IMPACT band to that of  $\beta$ -actin band in indicated cells co-cultured with either IDO-expressing (IDO) or control (NT) fibroblasts. ANOVA with Bonferroni correction post-hoc test was used to calculate statistical significance. Significant ( $p$  value  $<0.0001$ ) differences in IMPACT level between fibroblasts and immune cells are indicated by asterisks (\*),  $n=3$ .

## Time-Dependent Increase in IMPACT Expression Level in Fibroblasts Cultured in Tryptophan-Free Environment

To evaluate the long-term effect of IDO-induced low tryptophan environment on IMPACT expression in fibroblasts, these cells were cultured in a tryptophan-free media and harvested and lysed on days 0, 3, 5 and 7. As shown in Figure 2.5A, the level of IMPACT protein expression increased in a time-dependent manner. The quantitative analysis in Figure 2.5B showed that this increase was indeed significant (1.5-fold on day 5 and 3-fold on day 7 compared to that of day 0).



**Figure 2.5. Increased IMPACT expression level in fibroblasts cultured in tryptophan-deficient media.**

A) Fibroblasts were cultured in tryptophan-deficient media for a period of 0, 3, 5 and 7 days. Cells were then harvested and cell lysates were analyzed for IMPACT expression. Blots were stripped and reprobed for  $\beta$ -actin as equal loading control. A representative experiment is shown. B) The mean ratio of densities of IMPACT protein band to that of  $\beta$ -actin band in cells cultured for different time points in tryptophan-free media. ANOVA with Bonferroni correction post-hoc test was used to calculate statistical significance. Significant ( $p$  value  $<0.05$ ) time-dependent increasing levels of IMPACT expression are indicated by asterisks (\*),  $n=3$ .

### **Increased CHOP Expression Level Following IMPACT Knockdown in Fibroblasts in the Presence of IDO**

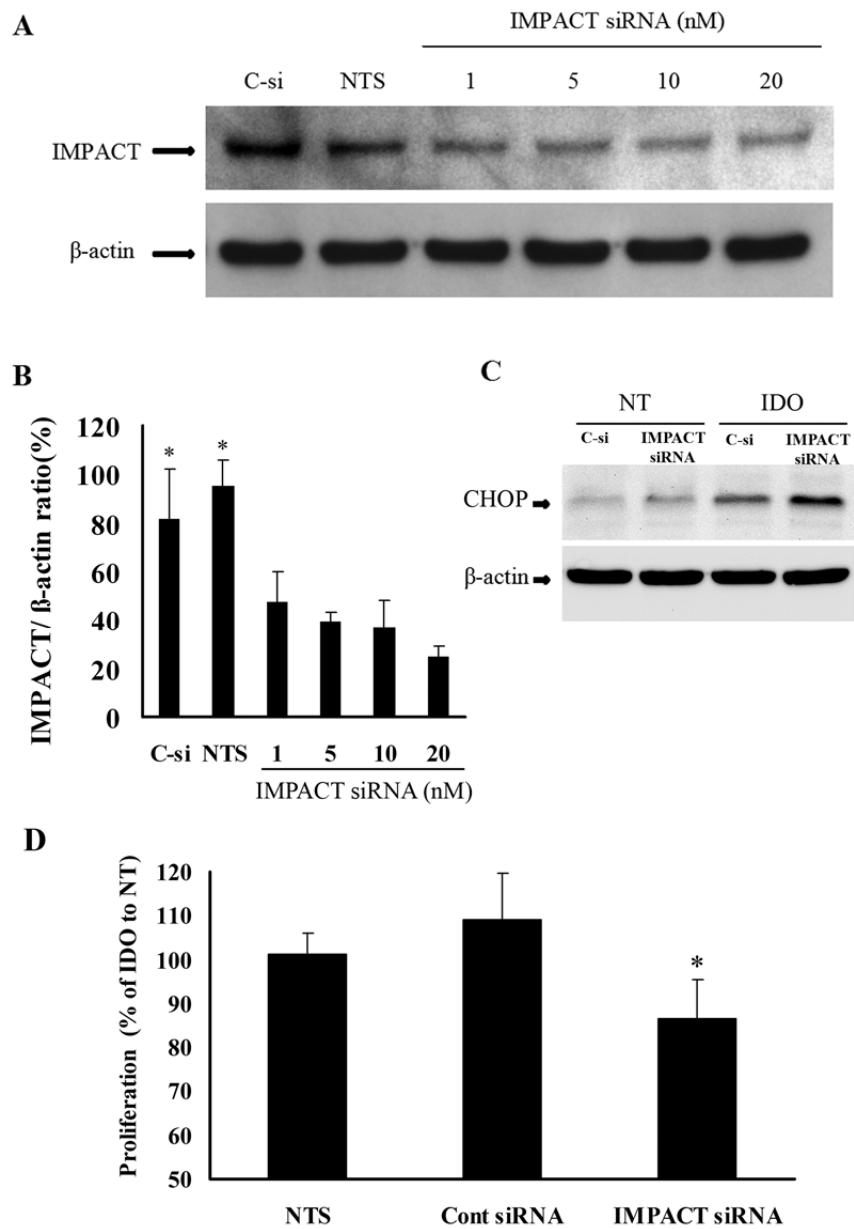
In order to assess whether there is a correlation between loss of IMPACT and CHOP expression levels, we knocked-down the expression of IMPACT protein in human fibroblasts using siRNA against IMPACT. Fibroblasts were either non-transfected (NTS) or transfected with a non-silencing control siRNA (C-si) or with increasing concentrations (0, 1, 5, 10 or 20 nM) of IMPACT siRNA (Figure 2.6A). Quantitative analysis shown in Figure 2.6B revealed that this method knocked-down the expression level of IMPACT by almost 4-fold in fibroblasts treated with 20 nM IMPACT siRNA. This concentration of IMPACT siRNA was used in subsequent experiments.

IMPACT knocked-down fibroblasts and non-silencing control siRNA transfected fibroblasts were co-cultured with IDO-expressing (IDO) or non-treated (NT) fibroblasts. Bystander fibroblasts were harvested and evaluated for the expression of CHOP protein. As shown in Figure 2.6C, in the presence of IDO, loss of IMPACT leads to marked upregulation of CHOP.

### **Proliferation Suppression in IMPACT Knocked-down Fibroblasts Co-Cultured with IDO-Expressing Fibroblasts**

In order to further confirm our hypothesis that in fact higher level of IMPACT expression in primary skin cells can act as a protective mechanism against IDO-induced GCN2 activation, we knocked-down the expression of IMPACT protein in human fibroblasts. IMPACT knocked-down fibroblasts were co-cultured with IDO-expressing or control fibroblasts for three days and MTT assay was performed for the IMPACT knocked-down and control fibroblasts. As shown in

Figure 2.6D, IDO-exposed IMPACT knocked-down fibroblasts proliferation (adjusted to the proliferation of corresponding non-IDO-exposed control) ( $86.5\% \pm 9.0$ ) was significantly lower than adjusted proliferation of IDO-exposed non-transfected ( $101.4\% \pm 4.5$ ,  $n=3$ ,  $p<0.05$ ) and non-silencing control siRNA transfected ( $109.0\% \pm 10.6$ ,  $n=3$ ,  $p<0.05$ ) fibroblasts.



**Figure 2.6. Effects of IDO expression on the level of CHOP expression and proliferation rates of IMPACT knocked-down fibroblasts.**

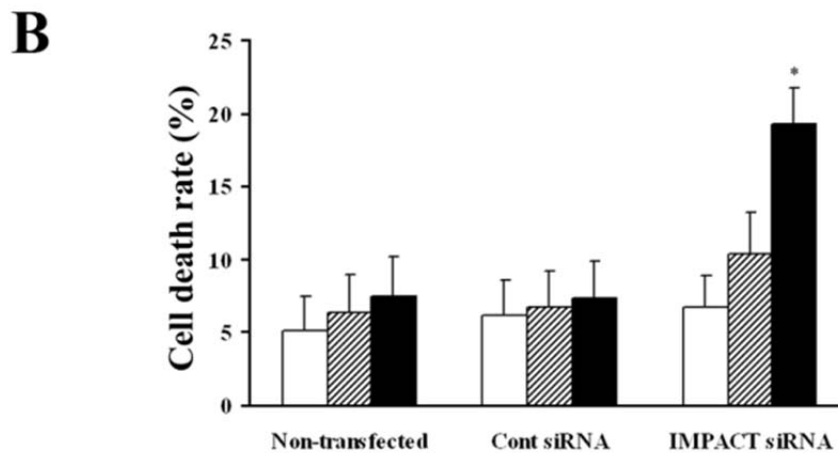
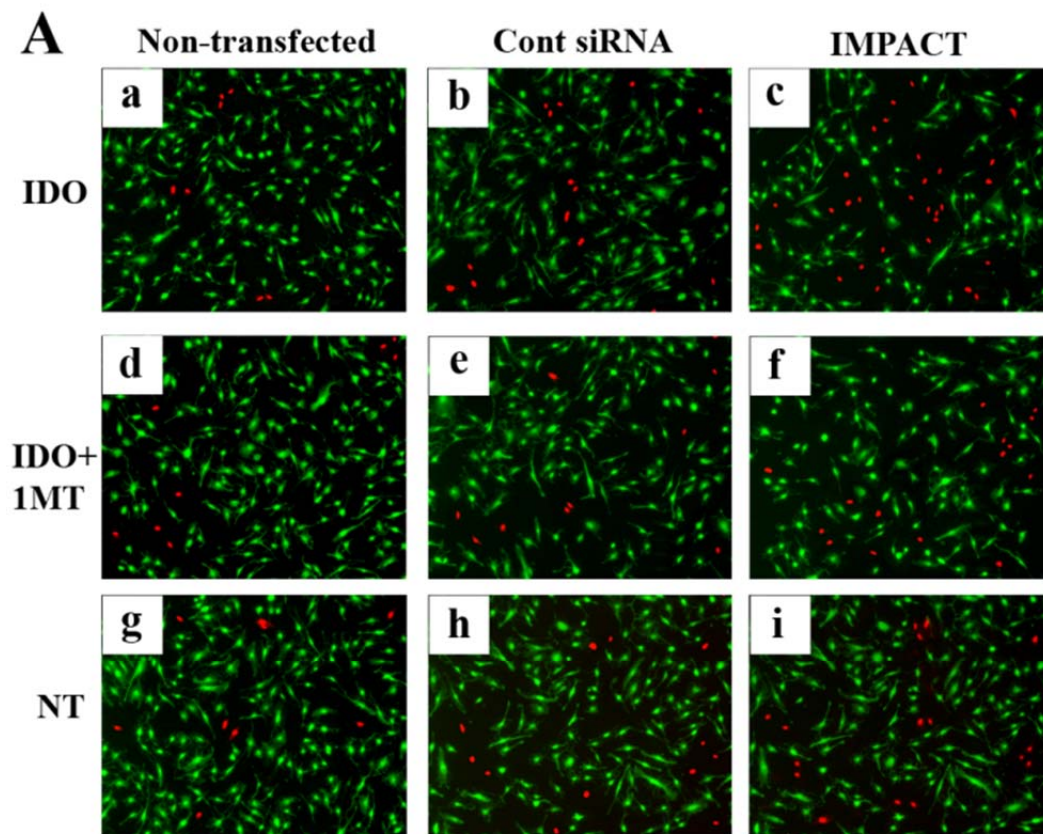
A) IMPACT siRNA was used to knock down the expression of IMPACT protein in primary human fibroblasts. Fibroblasts were left non-transfected (NTS) or transfected with a non-silencing control siRNA (C-si) or with increasing concentrations of IMPACT siRNA (IMPACT). Forty-eight hours after transfection, cells were harvested and cell lysates were prepared and analyzed for the expression of IMPACT protein by western blot. Blots were stripped and reprobed for  $\beta$ -actin as equal loading control. A representative experiment is shown. B) The mean ratio of densities of IMPACT protein bands to that of  $\beta$ -actin bands in non-transfected cells, cells transfected with IMPACT siRNA or control siRNA. ANOVA with Bonferroni correction post-hoc test was used to calculate statistical significance. The significant (p value <0.05) differences in IMPACT level between IMPACT siRNA treated and control groups have been indicated by asterisks (\*), n=3. C) IMPACT knocked-down fibroblasts (IMPACT siRNA) and non-silencing control siRNA (C-si) transfected fibroblasts were co-cultured with IDO-expressing (IDO) or non-treated (NT) fibroblasts. Bystander fibroblasts were harvested and evaluated for the expression of CHOP protein. Blots were stripped and reprobed for  $\beta$ -actin as equal loading control. A representative experiment is shown. D) IDO-expressing (IDO) or control non-treated fibroblasts (NT) were co-cultured with another strain of fibroblasts non-transfected, transfected with a non-silencing control siRNA or with 20 nM IMPACT siRNA in two chamber co-culture systems for 72 hours. MTT assay was then performed for the bystander cells. Data represent the ratio of proliferation rates of each indicated bystander IDO exposed group to its corresponding non-treated group. Non-transfected fibroblast (NTS), non-silencing control siRNA transfected fibroblasts (Cont siRNA), IMPACT siRNA transfected fibroblasts (IMPACT siRNA). ANOVA with Bonferroni correction post-hoc test was used to calculate statistical significance. Significant (p value <0.05) differences are indicated by asterisks (\*), n=3.

**Increased Cell Death in IMPACT Knocked-down Fibroblasts Co-Cultured with**

**IDO-Expressing Fibroblasts**

IMPACT knocked-down fibroblasts were co-cultured with IDO-expressing or control fibroblasts for three days and a live/dead cell viability assay was performed for the bystander fibroblasts. The results of Figure 2.7A showed the fluorescent microscopic images of non-transfected, non-silencing control siRNA (ContsiRNA), or IMPACT siRNA transfected fibroblasts co-cultured with IDO-expressing fibroblasts (IDO), IDO-expressing fibroblasts in the presence of 1-MT (IDO + 1-MT) or control non-treated fibroblasts (NT). The number of dead (red color) cells was higher in IMPACT knocked-down fibroblasts exposed to IDO-expressing cells in comparison with that of corresponding non-transfected or non-silencing control siRNA transfected fibroblasts. Furthermore, addition of 1-MT, a specific IDO inhibitor, resulted in

partial recovery of IMPACT knocked-down fibroblasts from the apoptotic effects of IDO. Quantitative analysis of the viability assays shown in Figure 2.7B indicated that the death rate of IDO-exposed IMPACT knocked-down fibroblasts was significantly higher (more than 2.5-fold) compared to the death rates of other indicated experimental conditions.



**Figure 2.7. Effects of IDO expression on the death rates of primary IMPACT knocked fibroblasts.**

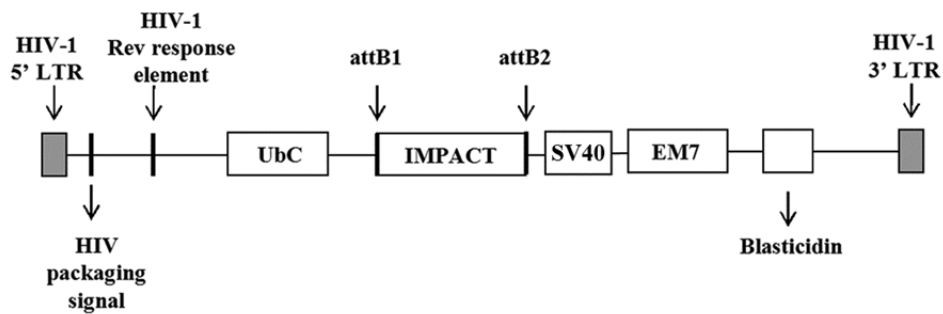
IDO- expressing fibroblasts (IDO), IDO-expressing fibroblasts in the presence of 1-MT (IDO + 1-MT) or control non-treated fibroblasts (NT) were co-cultured with another strain of fibroblasts, non-transfected, transfected with a non-silencing control siRNA (Cont siRNA), or with 20 nM IMPACT siRNA in two chamber co-culture systems for 72 hours. Cell viability was then analyzed using a fluorescence-based live/dead cell viability assay kit. A) Panels a-i show the fluorescent microscopy images of the indicated groups of bystander fibroblasts. In this assay, dead cells are stained in red color and live cells are stained in green. B) Cell death rates were determined for each indicated group. Non-transfected fibroblasts, non-silencing control siRNA transfected fibroblasts (Cont siRNA), IMPACT siRNA transfected fibroblasts (IMPACT siRNA). NT (open bars), IDO (solid bars), IDO+1-MT (hatched bars). ANOVA with Bonferroni correction post-hoc test was used to calculate statistical significance. Significant (p value <0.0001) difference is indicated by an asterisk (\*), n=3.

**IMPACT Overexpression Rescues Jurkat Cells From the Effects of Tryptophan Deficiency**

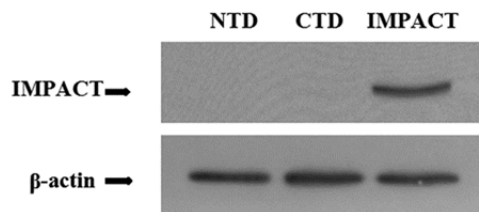
In order to further test our hypothesis, IMPACT gene was cloned into a lentiviral backbone (IMPACT/FUGWBW) (Figure 2.8A) to overexpress IMPACT protein in Jurkat cells. Empty vector was used as the control. Lentiviral vectors were then generated. Jurkat cells were either stably transduced with IMPACT vector (IMPACT) or with control empty vector (CTD) or left non-transduced (NTD) (Figure 2.8B). Proliferation assay was then performed for the IMPACT vector-transduced Jurkat cells and the non-transduced and control empty vector-transduced Jurkat cells in the regular or tryptophan-deficient environment in order to study the resistance of IMPACT over-expressing Jurkat cells to tryptophan deficiency. As shown in Figure 2.8D, proliferation of Jurkat cells transduced with IMPACT-expressing vector ( $30.2 \pm 1.8$ ) was rescued significantly in tryptophan-deficient environment in comparison with that of non-transduced Jurkat cells ( $17.9 \pm 1.3$ , n=3, p<0.0001) or control empty vector transduced Jurkat cells ( $18.3\% \pm 0.8$ , n=3, p<0.0001). The same procedure was also performed in the presence or absence of IDO. As shown in Figure 2.8E, presence of IDO was able to significantly inhibit the proliferation of Jurkat cells in all the groups. Although a trend was seen in rescue of IMPACT over-expressing Jurkat cells proliferation ( $12.5\% \pm 4.4$ ), the difference was not significant compared to that of the control groups of non-transduced Jurkat cells ( $9.2 \pm 2.8$ ) or control empty

vector transduced Jurkat cells ( $8.6 \pm 2.5$ ,  $n=3$ ,  $p<0.05$ ). This may be due to the high kynurenine levels in the IDO-conditioned medium that can cause apoptosis in Jurkat cells. Moreover, addition of 1-MT resulted in partial recovery of Jurkat cells from the inhibitory effects of IDO.

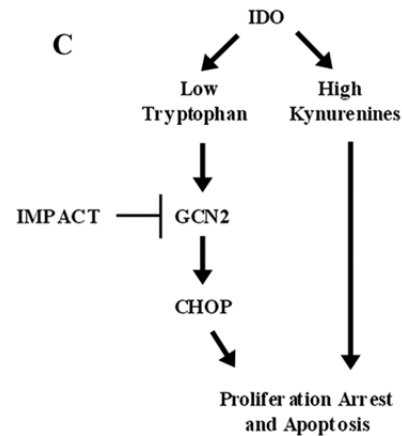
**A**



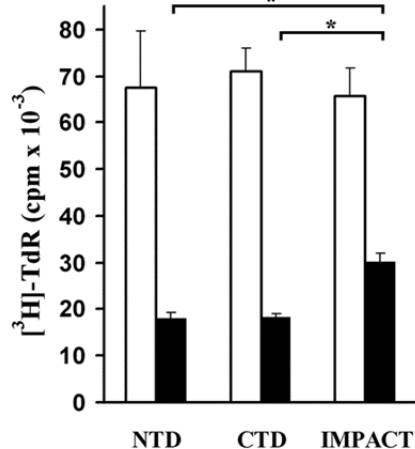
**B**



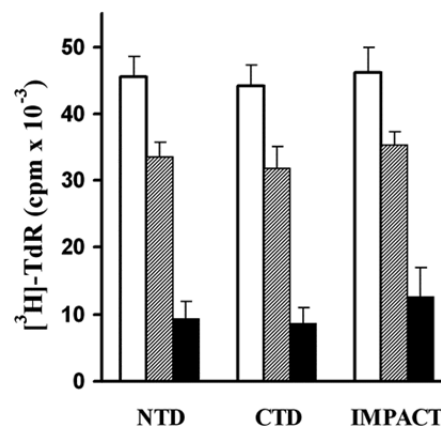
**C**



**D**



**E**





**Figure 2.8. Effects of tryptophan deficiency or IDO exposure on the proliferation of IMPACT-expressing Jurkat cells.**

A) Schematic diagram of the IMPACT/FUGWBW lentiviral construct. B) IMPACT protein expression in non-transduced Jurkat cells (NTD), control empty vector transduced Jurkat cells (CTD) and IMPACT-expressing vector transduced Jurkat cells (IMPACT). The blot was reprobated with a  $\beta$ -actin antibody as a loading control. A representative experiment is shown. C) Schematic diagram of suppressive effects of IDO on immune cells. D) IMPACT-expressing Jurkat cells and the control groups were cultured in regular (open bars) or tryptophan-deficient (solid bars) environment. Thymidine incorporation assay was performed to evaluate the cell proliferation. ANOVA with Bonferroni correction post-hoc test was used to calculate statistical significance. The significant ( $p$  value  $<0.0001$ ) difference are indicated by asterisks (\*),  $n=3$ . E) IMPACT-expressing Jurkat cells and the control groups were cultured in the absence (open bars) or presence (solid bars) of IDO or in the presence of IDO and 1-MT (hatched bars). Thymidine incorporation assay was performed to evaluate the cell proliferation ( $n=3$ ).

## **2.4. Discussion**

The main finding of this study is that protein IMPACT homolog, a GCN2 kinase pathway inhibitor, is highly and constitutively expressed in primary skin cells, making them more resistant to low tryptophan conditions caused by IDO expression. Data presented in this paper suggest that IMPACT abolishes the expression of the GCN2 pathway downstream target gene CHOP (GADD153) and therefore makes skin cells resistant to the environment generated by IDO expression. On the other hand, in immune cells where IMPACT is not expressed or expressed at a very low level, GCN2 kinase becomes activated under this stress condition and, as a result the apoptotic protein CHOP is highly expressed.

Several studies have confirmed the role of IDO in immunomodulation (e.g. in pregnancy) (30, 31) and autoimmune disease (37). It has also been shown that IDO expression in tumor cells can facilitate tumor escape and tumor resistance (74). Our group and others have shown that due to the potent immunoregulatory activity of IDO and its counter-regulatory effects to T cell stimulation, local expression of IDO may have promising potential in protecting allograft survival (6, 7, 145-148). Further, several studies conducted by our group confirmed that the suppressive effect of IDO expression is in fact selective and while IDO has potent suppressive

effects on immune cells, its effect on skin cells is minimal. Interestingly, the results also showed that even amongst different types of immune cells, bystander Jurkat cells co-cultured with IDO-expressing cells show significantly higher levels of apoptosis compared to that of primary CD4<sup>+</sup> as well as CD8<sup>+</sup> T cells (82% vs 20%) (10).

Although the selective effects of IDO on different cell types have been demonstrated, the downstream molecular mechanism(s) by which IDO suppresses T cells are poorly understood. GCN2 stress response kinase is critical for resistance to nutritional deficiencies as it results in repression of global protein synthesis while causing induced translation of the downstream target genes ATF4 and CHOP (143). It has recently been suggested that in murine T cells, GCN2 kinase pathway detects and responds to conditions generated by IDO (83). Our group has also shown that the selective suppressive effects of IDO expression on immune cells versus skin cells are at least in part due to the differential expression of CHOP, a well-accepted downstream marker gene of GCN2 kinase activation (11).

In this study, by detecting the level of phosphorylated as well as total GCN2, we have shown that there is a positive correlation between overexpression of CHOP with increased phosphorylation and activation of GCN2 kinase in response to IDO expression in Jurkat cells, a T cell line (Figure 2.2 B-D). We have also shown that under the same experimental conditions, GCN2 kinase is not activated in primary skin cells such as fibroblasts and keratinocytes. This study also shows that the level of GCN2 kinase activation as well as CHOP expression is significantly higher in Jurkat cells co-cultured with IDO-expressing cells compared to that of the control group. Moreover, our results show that Jurkat cells co-cultured in the presence of IDO-expressing cells express high levels of cleaved PARP as a marker for apoptosis. We suggest that

in the stressed low-tryptophan environment generated by IDO, Jurkat cells are not able to recover or survive, and therefore become apoptotic.

An important unanswered question, not addressed by previous studies, is why does GCN2 kinase pathway become selectively activated in immune cells and not in skin cells? According to the literature, protein IMPACT homolog was recently found to be an inhibitor for GCN2 kinase pathway (143). The human IMPACT protein is a conserved evolutionary protein located on chromosome 18q11.2–12.1 (141). It has been shown that IMPACT competes with GCN2 and binds to GCN1, the activator of GCN2, therefore inhibits the GCN2 kinase activation (143). IMPACT overexpression inhibits GCN2 activation under essential amino acid-deficient conditions and results in the inhibition of expression of downstream genes of GCN2 pathway such as CHOP and ATF4 (142, 143).

This study for the first time shows that the expression level of protein IMPACT homolog is indeed different in IDO-sensitive versus IDO-resistant cells. Our results indicate that IMPACT is expressed abundantly and constitutively at both the mRNA and protein levels in primary skin cells, i.e. fibroblasts and keratinocytes. IMPACT is expressed constitutively at a very low level in stimulated human primary T cells and is not detectable in Jurkat cells. The low presence of IMPACT protein in CD3<sup>+</sup> T cells may be due to a CD4<sup>+</sup> sub-population which has been previously shown by our group to be more resistant to the environment generated by IDO (101). Moreover, IMPACT expression level is independent of IDO-induced low tryptophan environment. These data collectively suggest that cells with high expression level of IMPACT are resistant to IDO-mediated activation of GCN2 kinase pathway due to IMPACT-induced inhibition of GCN2 kinase. As such, in these cells CHOP protein levels show no increase. On the other hand, in cells that express very low or no IMPACT protein,

GCN2 kinase pathway becomes activated in response to IDO-mediated stress environment and as a result, the level of CHOP increases. Moreover, differences between IMPACT expression levels in Jurkat cells compared to that of primary T cells explain our previous findings in regard to significantly higher apoptotic effects of IDO on Jurkat cells compared to that of primary T cells (10). This study also shows that knocking down the expression of IMPACT in fibroblasts co-cultured with IDO-expressing cells reduces the proliferation as well as viability of these cells. Moreover, this study shows that in the presence of IDO, IMPACT knocked-down fibroblasts demonstrate marked upregulation of CHOP protein. Primary fibroblasts are generally resistant to apoptosis induced in many conditions (179); therefore, cell death induction in these cells is remarkable.

As mentioned in the introduction and also shown in Figure 2.8C, suppressive effects of IDO on immune cells are attributed to: 1) IDO-induced tryptophan deficiency and 2) cytotoxic effects of tryptophan metabolites (kynurenines). The result of Figure 2.8D indicates that overexpression of IMPACT protein in IDO sensitive cells (Jurkat cells) makes them resistant to tryptophan deficiency and rescues their proliferation rates in tryptophan-deficient environment. On the other hand, IMPACT overexpression in Jurkat cells cannot completely rescue the cells from the cytotoxic effects of kynurenine products generated in the presence of IDO. Therefore, although a trend in augmented proliferation is seen in IMPACT-overexpressing Jurkat cells in the presence of IDO, we were not able to detect a significant increase in the proliferation rates of these cells (Figure 2.8E).

Although our data evidently show that IMPACT expression in primary skin cells makes them resistant to low tryptophan environment generated by IDO, other yet unknown additional mechanism(s) may exist in resistance of these cells to IDO effects.

Our findings indicate that the level of IMPACT expression in fibroblasts increases in a time-dependent manner under stress conditions generated by a tryptophan-deficient environment. Here we suggest that in this deprived environment, fibroblasts perhaps increase the expression of IMPACT in order to combat with the imposed stress and to ensure constant high level of protein synthesis and translation.

It should be mentioned that IDO inhibitors are currently being used in phase I clinical trials in order to improve cancer chemotherapy (74). Therefore, IMPACT inhibition if proven to be high in cancer cells, may be considered as a legitimate strategy in anti-cancer therapy.

In summary, our findings demonstrate for the first time that the differential expression of protein IMPACT homolog in primary skin cells versus immune cells makes them resistant to the stress environment generated by IDO expression.

## CHAPTER 3

### Highly Efficient Stable Expression of Indoleamine 2, 3-Dioxygenase Gene in Primary Fibroblasts <sup>2</sup>

#### 3.1. Introduction

Indoleamine 2, 3-dioxygenase (IDO) is a monomeric, heme-containing enzyme that catalyzes the rate-limiting step of conversion of tryptophan to kynurenine (60). Recently, tryptophan catabolism has been implicated in immunological tolerance. One theory proposes that degradation of tryptophan suppresses T cell proliferation by reducing the availability of this essential amino acid in local tissue environments, thereby sensitizing T cells to apoptosis (51). Another theory suggests that the major tryptophan metabolite, kynurenine, suppresses immune reactivity through direct interaction with effector T lymphocytes (180). The immunomodulatory effects of tryptophan deficiency and excess kynurenine caused by IDO are of particular interest in the field of transplantation (52).

A critical aspect of an immunological intervention using IDO is the requirement to achieve effective expression of this gene. However, genetic manipulation by non-viral transfection approaches has been challenging due to the issues of low transfection efficiency, loss of cell viability, and difficulty in obtaining stable transfection (181, 182). Previously, we have shown that by using dermal fibroblasts transduced with an IDO-expressing adenoviral vector, IDO functions as a local immunosuppressive factor (10), and local expression of IDO

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<sup>2</sup> A version of this chapter has been published. With kind permission from Springer Science+Business Media: Biol Proced Online, Highly Efficient Stable Expression of Indoleamine 2,3 Dioxygenase Gene in Primary Fibroblasts, 2010, 12:107-112, Rezakhanlou AM\*, Habibi D\*, Lai A, Jalili RB, Ong CJ, Ghahary A. \* Co-first Authors.

suppresses islet allogeneic immune response in mouse islet transplantation (145). Further, we have used the local immunosuppressive effect of IDO in the development of a non-rejectable skin substitute (6). These findings demonstrate that IDO has considerable potential for immunoregulation and induction of immunotolerance in transplantation.

Nevertheless, the immunogenicity and transient gene expression of adenoviral vectors may hinder its clinical use in transplantation. Thus, in order to sustain the efficacy of IDO activity in the local environment of transplanted organ, it is essential to prolong the expression of functional IDO protein. It was shown that lentiviral vectors can maintain efficient target gene expression *in vivo* for more than four years (183). It has been reported that polybrene can markedly enhance the retrovirus transduction efficiency (184). In this report, we constructed an IDO-expressing lentiviral vector and showed that treatment of cells with polybrene enhanced IDO transduction efficiency almost 10 times. To enrich IDO expressing cells and thereby optimize transplantation immunotolerance, we selectively isolated the IDO-positive cells by fluorescence-activated cell sorting (FACS), and obtained a greater than 95% pure population of IDO-expressing cells. This approach will allow us to have a pure population of IDO-expressing fibroblasts in order to be used in our future allograft models and is advantageous when detection of the exogenous gene expression is needed.

As our second approach, we also constructed an IDO-expressing lentiviral vector containing blastidicin resistance gene and used this vector in order to obtain a pure population of IDO-expressing fibroblasts without the need to perform FACS analysis. This approach is useful when a pure population of IDO-expressing fibroblasts are needed in a short period of time in order to develop non-rejectable grafts.

### 3.2. Materials and Methods

#### Plasmid Constructions

A lentiviral construct for expressing the IDO gene was generated using the pLC-E vector (175) modified from the lentiviral backbone FUGW (176). For visualization of the lentiviral-mediated IDO expression, a sequence encoding the red fluorescent mCherry protein under the control of the UbC promoter was incorporated into the vector. The human IDO gene (NM\_002164) (a generous gift from Dr. J.M. Carlin of Miami University) was generated by PCR using a full-length cDNA encoding the gene as template and the forward primer (5'-GGG GACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGCACACGCTATGGAAAACCTCCT GG-3') and reverse primer (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAA CCTTCCTTCAAAGGGATTCTC-3'). The amplified PCR product was first inserted into an entry vector (pDON201), and then Gateway (Invitrogen) cloned into a lentiviral pLC-E expression vector. The IDO gene is expressed under the control of EF1- $\alpha$  promoter and the mCherry red fluorescent gene, a reporter gene, is expressed under the control of a ubiquitin promoter. The plasmid was amplified in competent DH10-B bacteria and purified using the Qiagen Plasmid DNA Maxi-prep kit (Qiagen). Sequence of the IDO/pLC-E construct was confirmed by DNA sequencing analysis.

In addition, a lentiviral construct for expressing the IDO gene was generated using the FUGWBW vector modified from the lentiviral backbone FUGW where the GFP gene was replaced by the Gateway cassette and a blasticidin S resistance gene expression cassette was inserted downstream of the Gateway cassette (175, 176). The human IDO gene (NM\_002164) was generated by PCR using a full-length cDNA encoding the gene as template and the IDO forward and reverse primers as described above. IDO gene was sub-cloned into pDON201, and



then into the lentiviral FUGWBW expression vector. The plasmid was amplified in competent DH10-B bacteria and purified using the Qiagen Plasmid DNA Maxi-prep kit (Qiagen). Sequence of the IDO/FUGWBW construct was confirmed by DNA sequencing analysis. Empty FUGWBW vector was used as the control.

## **Cell Culture**

Skin samples were collected from 6-to 8-week-old male C57BL/6 (B6) mice according to the guidelines of the Animal Policy and Welfare Committee of the University of British Columbia. The samples were then washed in sterile DMEM (Invitrogen Life Technologies, Carlsbad, CA) supplemented with antibiotic-antimycotic preparation (100 U/ml penicillin, 100 mg/ml streptomycin, 0.25 mg/ml amphotericin B (Invitrogen). Cultures of fibroblasts were established as previously described (185), and grown in DMEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen Life Technologies, Carlsbad, CA). Confluent cells were released by trypsinization, reseeded onto 75 cm<sup>2</sup> cell culture flasks (BD Biosciences, MA), and incubated in a humidified incubator at 37°C supplied with 5% CO<sub>2</sub>. Fibroblasts at passages three to five were used in all experiments.

## **Lentiviral Vector Production and Cell Transduction**

Replication-defective lentiviral vectors were generated as previously described (177, 178) by transient transfection of 293T human kidney cells with either IDO/pLC-E vector or IDO/FUGWBW vector, R8.9 (packaging plasmid) and VSV-G (envelope plasmid). 293T cells were seeded the night before transfection in DMEM medium supplemented with 10% fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA). The cells were replaced with

DMEM plus 10% FBS, and transfected at 60-70% confluency using ProFectin (Mammalian Transfection System Calcium Phosphate; Promega). For each 100 mm<sup>2</sup> round plate, 10 µg IDO/pLC-E vector, 7.5 µg, R8.9 plasmid and 2.5 µg VSV-G plasmid were used. The culture medium was replaced with a fresh medium containing DMEM and 5% FBS 12-16 hours after transfection. Supernatant containing lentiviral vectors was harvested 30 hours after the medium change. The vector stocks were concentrated by centrifugation at 126,000 g for 90 min using a Beckman Ultracentrifuge and stored at -80°C until use.

For transduction, 293T cells or mouse fibroblasts were seeded on flat bottom 6-well cell culture plates (Corning Incorporated, Corning, NY, USA) and incubated with IDO/pLC-E lentiviral vector for 24 hours in the absence or presence of 10 µg/ml polybrene.

In the case of IDO/FUGWBW vector, seeded mouse fibroblasts were incubated with the same titer of IDO lentiviral vector or the control empty vector for 24 hours. Blasticidin selection (5 µg/ml) was subsequently performed for a period of two weeks prior to validation of the vector in order to obtain a stable population of IDO-expressing or control fibroblasts. Thereafter, transduced fibroblasts were continuously kept in blasticidin containing medium.

### **SDS-PAGE and Western Blotting**

For detection of the IDO protein expression, transduced cells were harvested 24 hours post-transduction and washed twice with PBS. Cells were then lysed in lysis buffer (50mM Tris-HCl, pH 7.4; 10 mM EDTA; 5 mM EGTA; 0.5% Igepal; 1% Triton X-100, and protease inhibitor cocktail (Sigma). Equal amounts of total protein from each individual 293T cell culture were separated by 10% SDS-PAGE. Proteins were then transferred to a PVDF membrane (Millipore Corp., Bedford, MA) and immunoblotted with a polyclonal anti-human IDO antibody

(Washington Biotechnology Inc., Baltimore, MD) at final dilution of 1:5,000. Horseradish peroxidase conjugated goat anti-rabbit IgG was used as the secondary antibody for the enhanced chemiluminescence detection system (ECL; Amersham Biosciences, UK). Blots were then stripped and reprobed for  $\beta$ -actin as a control for protein loading.

### **Fluorescence Microscopy**

Lentiviral-mediated IDO expression in IDO/pLC-E vector-transduced 293T cells and mouse fibroblasts were examined by fluorescence microscopy with a Zeiss Axiovert 200 M microscope. Images from identical areas of cultured cells were recorded using both fluorescence and bright-field microscopy. Images were captured using Northern Eclipse image analysis software.

### **Kynurenine Assay**

The biological activity of IDO was evaluated by measuring the level of tryptophan degradation product, L-kynurenine, present in the conditioned medium of transduced cells. The amount of L-kynurenine was measured by a previously established method (174). Proteins in the conditioned medium were precipitated by trichloroacetic acid. After centrifugation, 0.5 ml of supernatant was incubated with an equal volume of Ehrlich's reagent at room temperature for 10 min. The reaction mixture was measured spectrophotometrically at 490 nm. The concentration of kynurenine in the conditioned medium was calculated according to a standard curve of defined kynurenine concentration (0-20 mg/ml).

## Flow Cytometry

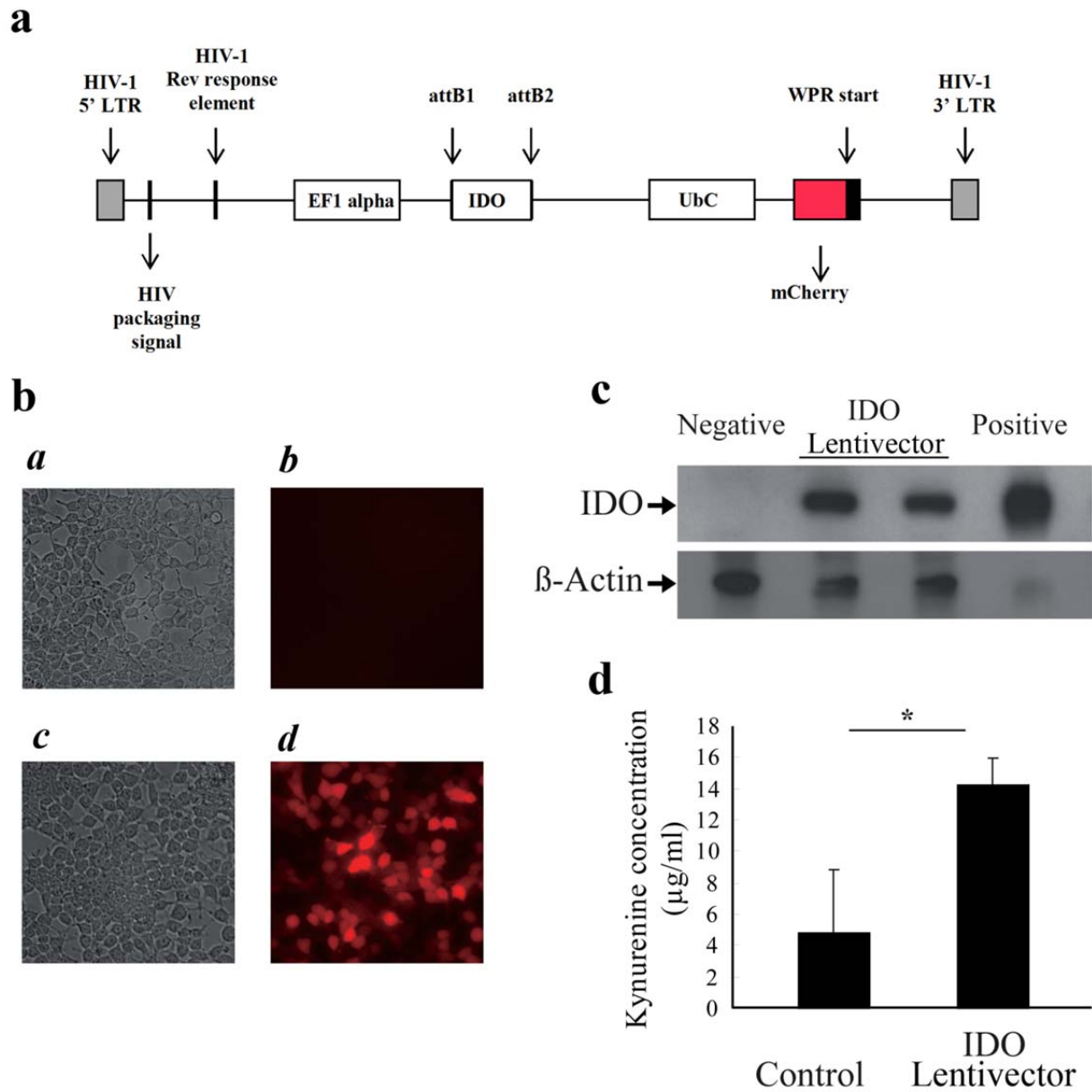
To determine the transduction efficiency of the IDO lentiviral vector, and in order to sort mCherry positive cells, non-transduced cultured fibroblasts, fibroblasts transduced with IDO lentiviral vector in the absence or presence of polybrene were trypsinized, collected, and centrifuged. The cell pellet was washed twice with PBS, and resuspended at  $10^6$  cells/ml for flow cytometry and at  $10^7$  cells/ml for sorting in PBS plus 2% FBS. For flow cytometry, a LSRII (BD) with 630 LP and 670/30 detectors were used. For sorting, a FACSAria (BD) with a blue laser detector was used. In each set, live cells were gated using Forward Scatter Channel (FSC) vs Side Scatter Channel (SSC) followed by gating on the IDO-expressing mCherry positive cells. The number of mCherry positive cells for fibroblasts transduced with IDO lentiviral vector in the presence of polybrene after sorting was also determined by flow cytometry analysis. The average purity was greater than 95%.

## 3.3. Results and Discussion

### Characterization of IDO/pLC-E Lentiviral-Mediated IDO Expression

A schematic diagram of the IDO-mCherry lentiviral vector is shown in Figure 3.1a. IDO-expressing lentiviral vector preparations were generated. To examine the efficacy of the generated lentiviral vector, the vector was first collected from the transfected 293T cells and used to transduce a fresh culture of 293T cells. The red-fluorescent mCherry protein acts as a reporter for the activity of the IDO gene, verifying that the IDO gene is present and expressed in the transduced cells (Figure 3.1b). As shown in Figure 3.1bd, majority of transduced cells express red fluorescent mCherry protein as a reporter gene for IDO expression compared to that of control cells (Figure 3.1bb). Panels *c* and *a* show the images of the same cells in bright field.

Further, to examine the protein expression of the exogenously introduced IDO gene, lysates of the transduced cells were subjected to SDS-PAGE and immunoblotted with an anti-IDO polyclonal antibody (Figure 3.1c). As seen in Figure 3.1c, the IDO expression was positive in both viral preparations, and the transduction was successful. Non-transduced cell lysate was used as a negative control and recombinant IDO protein was used as a positive control. In order to test whether the overexpressed IDO protein was functional, kynurenine assay was performed to measure the concentration of the major tryptophan degradation product (L-kynurenine) in the conditioned media. In comparison with non-transduced cells, the concentration of L-kynurenine in the conditioned media of transduced cells increased nearly three-fold (Figure 3.1d) and the result was significantly different from that of non-transduced cells ( $P < 0.05$ ,  $n=3$ ). The results show that the IDO-expressing lentiviral vector generated by transient transfection of 293T cells was functional and suitable for transduction of primary fibroblasts.



**Figure 3.1. Construction of a lentiviral-based vector for delivering the IDO gene.**

a) Schematic diagram of the IDO-mCherry lentiviral construct. b) Fluorescence microscopy analysis of IDO-expressing cells. Panels *a* and *b* as well as *c* and *d* represent bright-field and fluorescent images of the IDO/mCherry (red) expression in non-transduced and transduced 293T cells, respectively. c) Lentiviral vector-mediated IDO protein expression in transduced 293T cells. The arrow on the upper band shows the IDO protein. The IDO lentiviral vector lanes represent two separate viral preparations. The blot was reprobbed with a  $\beta$ -actin antibody as a loading control. d) Kynurenine assay. Functional IDO activity was evaluated by measuring the content of kynurenine in the conditioned media of non-transduced and IDO-lentiviral vector transduced cells. The data shown are the mean and standard deviation of kynurenine in conditioned media of three separate experiments.

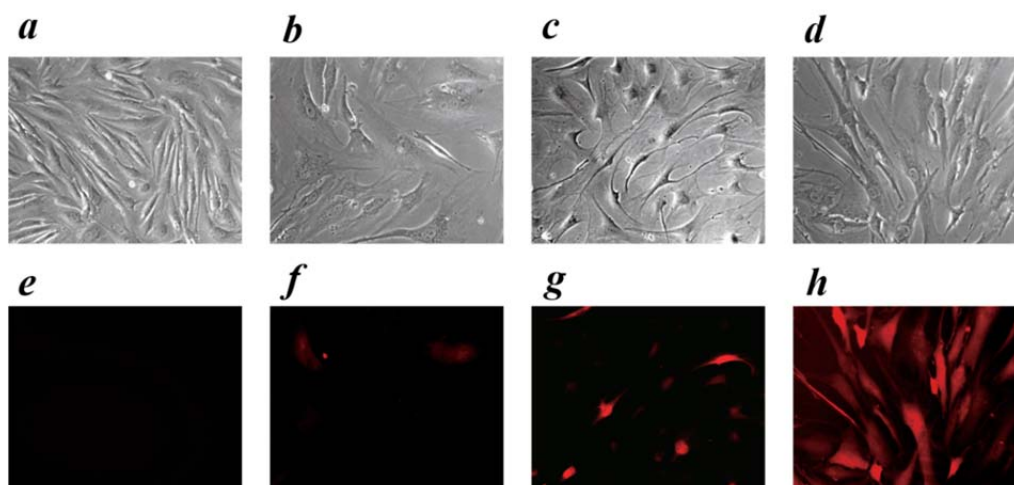
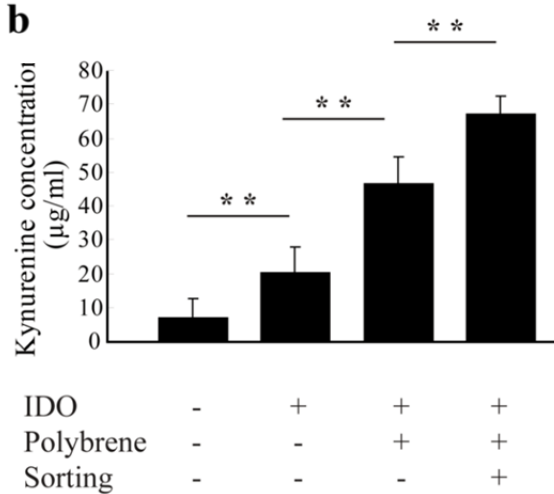
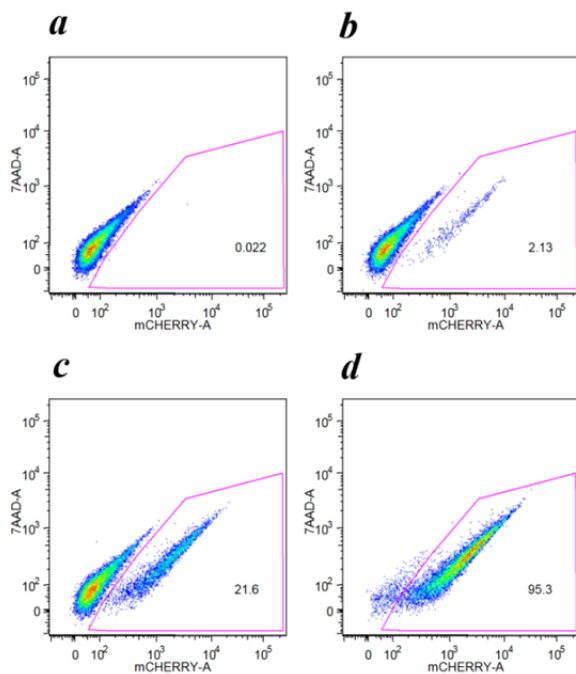
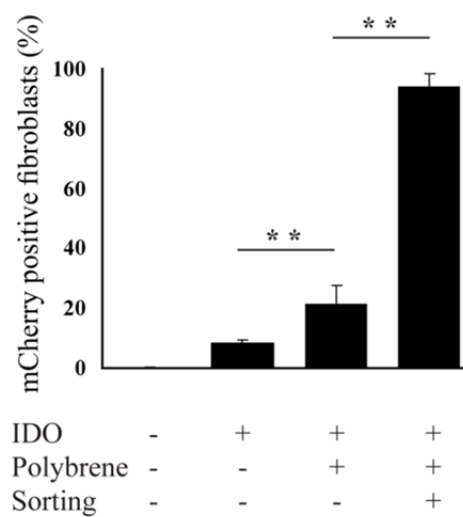
## Transduction of Primary Mouse Fibroblasts with IDO/pLC-E-Expressing Lentiviral Vector

Dermal fibroblasts were transduced with the IDO-expressing lentiviral vector. To analyze lentiviral-mediated IDO expression, transduced cells were visualized by fluorescence microscopy 24 hours post-transduction (Figure 3.2a). The upper and lower panels are bright-field images and fluorescence images, respectively. Fluorescence images are images of the same groups of cells captured by the bright-field microscope, and the fluorescence is due to expression of the mCherry gene. Panels *a* and *e* as well as *b* and *f* represent non-transduced fibroblasts and IDO-lentiviral vector transduced fibroblasts, respectively. To facilitate delivery of the IDO lentiviral vector, polybrene was added to cells at the time of transduction. The cells were similarly examined by fluorescence microscopy 48 hours post-transduction (Figure 3.2a, panels *c* and *g*). Transduced fibroblasts in the presence of polybrene were gated and sorted by FACS and examined by fluorescence microscopy (Figure 3.2a, panels *d* and *h*). As the fluorescent images show, the level of mCherry positive cells increased in the presence of polybrene and after sorting. The functionality of the overexpressed IDO in these cells was assessed by kynurenine assay (Figure 3.2b). The kynurenine content in the conditioned media of mouse fibroblasts transduced in the presence of polybrene increased approximately two-fold when compared to that of control with no polybrene treatment ( $P < 0.01$ ,  $n=3$ ). This finding revealed that polybrene significantly enhanced the transduction efficiency in primary mouse fibroblasts which resulted in enhanced IDO protein expression and induction in the levels of IDO enzymatic activity.

## **Analysis of Improvement of IDO/pLC-E Lentiviral Vector Transduction by Flow Cytometry**

To maximize the chances for successful transplantation and reduce the risk of immunorejection, it would be advantageous to have a highly pure population of IDO-expressing cells. By FACS, we sorted the IDO-mCherry fluorescent protein expressing cells (Figure 3.2c). Panel *a* of Figure 3.2c shows that there is less than 0.02% of autofluorescent cells in the negative-cell population. As shown in panels *b* and *c* of Figure 3.2c, treatment of IDO-lentiviral vector transduced cells by polybrene increased the number of mCherry positive cells by 10 fold. These cells were then gated and sorted by FACS. Figure 3.2c, panel *d*, shows that more than 95% of the cell population after sorting were expressing IDO, and this was confirmed by flow cytometry, fluorescence microscopy and kynurenine assay. The transduction efficiency was shown as percentage of mCherry positive fluorescent cells in the total cell population studied (Figure 3.2c, panel *e*). Transduction of mouse fibroblasts in the presence of polybrene showed a significant increase in the number of IDO-expressing cells ( $P < 0.01$ ,  $n=3$ ). As a result, we successfully obtained a cell population that contains more than 95% IDO-expressing primary fibroblasts (Figure 3.2c, panels *d-e*).



**a****b****c****e**

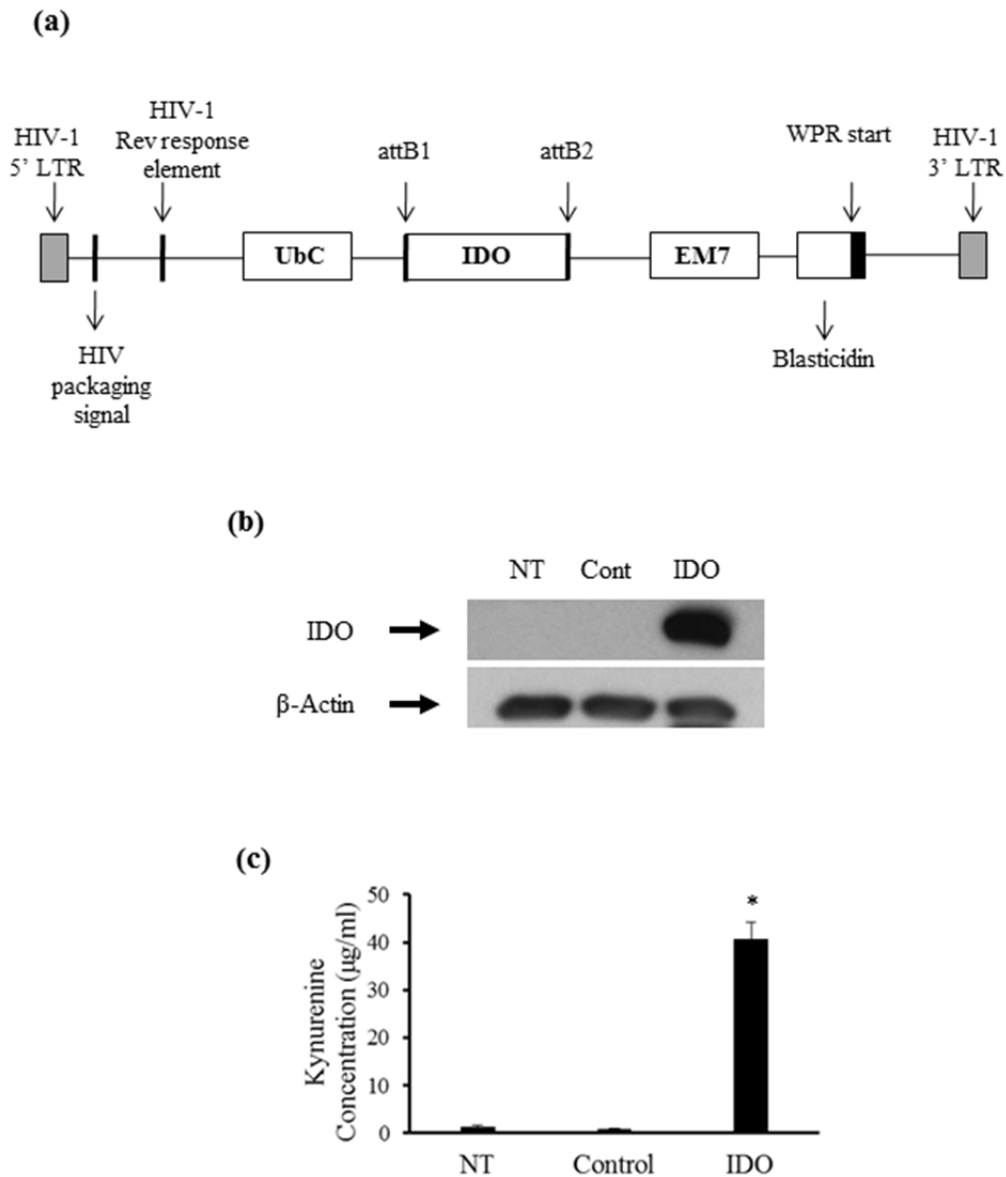
**Figure 3.2. Transduction of mouse fibroblasts with IDO/pLC-E -expressing lentiviral vector.**

a) Fluorescence microscopy analysis of IDO-lentiviral vector transduced fibroblasts. Cultured mouse fibroblasts were incubated with IDO lentiviral vector either in the presence or absence of polybrene. Cells that were transduced in the presence of polybrene were then sorted by FACS. Panels *a* and *e*, *b* and *f*, *c* and *g* as well as *d* and *h* represent bright-field and fluorescence images of non-transduced fibroblasts, IDO-lentiviral vector transduced fibroblasts, fibroblasts transduced with IDO-expressing lentiviral vector in the presence of polybrene, and fibroblasts transduced with IDO-expressing lentiviral vector in the presence of polybrene after sorting, respectively. b) Kynurenine levels in different indicated cells. The data shown are the mean and standard deviation of kynurenine measurements obtained from 3 different experiments. c) Flow cytometry analysis of the IDO-expressing cell population. Fibroblasts were left either non-transduced or transduced with IDO-expressing lentiviral vector in the absence or presence of polybrene and flow cytometry was then conducted by gating the cells based on their mCherry fluorescence. The selected cell population was prepared by cell sorting and the number of mCherry positive cells was greater than 95%. Panel *a* shows non-transduced fibroblasts, *b*, transduced IDO-expressing fibroblasts in the absence of polybrene, *c*, transduced IDO-expressing fibroblasts in the presence of polybrene, and *d*, FACS-sorted IDO-expressing fibroblasts (in the presence of polybrene). Panel *e* shows the percentage of mCherry positive fluorescent cells in the total cell population studied as detected by flow cytometry. The data shown are the mean and standard deviation of mCherry positive cells obtained from 3 separate experiments.

**Transduction of Primary Mouse Fibroblasts with IDO/FUGWBW-Expressing**

**Lentiviral Vector**

The IDO gene was cloned into the FUGWBW lentiviral vector (IDO/FUGWBW; Figure 3.3a). Empty vector was used as the control. Lentiviral vectors were generated. In order to analyze lentiviral-mediated IDO expression, primary mouse fibroblasts were stably transduced with IDO vector (IDO), or with control empty vector (Cont), or left non-treated (NT; Figure 3.3b) and blasticidin selection was performed. As shown in Figure 3.3b, western blot results confirm high levels of IDO protein expression in the IDO-transduced fibroblasts. In addition, kynurenine measurement was performed in order to confirm the functionality of the expressed IDO protein (Figure 3.3c). The concentration of L-kynurenine in the conditioned media of IDO-transduced cells increased significantly compared to that of non-transduced or control vector-transduced cells ( $P < 0.0001$ ,  $n=3$ ). As a result, using blasticidin selection, we obtained an enriched population of IDO-expressing primary fibroblasts without the need to perform sorting.



**Figure 3.3. Transduction of primary mouse fibroblasts with IDO/FUGWBW-expressing lentiviral vector.**

a) Schematic diagram of the IDO/FUGWBW lentiviral construct. b) Result of western blot analysis for IDO/FUGWBW-transduced primary mouse fibroblasts. Blots were stripped and reprobed for  $\beta$ -actin as equal loading control. A representative experiment is shown. c) The level of kynurenine in the conditioned media of non-treated (NT), control empty vector-transduced and IDO-transduced (IDO) fibroblasts. The data shown are the mean and standard deviation of kynurenine in conditioned media of three separate experiments.

As efficient gene delivery is a prerequisite for successful gene therapy, we believe that a) in our first approach polybrene significantly increases lentiviral-mediated delivery of the IDO gene into target cells and mCherry expression is advantageous when detection of the exogenous gene is required b) using blasticidin selection provide a simple and fast yet high-efficient approach to generate a pure population of IDO-expressing fibroblasts without the need to perform FACS analysis. These IDO delivery approaches make this enzyme a promising and highly attractive strategy for improving the outcome of cell and possibly organ transplantation.

## CHAPTER 4

### **Borrelidin, a Small Molecule Nitrile-Containing Macrolide Inhibitor of Threnoyl-tRNA Synthetase, is a Potent Inducer of Apoptosis in Acute Lymphoblastic Leukemia <sup>3</sup>**

#### **4.1. Introduction**

Acute lymphoblastic leukemia (ALL) is a clonal proliferation and expansion of immature lymphoid cells which results in an accumulation of leukemic blasts in the bone marrow and various extramedullary sites (152-154). It affects both adults and children and each year 5,000 cases of ALL are diagnosed in the United States (153-156). Improvements in primary therapy for childhood ALL, has led to an overall complete remission rate of about 80%. However, although rates of complete remission in adult ALL are high (80% to 90%), long-term disease-free survival ensues in only approximately 40% and the rest of the adult patients relapse within first two years (152, 153, 156). This has been attributed to poor tolerance of and compliance with treatment, an increased frequency of high-risk leukemia with greater drug resistance, less effective treatment regimens, and reluctance to accept temporary toxic effects, as compared with childhood ALL (157). Therefore, development of new therapies that can work alone or in combination with existing approved drugs is much needed in order to extend the complete remission time or be used in the refractory adult ALL patients.

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<sup>3</sup> A version of this chapter has been published. With kind permission from Springer Science+Business Media: Invest New Drugs, Borrelidin, a Small Molecule Nitrile-Containing Macrolide Inhibitor of Threonyl-tRNA synthetase, is a Potent Inducer of Apoptosis in Acute Lymphoblastic Leukemia, 2011, DOI 10.1007/s10637-011-9700-y, Habibi D, Ogloff N, Jalili RB, Yost A, Weng AP, Ghahary A, Ong CJ.

ALL therapy is among the most complex anticancer programs, comprising 6-12 drug treatments (152, 155). L-asparaginase (ASNase) has been used for both pediatric and adult ALL therapies over the past 30 years. This enzyme hydrolyzes the deamination of L-asparagine (Asn) to ammonia and aspartic acid (155, 158). Asn depletion leads to an increase in intracellular levels of uncharged tRNA, nutritional deprivation and inhibition of RNA and DNA synthesis, resulting in protein biosynthesis inhibition and subsequent apoptotic cell death of the leukemic cells (155, 158). The use of ASNase in ALL therapy suggests that other amino acid limitation strategies might be used in treatment of ALL. Moreover, amino acid deprivation strategies have previously been used in treatment of several tumors including hepatocellular carcinomas and melanomas (161, 162).

Mechanistically, ASNase treatment induces the activation of the stress-responsive kinase general control nonderepressible 2 (GCN2) (159, 160). Amino acid deprivation, Asn in case of ASNase, causes a rise in the amount of uncharged transfer RNA (tRNA) in cells that activates the regulatory domain of GCN2 triggering its kinase activity and subsequently phosphorylation of eukaryotic initiation factor 2 (eIF2 $\alpha$ ). Activation of the GCN2 kinase pathway and eIF2 $\alpha$  phosphorylation can result in apoptosis, cell cycle arrest, differentiation, or compensatory adaption depending on the initiating stress and cell type (132, 170, 171, 186). The CCAAT/enhancer-binding protein homologous protein (CHOP, also known as GADD 153) is a DNA damage-inducible nuclear leucine zipper protein that has role in induction of apoptosis (186). CHOP is a downstream target gene in the GCN2 pathway and is as a well-accepted marker for GCN2 activation (83, 132). While CHOP can be induced by other stresses as well, its induction by amino acid deficiency is known to be specifically mediated by GCN2 (83, 132).

The small molecule drug, borrelidin, is a nitrile-containing macrolide antibiotic isolated from *Streptomyces*. Borrelidin was originally identified as an anti-bacterial, anti-viral, and anti-malarial agent. Borrelidin was also found to be a potent and selective inhibitor of bacterial and eukaryal threonyl-tRNA synthetase (ThrRS), an enzyme which functions to couple the essential amino acid threonine with its cognate tRNA. Since aminoacyl-tRNAs are the products of the first step in protein biosynthesis, borrelidin-mediated inhibition of aminoacyl-tRNA synthesis leads to an induction in the levels of uncharged tRNA and ultimately inhibition of protein synthesis (165, 166). Indoleamine 2, 3-dioxygenase (IDO), similarly to ASase and borrelidin, induces amino acid deprivation-mediated stress in cells through catabolism of tryptophan. It has been shown that the apoptotic effects of IDO on immune cells are at least in part due to the selective activation of GCN2 kinase pathway in these cells (7, 11, 187).

Borrelidin can mimic the effects of amino acid deprivation by inducing an increase in uncharged tRNAs. As such, in this study we hypothesize that borrelidin-induced nutritional stress will block ALL cell proliferation and therefore may provide a potential therapy in treating ALL. We aimed to investigate if borrelidin could have a potential role in targeting ALL cell lines and whether the GCN2 kinase pathway activation is involved as one of the downstream mechanisms by which borrelidin affects lymphoblastic cells. Our results indicate that borrelidin can selectively impair proliferation, and induce cell cycle arrest and apoptosis of the malignant ALL cell lines. Borrelidin treatment resulted in a marked activation of GCN2 kinase pathway and induction of CHOP protein in ALL cell lines.

## **4.2. Materials and Methods**

### **Cell Culture**

Malignant human ALL cell lines, Jurkat and CEM cells, (ATCC, Manassas, VA) were used for majority of the experiments in this study as these cells are widely used to study acute lymphoblastic leukemia. These cells were maintained in RPMI 1640 (Hyclone, UT) containing 10% fetal bovine serum (FBS) and 2 mM L-glutamine and antibiotic-antimycotic preparations (100 U/ml penicillin, 100 mg/ml streptomycin, 0.25 mg/ml amphotericin B (Invitrogen, Carlsbad, CA)). A custom made tryptophan-deficient RPMI 1640 (Hyclone, UT) was used as a positive control.

Cultures of human fibroblasts were established and the procedure was done based on the approval of Ethics committee of the University of British Columbia as previously described (173). Cells were grown in DMEM supplemented with 10% FBS, and 2 mM L-glutamine and antibiotic-antimycotic preparations (100 U/ml penicillin, 100 mg/ml streptomycin, 0.25 mg/ml amphotericin B (Invitrogen)). Confluent cells were released by trypsinization, reseeded onto 75 cm<sup>2</sup> cell culture flasks (BD Biosciences, MA). Fibroblasts at passages three to five were used in all experiments. Cells were cultured at 37° C in a 5% CO<sub>2</sub> atmosphere.

### **Borrelidin Treatment of Jurkat and CEM Cells**

Jurkat and CEM cells were seeded in 6-well plates. Dose response was performed using different concentrations of borrelidin (Alexis Biochemicals, PA) and 20, and 40 ng/ml were chosen for subsequent experiments as submaximal doses that reflect the effects of borrelidin. In order to inhibit threonyl-tRNA synthesis and to induce nutritional stress, Jurak and CEM cells were treated with 0 (DMSO as vehicle), 20, or 40 ng/ml borrelidin.



### **Tritiated Thymidine Incorporation Assay**

Cell proliferation was measured by [<sup>3</sup>H] thymidine incorporation assay. Fibroblasts, Jurkat and CEM cells were each seeded in 96-well plates, treated with a titration of borrelidin and incubated for 48 hours at 37°C in a 5% CO<sub>2</sub> atmosphere. Cells were then pulsed by [<sup>3</sup>H] thymidine (1 µCi/well, PerkinElmer, MA) and incubated for 16 hours. Cells were lysed by freezing and thawing at -80° C and harvested on a Harvester 96 Mach III M (TOMTEC, Milano, Italy). The amount of incorporated thymidine was measured using a Wallac-1450-MicroBeta Jet β-scintillation machine (American Laboratory Trading, Inc. CT).

### **Cell Growth Assay**

In order to study the cell growth in the absence or presence of borrelidin, Jurkat cells were seeded in 96-well plates and treated with either vehicle or 40 ng/ml (as a submaximal dose) borrelidin. The number of cells in each well were counted using a hemocytometer every day for 7 days.

### **Apoptosis Assay and Cell Cycle Progression Analysis**

In order to study the effects of borrelidin treatment on the level of apoptosis and on the cell cycle distribution of Jurkat and CEM cells, cells were seeded in 6 well plates and cultured in the presence of 0 (vehicle), 20, or 40 ng/ml borrelidin. After 72 hours, cells were diluted 10-fold in cold PBS, pelleted by centrifugation and fixed in 1.0 ml 80% ethanol. Fixed cells were incubated at 4°C overnight, rinsed in 1.0 ml PBS, and then washed in 1.0 ml staining buffer (PBS/0.1% Triton X-100) followed by a 30 min incubation in 0.5 ml staining buffer supplemented with RNaseA 50µg/ml and propidium iodide (PI) 50 µg/ml (Sigma Aldrich Inc.,

St. Louis, MO). The proportion of cells containing sub-G<sub>0</sub>/G<sub>1</sub>, G<sub>1</sub>, and S phases were determined by FACSCanto II flow cytometer (Becton Dickinson, San Jose, CA) using FlowJo software (Tree Star, Inc. Ashland, OR).

### **Western Blot Analysis**

Jurkat and CEM cells were harvested after treatment with borrelidin, washed twice with PBS, and lysed in lysis buffer (50mM Tris-HCl, pH 7.4; 10 mM EDTA; 5 mM EGTA; 0.5% Igepal; 1% Triton X-100, and protease inhibitor cocktail (Sigma)). Cell lysates were centrifuged at 14,000 g for 10 min. The protein concentration of supernatant was measured by BCA protein assay kit (Pierce, Rockfield, IL). Equal amounts of cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to a PVDF membrane (Millipore Corp., Bedford, MA). After blocking with 5% skim milk, the blots were probed with the following antibodies: anti phospho-eIF2 $\alpha$  (Ser51, 1:1,000 dilution, Cell Signaling Technology INC., Beverly, MA), anti eIF2 $\alpha$  (1:1,000 dilution, Cell Signaling), anti phospho-GCN2 (Thr898, 1:1000 dilution, Cell Signaling), anti GCN2 (1:1000 dilution, Cell Signaling), anti-CHOP (1:1000 dilution, Cell Signaling) and anti-cleaved PARP (Asp214) antibody (1:1,000, Cell Signaling). Horseradish peroxidase conjugated goat anti-rabbit IgG and horseradish peroxidase conjugated goat anti-mouse IgG served as secondary antibodies for the enhanced chemiluminescence detection system (ECL; Amersham Biosciences, UK). Blots were then stripped and reprobed for  $\beta$ -actin (1:25,000 dilution, Sigma) and used as a control of protein loading.

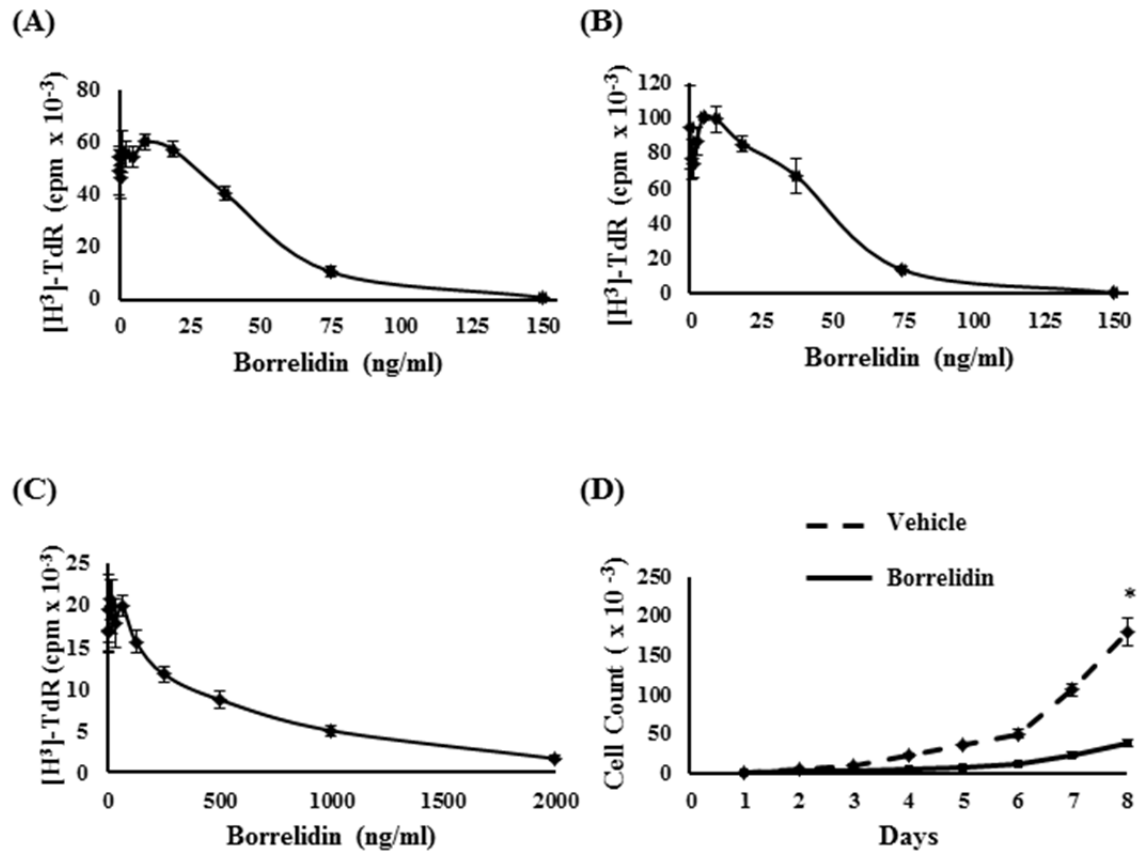
## Statistical Analysis

All data are reported as mean  $\pm$  SD. Statistical significance was calculated using two-tailed unpaired Student's *t*-test or one-way analysis of variance (ANOVA) with post-hoc test using Bonferroni correction in case of multiple comparisons. *p*-values  $< 0.05$  were considered to be statistically significant in this study.

## 4.3. Results

### Borrelidin Treatment Selectively Inhibits the Proliferation and Growth of Malignant ALL Cell Lines

To study the effects of threonyl-tRNA synthesis inhibition, Jurkat, CEM cells and primary human fibroblasts were treated with a titration of borrelidin and then proliferation was monitored using the [ $^3\text{H}$ ] thymidine incorporation assay. Quantitative analysis shown in Figure 4.1a & b indicated that at very low concentrations, borrelidin was able to potently and significantly inhibit the proliferation of both Jurkat cells and CEM cells, with a half-maximal inhibitory concentration ( $\text{IC}_{50}$ ) of 50 ng/ml. These data show that there was a strong negative correlation between borrelidin concentration and proliferation rates of ALL cell lines ( $r = -0.91$ ). Meanwhile, primary human fibroblasts were relatively resistant to the suppressive effects of borrelidin and very high concentrations of borrelidin ( $\text{IC}_{50}$  of 400 ng/ml) were needed to inhibit the proliferation of these primary cells (Figure 4.1c). Moreover, quantitative analysis showed that borrelidin was able to significantly (more than 4-fold) inhibit the growth rate of Jurkat cells as compared with that of the control vehicle-treated group (Figure 4.1d) ( $n=3$ ,  $p<0.00001$ ).



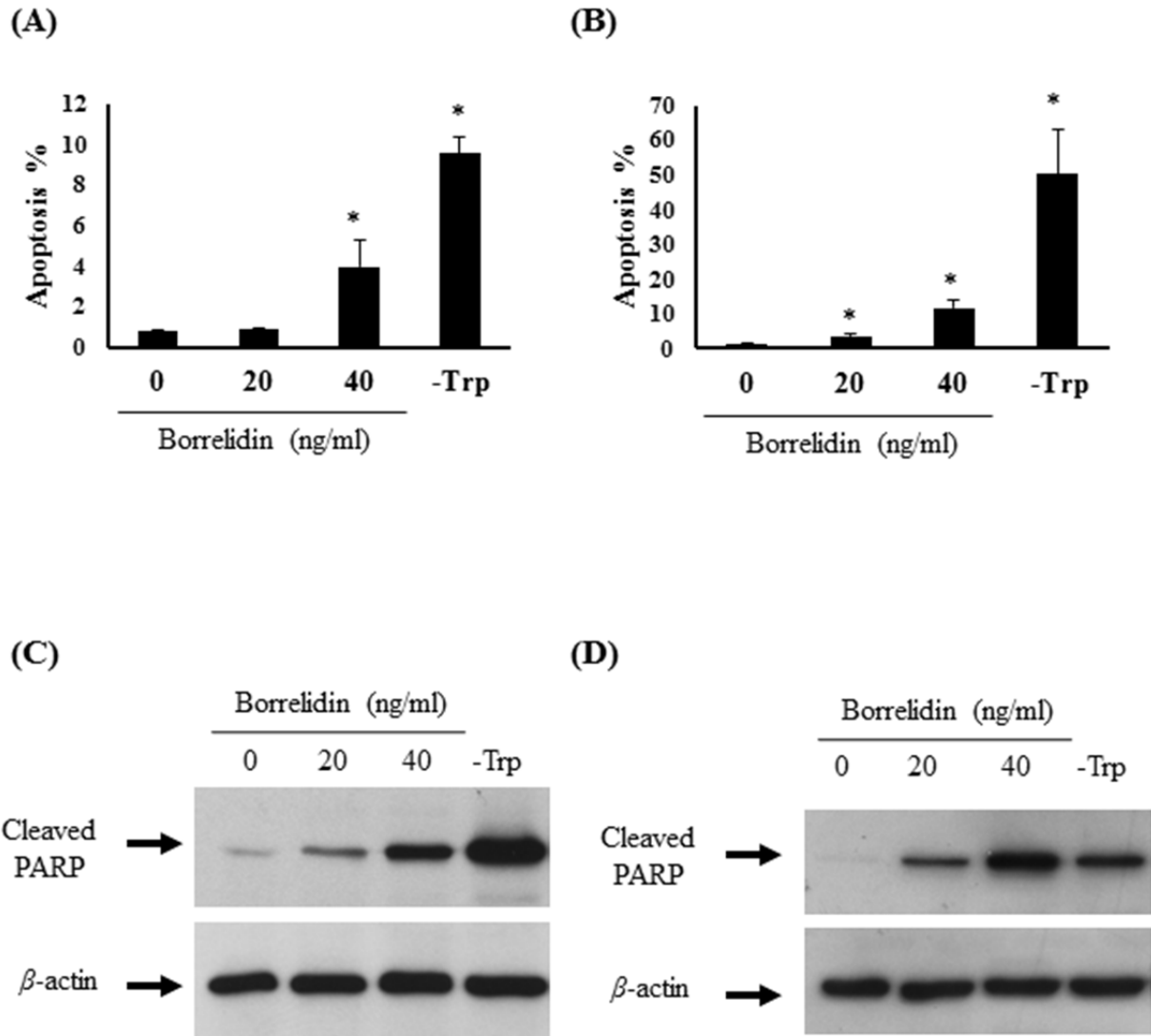
**Figure 4.1. Selective effects of borrelidin treatment on the proliferation and growth of ALL cell lines.**

(a) Jurkat cells, (b) CEM cells, and (c) primary human fibroblasts were treated with a titration of borrelidin. Thymidine incorporation assay was performed to evaluate the cell proliferation. (d) Jurkat cells were grown in the presence (40 ng/ml) or absence (vehicle) of borrelidin. Growth rate of each group was determined for a period of 7 days. Significant (p value  $<0.00001$ ) difference is indicated by an asterisk (\*),  $n=3$ .

## **Borrelidin Induces Apoptosis in Jurkat Cells and CEM Cells**

Previous studies show that cells undergo an initial repression in protein synthesis in response to nutritional stress. This will provide the cells with an opportunity to respond to the changes in the environment in order to recover (188). However, if the stress conditions continue, attenuation in translation is followed by the induction of apoptosis (189). To evaluate the potential apoptotic effects of borrelidin, Jurkat and CEM cells were either treated with vehicle or borrelidin (20 ng/ml, 40 ng/ml) or were cultured in tryptophan-deficient media (-Trp) and propidium iodide staining and flow cytometry was performed. As mentioned in the introduction, IDO generates a tryptophan-deficient environment through catabolism of tryptophan. In immune cells, this environment mediates the activation of the GCN2 kinase pathway. Therefore, in our experiments, we also used tryptophan-deficient media as a positive control in order to mimic the effects of IDO. The proportion of cells containing subdiploid DNA was determined by flow cytometry. The quantitative analysis shown in Figure 4.2a indicated a significantly higher level of apoptosis in Jurkat cells treated with 40 ng/ml borrelidin as well as in Jurkat cells cultured in tryptophan-deficient media ( $3.9 \pm 1.3$  and  $9.6 \pm 0.8$  respectively) compared to that of the vehicle-treated group ( $0.8 \pm 0.06$ ,  $n=3$ ,  $p<0.01$ ). A significantly higher level of apoptosis was also measured in CEM cells treated with 20, and 40 ng/ml borrelidin ( $3.6 \pm 0.4$ , and  $11.5 \pm 2.5$ , and respectively) compared to that of the vehicle-treated group ( $1.0 \pm 0.3$ ,  $n=3$ ,  $p<0.0001$ ) (Fig. 2b).

Moreover, the same experiment was performed and cell lysates were used to evaluate the levels of cleaved PARP (as a marker for apoptosis). Western blot analysis demonstrated a marked concentration-dependent higher expression of cleaved PARP in both Jurkat cells (Figure 4.2c) and CEM cells (Figure 4.2d) treated with borrelidin in compare with that of the control group.



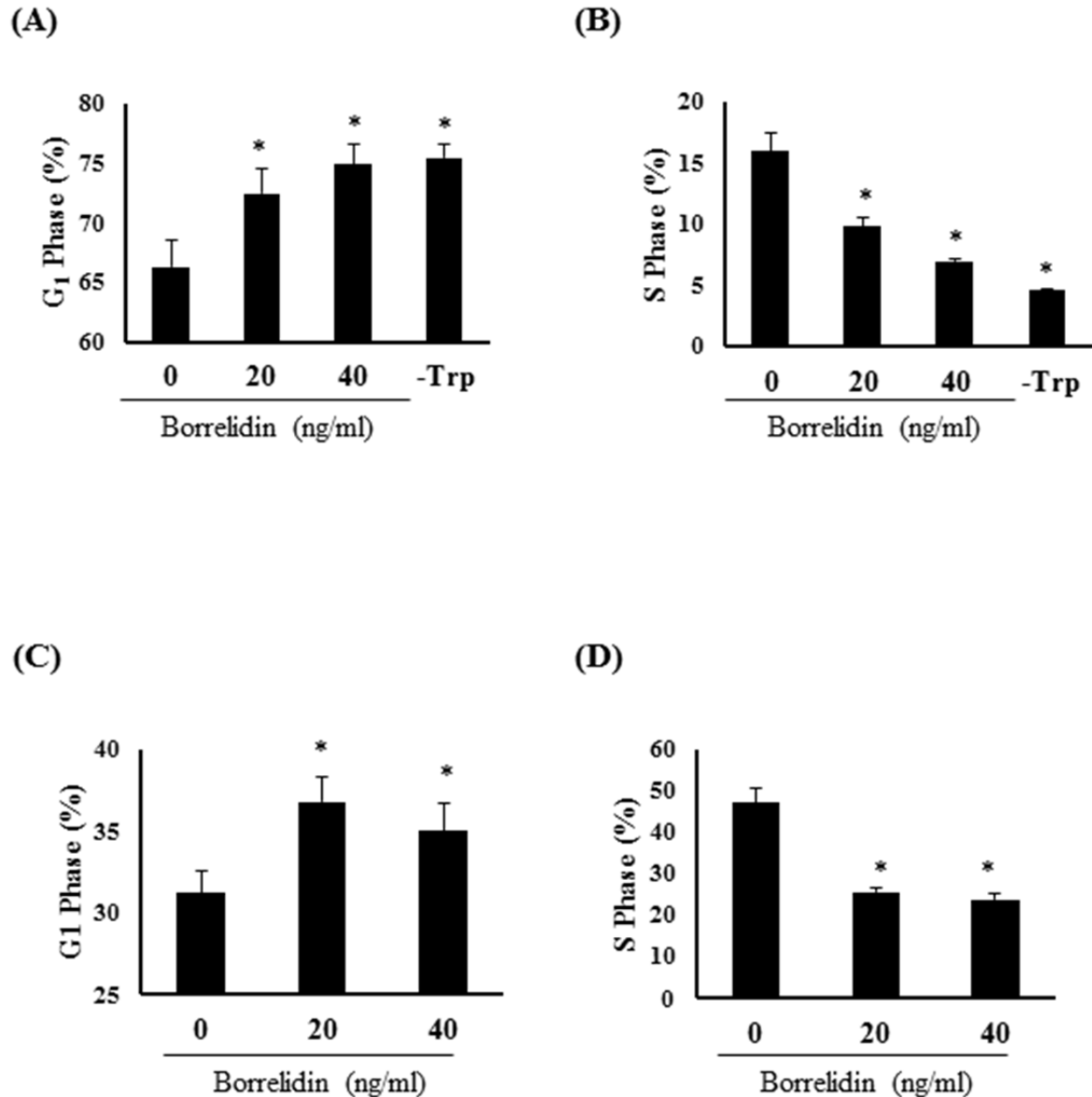
**Figure 4.2. Increased apoptosis in borrelidin-treated Jurkat cells and CEM cells.**

(a) Jurkat cells and (b) CEM cells were grown in the presence (20 or 40 ng/ml) or absence of borrelidin or cultured in tryptophan-deficient media (-Trp). Propidium iodide staining and flow cytometry was performed to study the apoptotic effects of borrelidin on these cells. Data represent flow cytometric quantification of sub-G<sub>0</sub>/G<sub>1</sub> DNA content. Significant (p value <0.01) differences are indicated by asterisks (\*), n=3. (c) Jurkat cells and (d) CEM cells were treated with vehicle, borrelidin (20 ng/ml, 40 ng/ml) or were cultured in tryptophan-deficient media. Cells were harvested and cleaved PARP protein expression was analyzed by Western blot. Blots were stripped and reprobbed for  $\beta$ -actin as equal loading control. A representative experiment is shown.

### **Borrelidin Treatment Induces G<sub>1</sub> Arrest in Jurkat cells and CEM Cells**

To study the effects of borrelidin on the cell cycle progression, Jurkat and CEM cells were treated with borrelidin followed by propidium iodide staining. The percentage of cells in G<sub>1</sub> or S phase was then measured. Quantitative analysis as shown in Figure 4.3a indicates that there was a significant increase in G<sub>1</sub> phase of Jurkat cells when treated with borrelidin (20 or 40 ng/ml) or cultured in tryptophan-deficient media ( $72.5 \pm 2.0$ ,  $74.9 \pm 1.8$  and  $75.4 \pm 1.2$  respectively) compared to that of vehicle (0 ng/ml borrelidin) treated group ( $66.3 \pm 2.3$ , n=3, p<0.01). The level of S phase decreased significantly in Jurkat cells in the presence of borrelidin (20 or 40 ng/ml) or in the presence of tryptophan-deficient media ( $9.7 \pm 0.8$ ,  $6.9 \pm 0.2$  and  $4.5 \pm 0.1$  respectively) when compared to the vehicle-treated group ( $15.9 \pm 1.4$ , n=3, p<0.001) (Figure 4.3b).

Similarly, quantitative analysis as shown in Fig. 4.3c showed a significant concentration-dependent G<sub>1</sub> induction in CEM cells treated with borrelidin (20 or 40 ng/ml) ( $36.8 \pm 1.5$ ,  $35.0 \pm 1.6$  respectively) compared to that of control group ( $31.2 \pm 1.4$ , n=3, p<0.01). The levels of S phase significantly decreased in CEM cells treated with borrelidin (20 or 40 ng/ml) ( $25.5 \pm 1.1$ , and  $23.8 \pm 1.5$  respectively) when compared to the vehicle-treated group ( $47.0 \pm 3.5$ , n=3, p<0.001) (Fig. 4.3d).



**Figure 4.3. Induced G<sub>1</sub> arrest in borrelidin-treated Jurkat and CEM cells.**

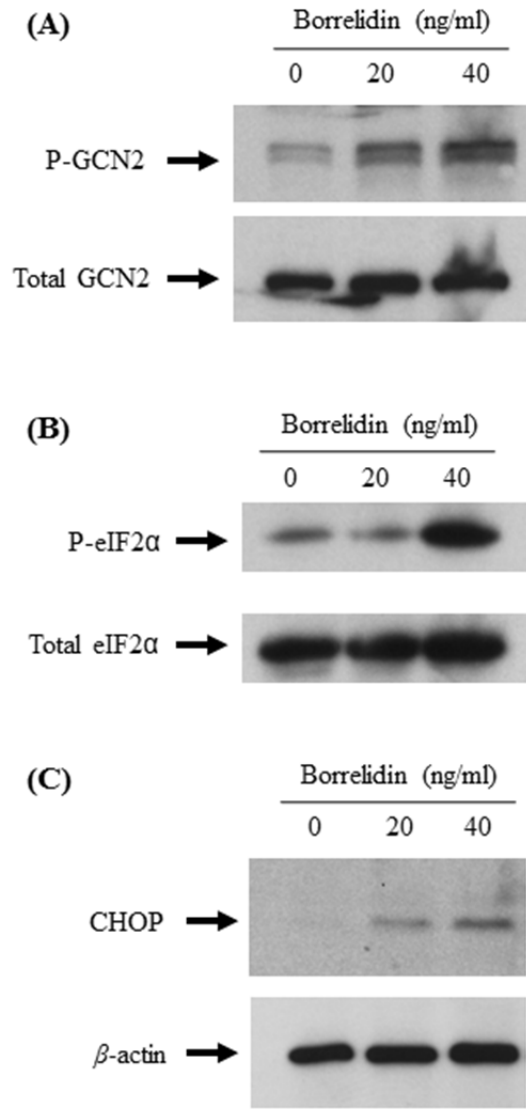
Jurkat cells and CEM cells were treated with vehicle, borrelidin (20 or 40 ng/ml) or were cultured in tryptophan-deficient media (-Trp). Propidium iodide staining and flow cytometry was performed to study the effects of borrelidin on cell cycle progression. (a) Effects of borrelidin treatment on G<sub>1</sub> phase in Jurkat cells. (b) Effects of borrelidin treatment on S phase in Jurkat cells. (c) Effects of borrelidin treatment on G<sub>1</sub> phase in CEM cells. (d) Effects of borrelidin treatment on S phase in CEM cells. Significant (p value < 0.01) differences are indicated by asterisks (\*), n=3.



## **GCN2 Kinase is Activated and CHOP is Induced in Jurkat Cells and CEM Cells Treated with Borrelidin**

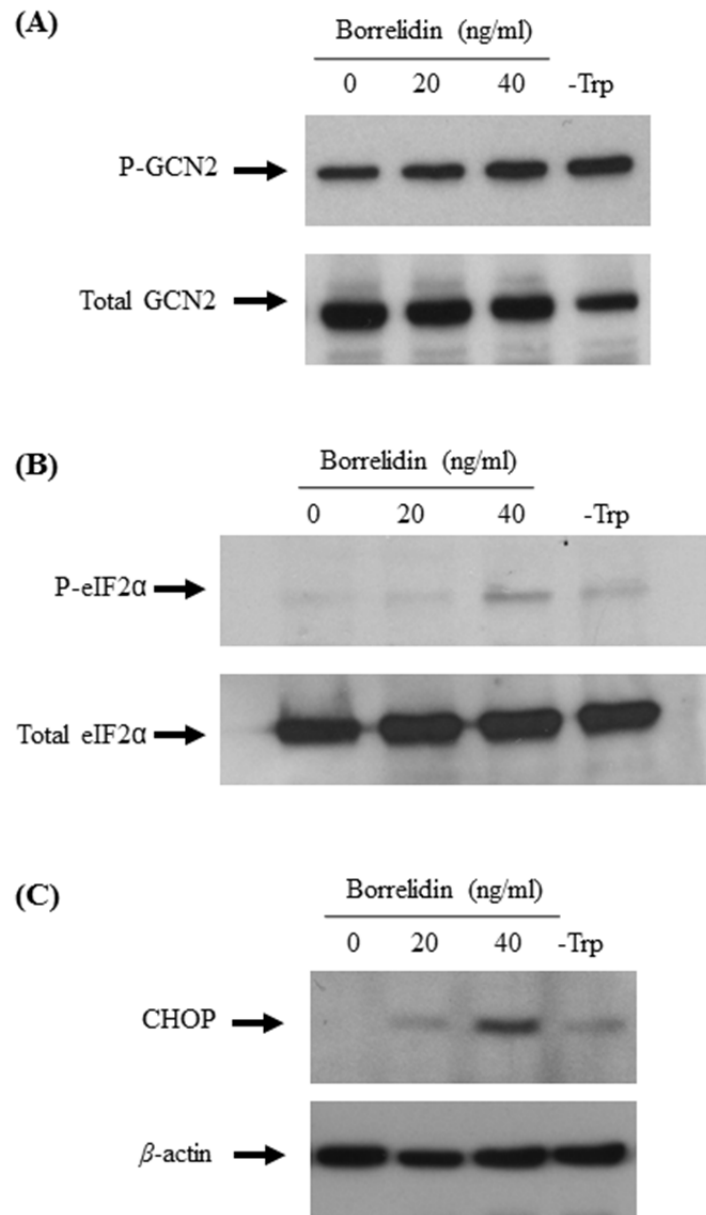
Activation of the GCN2 kinase pathway by uncharged tRNAs acts as a downstream mechanism to sense amino acid deprivation. Therefore, we asked whether borrelidin-induced nutritional stress activates GCN2 kinase pathway and induces CHOP in ALL cell lines. Jurkat cells were treated with either vehicle, or borrelidin (20 or 40 ng/ml). Cells were harvested and evaluated for the expression of phosphorylated GCN2, total GCN2, phosphorylated eIF2 $\alpha$ , total eIF2 $\alpha$  and CHOP. Western blot analysis (Fig. 4.4) revealed that by increasing the concentration of borrelidin, the GCN2 kinase pathway was markedly activated in Jurkat cells when compared with that of the vehicle-treated group. Jurkat cells treated with borrelidin showed a marked concentration-dependent induction of phosphorylated GCN2, phosphorylated eIF2 $\alpha$  and CHOP relative to that of Jurkat cells treated with vehicle (Figure 4.4a-c).

To provide further evidence, the same set of experiments were performed using another ALL cell line (CEM) as shown in Figure 4.5a-c. As shown in western blot results in Figure 4.5a, CEM cells in the presence of borrelidin as well as in tryptophan-deficient media showed a marked GCN2 kinase pathway activation in compare with that of the vehicle-treated group. These results revealed a marked concentration-dependent induction of phosphorylated GCN2, phosphorylated eIF2 $\alpha$  and CHOP in borrelidin-treated CEM cells and CEM cells cultured in tryptophan-deficient media (Figure 4.5a-c).



**Figure 4.4. Activation of the GCN2 kinase pathway and CHOP induction in borrelidin-treated Jurkat cells.**

Jurkat cells were treated with vehicle, 20 or 40 ng/ml borrelidin. Cells were harvested and evaluated for the expression of (a) phospho-GCN2, total GCN2, (b) phospho-eIF2 $\alpha$ , total eIF2 $\alpha$  and (c) CHOP. Blots were stripped and reprobed for  $\beta$ -actin as equal loading control. A representative experiment is shown. P-GCN2: phosphorylated GCN2, P- eIF2 $\alpha$ : phosphorylated eIF2 $\alpha$ .



**Figure 4.5. Activation of GCN2 kinase pathway and CHOP induction in borrelidin-treated CEM cells.**

CEM cells were treated with vehicle or 20 ng/ml or 40 ng/ml borrelidin or were cultured in tryptophan-deficient media (-Trp). Cells were harvested and evaluated for the expression of (a) phospho-GCN2, total GCN2, (b) phospho-eIF2 $\alpha$ , total eIF2 $\alpha$  and (c) CHOP. Blots were stripped and reprobed for  $\beta$ -actin as equal loading control. A representative experiment is shown.

#### 4.4. Discussion

The main finding of this study is that low concentrations of borrelidin, a threonyl-tRNA synthetase inhibitor, are able to selectively impair the proliferation and finally induce apoptosis in malignant ALL cell lines, Jurkat cells and CEM cells. Furthermore, our results show that borrelidin treatment is correlated with activation of the GCN2 kinase pathway and induction of CHOP.

The overall cure rate of childhood ALL has significantly increased over the last few decades. However, due to the poor complete remission rate in adult ALL, development of novel drugs is vital. The use of L-asparaginase, an asparagine hydrolyzing enzyme, in ALL therapy for more than 3 decades suggests that amino acid limitation may be used as a therapeutic approach to treat ALL (155, 190). The small molecule drug, borrelidin, is a selective inhibitor of threonyl tRNA synthetase (165, 166). This drug has attracted attention because it displays strong nanomolar antiangiogenic activity and shows anti-metastatic effects in animal models (165-167). Borrelidin potently inhibits capillary formation by inducing apoptosis of the tube-forming cells via activation of the caspase-3 and caspase-8 pathways (165). It is widely accepted that small molecule drugs are less immunogenic, less allergenic, and more stable as compared with large proteins (191-193). Small molecule drug borrelidin (489.7) can also be used orally which results in better patient compliance as opposed to ASNase that has to be administered via intramuscular injection. As borrelidin increases the levels of uncharged tRNA and induces nutritional stress, we asked whether borrelidin treatment will inhibit ALL cell lines proliferation and induces apoptosis in these cells.

This study shows for the first time that low concentrations of borrelidin are able to significantly impair the proliferation and growth of ALL cell lines which suggests the potential efficacy of this drug in inhibiting leukemic cell growth in treating ALL. Our results show that the inhibitory effect of borrelidin on ALL cell lines is selective and primary human fibroblasts are resistant to these effects of borrelidin. These results indicate that low concentrations of borrelidin have potent selective and suppressive effects on leukemic cells. Our group has also previously shown that IDO has the same selective apoptotic effects on immune cells and that primary dermal fibroblasts, keratinocytes, and endothelial cells are resistant to the IDO-mediated tryptophan-deficient environment due to the selective activation of the GCN2 kinase pathway in immune cells (10, 101, 169).

Our flow cytometry results show that borrelidin treatment increases the level of apoptosis in Jurkat cells and CEM cells. Moreover, Jurkat cells and CEM cells treated with borrelidin have higher levels of cleaved PARP, a marker for apoptosis.

Cell cycle deregulation, specifically at cell-cycle check points have previously been highlighted as a common modality that leads to cancer. Therefore, targeting these check points with various compounds may lead to development of effective anti-tumor drugs (194). The cell cycle results in this study indicate that borrelidin treatment is able to significantly induce the G<sub>1</sub> arrest in Jurkat cells and CEM cells in a concentration-dependent manner.

Another important question is the mechanism by which borrelidin can mediate its apoptotic effects on ALL cell lines. As borrelidin increases the level of uncharged tRNA, we suggest that the GCN2 kinase pathway is one of the downstream mechanisms of borrelidin effects. GCN2 kinase is a complex stress pathway: In response to environmental stresses such as amino acid deprivation, it becomes activated to remedy cellular damage by repression of global

protein synthesis while stimulating translation of selected genes responsible for amino acid biosynthesis to relieve the particular cell stress and allow them to survive (139, 195, 196). However, if the stress cannot be relieved, gene expression switches to promoting cell death via transcription of the pro-apoptotic transcription factor CHOP (159, 186, 197).

In this study, by detecting the level of phosphorylated GCN2, total GCN2, phosphorylated eIF2 $\alpha$ , total eIF2 $\alpha$  and CHOP we have shown for the first time that there is a positive concentration-dependent correlation between overexpression of CHOP with increased phosphorylation and activation of GCN2 kinase pathway in response to borrelidin treatment in ALL cell lines, Jurkat cells and CEM cells. We suggest that in the stress environment generated by borrelidin, Jurkat cells and CEM cells are not able to recover or survive, and therefore induce CHOP expression and become apoptotic. These data suggest that the apoptotic effects of borrelidin treatment on ALL cell lines is mediated by CHOP induction, possibly due to the activation of the GCN2 kinase pathway. However, other yet unknown additional mechanism(s) may exist that lead to the apoptosis of these cells upon borrelidin treatment.

In summary, our findings demonstrate for the first time that low concentrations of borrelidin have significant and selective effects on proliferation and apoptosis of ALL cell lines which suggests the potential use of borrelidin in treatment of ALL. This study produced several novel and potentially clinically important findings that have to be further confirmed. These findings advance our understanding of the basic biological mechanism of borrelidin action and further reveal the role of GCN2 and CHOP as one of the mechanisms involved in managing the stress response to this potentially therapeutic agent.

## **CHAPTER 5. Conclusion and Suggestions for Future Work**

### **5.1. General Discussion and Conclusion**

Allograft rejection, which is due to the T cell-mediated response of the host, is a major problem in any type of organ transplantation (198). Therefore, transplant patients need lifelong use of systemic immunosuppressive drugs that function as anti T-cell proliferation agents. Unfortunately, these drugs have multiple serious side effects such as nephrotoxicity, hyperlipidemia, mouth ulcers, increased risk of infection, and increased risk of development of malignant diseases such as lymphomas (199-201). Adverse side effects of current systemic immunosuppressive drugs are a major source of concern for the patients, physicians and researchers. As such, new therapies should be developed in order to establish and maintain local immunosuppression at the graft site.

Immune cells are susceptible to amino acid deprivation or metabolites generated from amino acid catabolism (202). Different enzymes regulate these catabolic pathways and have been shown to have the potential to be used as targeted intervention in order to treat different diseases of the immune system. In recent years, the immunosuppressive effects of some of these enzymes including IDO have been widely studied (31, 63, 79, 90, 92).

IDO is a potent immunomodulatory enzyme that breaks down tryptophan. IDO has a regulatory effect on T cells due to a tryptophan-deficient microenvironment and/or accumulation of toxic metabolites of tryptophan (51, 80). IDO expression in the placenta is crucial in preventing immunological rejection of the fetal allograft (31). It is suggested that the stress kinase GCN2 kinase pathway senses and responds to the stress environment generated by IDO (83).

In recent years and in the light of large amount of studies on IDO, the role of this enzyme has evolved from only a regulator of tryptophan degradation to one of the central regulators of immune system. Different studies suggest the immunoregulatory effects of IDO in different physiological and pathological conditions (32-35, 48, 78). Translating this naturally occurring immunosuppressive effect of IDO is particularly of interest in potential medical implications of this enzyme as an ideal target for therapeutic intervention. Strategies to regulate IDO expression can be advantageous in inhibiting any undesirable activation of T cells during transplantation, inflammatory or autoimmune diseases (23, 85, 203).

Our group has previously used the immunosuppressive effects of IDO in the development of a non-rejectable skin substitute as well as non-rejectable islet grafts. We showed that local induction of IDO in the allografts creates a tryptophan-deficient microenvironment. In such an environment infiltrated immune cells, but not allogeneic cells, are unable to survive, proliferate and destroy the graft (7-10). Our group also showed that IDO expression in fibroblasts selectively induces apoptosis in immune cells but not in primary skin cells (11). However, the mechanism(s) of this selective effect of IDO-induced low tryptophan environment were poorly understood. Due to the importance of IDO as one of the central regulators of the immune system, understanding its mechanism of action is of great importance. Therefore, as a part of this thesis, we aimed to elucidate the cellular mechanism(s) that explain the phenomenon of selective IDO-mediated apoptotic effects of immune cells that contributes its potent immunosuppressive effects.

To address this question, as described in Chapter 2, we performed a series of experiments in which we showed that activation of the GCN2 kinase stress-responsive pathway and expression of pro-apoptotic CHOP protein were significantly higher in immune cells exposed to



an IDO-expressing environment compared to that of primary skin cells. In contrast, protein IMPACT homolog, a GCN2 kinase inhibitor, was highly and constitutively expressed in skin cells while its expression was very low in stimulated T cells and undetectable in Jurkat cells. Low levels of IMPACT in primary T cells could be due to a sub-population of CD4<sup>+</sup> T cells which have been shown previously by our lab to be more resistant to an IDO-induced stress environment (101). We have also previously shown that in the presence of IDO, bystander Jurkat cells show significantly higher levels of apoptosis as compared with that of primary CD4<sup>+</sup> or CD8<sup>+</sup> T cells (10). These results can be explained by the differences between IMPACT expression levels in Jurkat cells compared to that of primary T cells. IMPACT expression level in bystander cells was shown to be independent of IDO-induced stress environment.

It has been shown that IMPACT overexpression in amino acid-deprived conditions leads to GCN2 kinase inhibition and abolishes the expression of CHOP (142, 143). As such, our results also showed that marked upregulation of CHOP protein occurred in the IMPACT knocked-down fibroblasts. In addition, a significant IDO-induced suppressive as well as apoptotic effect was demonstrated in IMPACT knocked-down fibroblasts co-cultured with IDO-expressing fibroblasts (Figure 2.6 and Figure 2.7, Chapter 2). It should be mentioned that due to the resistance of primary fibroblasts to cell death, induction of apoptosis in these cells is very remarkable (179). We showed that proliferation of Jurkat cells, stably transduced with IMPACT-expressing vector, was rescued in tryptophan-deficient but not IDO-expressing environment (Figure 2.8, Chapter 2). This may be due to the ability of IMPACT to recover the effects of IDO-mediated tryptophan depletion (GCN2 dependent) but not the effects of IDO-generated cytotoxic metabolites. We suggest that high levels of IMPACT expression in primary skin cells abolishes the activation of the GCN2 kinase pathway and therefore inhibits the expression of CHOP and

makes skin cells more resistant to the IDO-induced environment. On the other hand, in immune cells where IMPACT is expressed at very low levels, the GCN2 kinase pathway has the ability to become activated in a stress environment and this leads to the expression of the pro-apoptotic protein CHOP. We believe that in such an IDO-induced stress environment, immune cells are not able to recover and survive and therefore they go under apoptosis.

These findings collectively suggest for the first time that high expression of the protein IMPACT homolog in non-immune cells such as skin cells acts as a protective mechanism against IDO-induced GCN2 activation therefore makes them resistant to the amino acid-deprived environment caused by IDO. The findings of this study help us to have a better understanding of the role and mechanism of IDO effects in suppressing the allogeneic immune response in transplantation. Selective activation of the GCN2 kinase pathway as a result of differential expression of IMPACT protein is an important piece of evidence as it further confirms the differential inhibitory effects of IDO on immune cells and its safety for developing allografts. As such, IDO expression can function as a local immunosuppressive factor to protect skin allografts without compromising skin cell viability.

Different findings support the idea that IDO expression in tumor cells, promotes the tumor growth and metastasis, however there are also contradictory evidence in this regard (109, 110, 112). As such, we believe that a better understanding of the biology of IDO is certainly needed in order to use this enzyme or its inhibitor in treatment of different diseases such as cancer. The complex nature of IDO function in immune system should be carefully considered in using IDO inhibitors as a cancer therapy strategy as their use may in fact lead to infection and autoimmunity. Therefore, it would perhaps be beneficial to use IDO inhibitors to disrupt only the local immune privilege site that develops in cancer. D-1-MT, an IDO inhibitor isomer is

currently being used in clinical trials as an adjuvant cancer therapy in combination with chemotherapy regimens (53). However, there are controversies over the efficacy of this isomer. In fact, it has been shown that in most cases, D-1-MT was not able to block activity of recombinant IDO or IDO-expressing cell lines (204). Nevertheless, D-1-MT was able to inhibit the degradation of tryptophan by IDO-expressing DCs (53). Therefore, D-1-MT has the ability to block IDO activity only in some cells. Due to these controversies, research into identifying novel inhibitors of IDO function needs to be performed.

Our results in Chapter 2 have shown that IMPACT protein, a GCN2 kinase inhibitor, is expressed abundantly in IDO-resistant cells such as primary skin cells and this makes skin cells resistant to the apoptotic effects of IDO. On the other hand, we showed that IMPACT is expressed at very low levels or is undetectable in IDO-sensitive cells such as immune cells (Figure 2.3, Chapter 2). Interestingly, our data suggest cells that have the potential to express IDO including DCs, primary fibroblasts, and tumor cells are themselves resistant to the apoptotic effects of IDO. In this regard, we propose that perhaps IMPACT expression in tumor cells may be a reason for the resistance of these cells to IDO's effects. As such, studies are needed to be done in order to evaluate the levels of IMPACT expression in human tumor cells. If IMPACT is proved to be expressed abundantly in tumor cells, IMPACT inhibition can then be considered a legitimate strategy in cancer therapy.

Our group has previously shown that IDO-expressing fibroblasts populated in collagen matrix can be applied successfully in order to generate non-rejectable allografts (6-9). We have also shown that the immunosuppressive effect of IDO is achieved as long as high level of IDO is expressed in the graft (9, 205). Therefore, we believe that stable expression of IDO in bystander fibroblasts will lead to long-term graft survival. Previously, our research group has used IFN- $\gamma$  or

adenoviral vectors in order to induce IDO expression (7, 10, 11, 104). However, the immunogenicity and temporary gene expression of adenoviral vectors may impede with its clinical use in transplantation. IFN- $\gamma$ -induced IDO expression is also transient and IFN- $\gamma$  is known to be a proinflammatory factor which limits its clinical application. As such, using appropriate stable gene transfer strategies (e.g. application of a lentiviral vector) in bystander hard-to-transfect primary fibroblasts was needed to extend graft survival. Therefore, in Chapter 3, we aimed to develop two new simple yet effective approaches in order to stably deliver the IDO gene to bystander primary fibroblast cells to be used in future transplant models. As such, two lentiviral gene delivery systems were generated and employed to transduce primary fibroblasts.

In the first approach, an IDO-expressing lentiviral vector containing red-fluorescent mCherry protein was generated. Using this system in combination with polybrene and FACS, a 95% pure population of IDO-expressing fibroblasts was successfully obtained (Figure 3.2, Chapter 3). This vector is useful in our transplant settings where the detection of transgene expression is important. In the second approach, IDO gene was cloned into a lentiviral backbone containing blasticidin S resistance gene. Transduction of primary fibroblasts with this vector and blasticidin selection generated a pure population of IDO-expressing cells without the need to perform sorting (Figure 3.3 Chapter 3). This pure population of cells may be very useful in our transplant settings when very pure populations of IDO-expressing cells are needed in a short period of time in order to be grafted to the recipient.

The findings of this study would potentially assist us to use IDO as an immuno-suppressive agent in different types of allograft transplantation and to promote the rates of allograft survival in burned patients as well as non-healing wounds frequently seen in diabetic

patients. This is a new approach which can be extended into different areas of cell and organ transplantation in the future.

As noted earlier, IDO can act as a double-edged sword in promoting or inhibiting cancer. On the other hand, asparaginase has been used for few decades in treatment of ALL (113, 152, 206). Therefore, amino acid deprivation strategies have been shown to have the potential to either inhibit or promote cancer. One explanation for these contradictory effects of IDO would be that in solid tumors, IDO expression may be advantageous for the tumor in order to escape the immune system. However, immune cells are sensitive to amino acid deprivation and need high levels of nutrients to maintain their proliferation rate. These differential effects of IDO in solid tumors versus immune cells may be due to the potential high levels of IMPACT in solid tumors which can make them resistant to the effects of IDO. As such, we suggest that in malignancies of the immune system such as ALL, amino acid deprivation strategies can in fact be useful in treatment of such diseases.

Amino acid deprivation strategies have proved to be advantageous as new therapies to regulate the immune system. However, researchers are still facing different challenges in this regard. For instance, IDO needs to be expressed intracellularly in order to mediate its immunosuppressive effects; making its use limited in clinical settings. In addition and as noted earlier, although bacterially-derived asparaginase has been successfully used in treatment of ALL, it generated allergic reactions in patients which ultimately leads to its discontinuation (154). It is widely accepted that large proteins are more immunogenic, more allergenic, and less stable as compared with small molecule drugs (191-193). Therefore, there is a clear need to develop small molecule drugs that can mimic the effects of these enzymes in order to be able to find novel therapeutics to treat diseases of the immune system.

Borrelidin, a small molecule nitrile-containing macrolide, is an inhibitor of bacterial and eukaryal threonyl-tRNA synthetase. Borrelidin-mediated inhibition of aminoacyl-tRNA synthesis leads to an induction in the levels of uncharged tRNA, nutritional stress and ultimately inhibition of protein synthesis (165, 166). Borrelidin can be easily obtained chemically and contrary to IDO, it does not need to be expressed intracellularly in order to mediate its function (166). As such, in Chapter 4, we hypothesized that aminoacyl-tRNA synthesis inhibitor borrelidin would inhibit the proliferation of malignant ALL cell lines, Jurkat and CEM cells, induce apoptosis, and activate the GCN2 kinase stress-responsive pathway in these cells.

Our results in Chapter 4 showed that borrelidin is able to potently inhibit the proliferation of ALL cell lines with a half-maximal inhibitory concentration of 50 ng/ml which suggests the potential efficacy of this drug in inhibiting the growth of malignant leukemic cells in ALL. Apoptotic effects of borrelidin were selective as it showed a greater inhibitory effect on ALL cell lines compared to that of primary fibroblasts (Figure 4.1, Chapter 4). As such, we suggest that high levels of IMPACT expression in fibroblasts may play a role in protecting these cells from borrelidin-induced GCN2 activation. These results suggest the advantage of low concentrations of borrelidin with selective high efficacy on leukemic cells.

Our flow cytometry and western blot analysis indicated that borrelidin was able to increase the level of apoptosis and cause G<sub>1</sub> arrest in ALL cell lines. Activation of the GCN2 kinase stress responsive pathway and induction of CHOP protein was significantly higher in ALL cell lines treated with borrelidin (Figure 4.4 and Figure 4.5, Chapter 4). These findings collectively suggest for the first time that very low concentrations of borrelidin may have promising potential in treating ALL by inducing apoptosis and mediating G<sub>1</sub> arrest and that borrelidin treatment in ALL cell lines is correlated with activation of the GCN2 kinase

pathway. We believe that the results of this study may lead to the potential application of borrelidin as a therapeutic agent for treatment of ALL. This drug may work well as a single agent therapy or in combination with existing approved drugs or with emerging new therapies for ALL.

It has only been in recent years that the potential applications of aminoacyl-tRNA synthetase inhibitors in humans have been described. Vam de Vijver et al. have recently established a panel of these compounds with immunosuppressive activity (207). In addition, it has been also shown that Ochratoxin A, a toxin of *Aspergillus ochraceus*, causes immunosuppression (208). We speculate that other aminoacyl-tRNA synthetase inhibitors may also show selective suppressive effects by inducing the levels of uncharged-tRNA and selectively activating the GCN2 kinase stress pathway. As such, these compounds also have the potential to be used as novel therapies in treatment of ALL.

In summary, in this thesis we were able to 1) demonstrate the role of IMPACT protein as an important factor that promotes resistance to the apoptotic effects of IDO, 2) generate a simple yet high efficient strategy through which an efficient and stable expression of IDO can be achieved for primary cells, 3) discover a potent pharmacomimetic of IDO with potential to be used in treating ALL and study its mechanism of effects on ALL cell lines.

## 5.2. Suggestions for Future Work

Although we believe that our work has generated important data in regards to the mechanism of immunosuppressive effects of IDO and borrelidin and their potential applications in treatment of diseases of the immune system, there is still a considerable amount of study that needs to be performed in this area of research. The following are some of my suggested studies to be done in order to improve upon our current findings.

- 1- In Chapter 2, we showed that high expression of protein IMPACT in primary skin cells makes them resistant to the stress environment generated by IDO expression. As such, we suggest evaluating the levels of protein IMPACT expression in different types of human cancer cells. If IMPACT is expressed at high level in these cells, IMPACT inhibition may then be considered as a legitimate strategy in anti-cancer therapy.
- 2- In Chapter 3, we generated a lentiviral-based gene delivery method in order to stably and efficiently express the IDO gene in hard-to-transfect primary fibroblasts to be used in generating non-rejectable skin substitute and islet allografts. The ultimate goal of this part of the project is to pave the way toward using this technology in a pre-clinical setting. Therefore, we suggest to define a safety profile for these cells to identify any potential risk factors prior to their engraftment into patients in future. We suggest 1) to perform *in vitro* and *in vivo* safety screening of IDO lentiviral vector-transduced fibroblasts; 2) to apply these cells in order to develop non-rejectable IDO-expressing skin grafts to test in appropriate animal models.
- 3- In parallel with using IDO-expressing lentiviral vectors, we also suggest establishing non-viral gene delivery methods, such as pNUT expression vector (209) and zinc finger



nuclease system (210), for IDO gene transfer. This will make our approach more translatable to clinic.

- 4- In Chapter 4, our data indicated that very low concentrations of the small molecule drug borrelidin are able to suppress the proliferation of ALL cell line *in vitro*. These results suggest the potential application of borrelidin in treatment of ALL. Therefore, we suggest 1) to determine the pharmacokinetic profile of borrelidin; 2) to examine its toxicology; 3) to examine the *in vivo* efficacy of borrelidin in treatment of animal models of ALL.
- 5- Finally, as T cells are involved in other diseases of the immune system, we suggest further investigating the effects of borrelidin on other diseases of the immune system such as autoimmune diseases and transplant rejection.

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