Qualitative and Hybrid Modeling and Analysis of the Regulatory Network of IDO in Tumour Immune Escape

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FINAL APPROVAL

It is certificate that we have read the thesis submitted by Ms. Javaria Ashraf and it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the M.S Degree in Bioinformatics.

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Dedicated to my loving Parents and Family.

Their support and love have always been a source of strength for me.

DECLARATION

I hereby declare that the work presented in the following thesis is my own effort, except where otherwise acknowledged, and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Dated: 10-02-2012

Javaria Ashraf

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LIST OF ABBREVIATIONS

Acronym	Abbreviation
Hepatocellular Carcinoma	HCC
Indoleamine2, 3-Dioxygenase	IDO
Intermediate Neglect Of Differential Overlap	INDO
Tryptophan 2, 3-Dioxygenase	TDO
Hydrogen Peroxide	H_2O_2
Non-Small Cell Lung Cancer	NSCLC
Immunohisto-Chemistry	IHC
Dendritic Cells	DCs
1-Methyl-Tryptophan	1MT
Small Cell Lung Cancer	SCLC
Toll-Like Receptors	TLRs
General Control Nonrepressed-2	GCN2
Interferon gamma	IFNγ
Interferon-alpha	IFN-α
Interferon-beta	IFN-β
Interlukin-10	IL-10
Cytotoxic T Lymphocytes Antigen-4	CTLA4
Nitric Oxide	NO
Nitricoxide Synthase	NOS
Inducible NOS	iNOS
Endothelial NOS	eNOS
Neuronal NOS	nNOS
Transforming Growth Factor-Beta	TGFβ

Cyclooxygenase-2	COX2
Prostaglandin E2	PGE2
Cluster of Differentiation 28	CD28
Major Histocompatibility Complex	MHC
Antigen Presenting Cell	APC
Suppressor of Cytokine Signaling-3	SOCS3
SrcHomology 2	SH2
Cluster of Differentiation 80	CD 80 / B7-1
Cluster of Differentiation 86	CD 86 / B7-2
Janus Kinase,andSignal Transducer And Activator of Transcription	JAK/ STAT
Gene Regulatory Networks	GRNs
Generalized Logical Networks	GLNs
Protein Signaling Pathways	PSPs
If and Only If	Iff
Hybrid Automaton	HA
Linear Hybrid Automaton	LHA

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ABSTRACT

A biological network is a global representation of multiple interactions taking place in a cell. These processes comprise genes, RNA and proteins acting as regulators. Regulatory networks act as skeleton offering a qualitative structure, on which kinetic logics rules applied to carry out quantitative modeling and simulation. Indoleamine 2,3-dioxygenase, a heme containing enzyme, has emerged as an important factor in tumor associated immune tolerance. IDO main function is immunosuppression, through breakdown of tryptophan, in the tumor microenvironment and tumor-draining lymph nodes. The modeling of the IDO network using discrete framework and through discrete automata helped in understanding fully the regulatory mechanism involved in the working of IDO in cancer studies. This has given forth a comprehensive analysis of dynamical phenomena;IDO is detrimental at high concentrationgiving epigenetic switches and regulation of system is done by maintaining IDO in homeostasis. The validation of model was done by the model checking tool. Model checker toolhelped in finding all possible time delay constraints from a specific initial state to epigenetic or homeostasis. It also give invariance kernel, strictly following these constraints helped in maintaining system in homeostasisand avoiding specific state i.e. diseased state. Thus, these temporal constraints will help in controllingex-vivo experimentsand designing drug targets. A new technique called protein knock out was applied which checked the stability of the system after knocking out specific protein from the system, knockout of COX2 showed the same homeostasis as that of the second IDO network, it proved that COX2 is the main elementwhich enhances IDO activity in cancer.TGFβ is a two

edged sword as it can be a tumor suppressor as well as immunosuppresor by promoting tumor development.Inhibition of iNOS accompanied silencing of COX2 as well as it is a main inducer of COX2 in the IDO BRN.The results are helpful and have futuristic prospects as on the basis of this clinical trials and experiments for immunotherapy can be designed and it will save time and money. It can bring fruitful results in tumor control and in increasing life expectancy after tumor invasion.

CHAPTER 1

INTRODUCTION

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers prevailing universally. It is associated with hepatitis B and C virus infection (Carr, 2009). The cure of HCC is poor even after surgery because of role of molecular mechanism to tumor development. Some of the causes may be tumor antigen-specific immune tolerance, tumor immunosuppressive elements. Considering the immunosuppressive as a cause of HCC progression, Indoleamine 2, 3-dioxygenase (IDO) got significant attention as a cause of tumor. IDO degrades the important amino acid tryptophan (Trp) in the kynurenine pathway and produce *N*-formyl kynurenine (Takikawa *et al.*, 1986). This will halt the proliferation of alloreactive T cells, which arrest the cell cycle at the G1 phase of the cell via local Trp deficiency and the accumulation of toxic, proapoptotic catabolites (Munn *et al.*, 1999). It was then recommended that IDO may be a microenvironmental factor that could play a role in tumor evasion from T cell-mediated rejection (Friberg *et al.*, 2002; Mellor and Munn, 1999). It was first presented by Uyttenhove (2003) that IDO was expressed in various human cancer tissues and cell lines, and that IDO was involved in shielding tumors from attack by tumor-associated antigen specific cytotoxic T cells (Uyttenhove *et al.*, 2003).

1.1. Immune Surveillance

Paul Ehrlich (1909) was the first to put forward concept of immune surveillance in tumor that cancer will proliferate rapidly if there is no defense system to control the outgrowth of endlessly arising malignant cells (Ehrlich, 1909). It gives the concept of tumor immunology to be used in tumor prevention and therapies for cure. Malignant

tumor immunology to be used in tumor prevention and therapies for cure. Malignant tissues are recognized by the immune system, it is a key concept which can be used to develop novel and new systems for the treatment of cancer (Disis, 2006). The aim of immunotherapy is to generate T cells to combat and kill tumor cell growth after recognizing tumor antigen. The success of T cell action depends on the activation of sufficient numbers of tumor reactive T cells and long term persistence of such T cell responses that will be necessary to prevent tumor recurrence (Disis, 2006).

1.1.1. Immune Response

Lewis Thomas (1959) first presented the theory of immune surveillance of neoplasia also supported by Burnet (Burnet, 1970). Immune system is very active and its major function is to search the body for the malignancy and to eradicate tumors as they ascend (Lawrence, 1959). Immune system recognizes the antigen at later stages when T cell are fully activated and converted from naive to mature type. It is the most important fact about immune surveillance theory so a question put forward by cancer researchers is that how can tumor overtake the immune system and escape through the immune surveillance mechanism (Fuchs and Matzinger, 1996). The answer lies in discovering and studying the protein of interest which is Indoleamine 2, 3 dioxygenase (IDO), an immunosuppressor and has a role in immune tolerance and in infectious diseases control (Mellor and Munn, 1999).

1.1.2. Immunosuppression

Cancer and immune response are closely related and investigation of the human immune response in cancer studies has had a long history (Oettgen, 1991). Immune

system can distinguish tumor cells from normal tissues. Tumors are considered non-self when any unique antigens are expressed by them; it is supposed that most tumors arise after the immune system is fully developed (Fuchs and Matzinger, 1996). This ability is critical for enabling active tumor control and destruction also minimizing toxicity threat (Hung *et al.*, 1998). Initial efforts for immune recognition in case of cancer were mainly depend on serological methods then advances in cell mediated immunity taken the study towards exploration of T cell role in cancer studies. It has always been a critical issue to study a specific role of cellular immunology in cancer research (Boon *et al.*, 1996).

It has been reviewed by Kersey (1973) that tumor cause immunodeficiency and this is implied in this way that tumors may override immune surveillance by destroying immune system (Kersey *et al.*, 1973). It is proved using syngeneic mouse that both virus-induced and chemical suppress the immune system and is called immunosuppressive (Plescia *et al.*, 1975). It was further reported by in vitro experimentations that tumor cells made the spleen cells immunologically insensitive (Wong *et al.*, 1974).

1.1.3. Tumor Progression

"Progression" is a term used when tumor develops aggressive behavior for growth and malignant characteristics in their life span. This will eventually lead to stable (stable steady state behavior) and irreversible qualitative change in its nature this is known as tumor progression (Foulds, 1975; Foulds, 1957). It is said progression and extension are two different terms taken in space and time but having no change in qualitative properties (Weiss, 1950; Weiss, 1939).

Tumor subverts the immune system and it can be a viable escape mechanism for them. It will grow and progress without check and balance. Immunotherapy cannot be used to cure and control tumor unless we do not know the whole mechanism that how tumor escape immune attack and start proliferation. Knowing the fact that IDO is produce excessively by tumor cells, in the present study evidence have been gathered that one cause of subversion of the immune system as well as progression of tumor is mediated by overexpression of IDO.

1.2. Indoleamine-2, 3-dioxygenase..... A Biological Entity

An important intracellular enzyme, IDO has the ability to catalyze the primary and rate-restricting step in kynurenine pathway in which IDO degrades the Trp. Its properties as immunosuppressive agent have been connected in

- Mother fetal relationship (Munn *et al.*, 1998),
- Immune response in tumor (Muller *et al.*, 2005)
- Incurable infection (Potula *et al.*, 2005) and
- Autoimmune reactions (Szanto *et al.*, 2007; Gurtner *et al.*, 2003)

How IDO effect immune system is controversial to some extent, one mechanism involves Trp depletion and/ or other is accumulation of the Trp degradation products called catabolites.

1.2.1. Biological Significance of Indoleamine-2, 3-dioxygenase

In recent years, IDO has attracted much attention as an important negative regulator of immune system. IDO was first identified in rabbit intestines in 1963 (Shimizu *et al.*, 1978; Higuchi *et al.*, 1963). IDO is a cytosolic heme containing protein (EC

1.13.11.42), (Taylor and Feng, 1991). It is expressed by the Intermediate Neglect of Differential Overlap (INDO) gene located at human chromosome 8p12 (Katz *et al.*, 2008). The gene INDO has ten exons covering approximately 15 kb length and encodes a polypeptide of 403 amino acids (Najfeld *et al.*, 1993; Kadoya *et al.*, 1992). It is the only rate-limiting enzyme found outside liver, where it works just like the hepatic enzyme Tryptophan 2, 3-dioxygenase (TDO). Both degrade Trp and other indole derivatives, by oxidative catalysis of the indole ring, into kynurenines L-kynurenine, picolinic acid. Quinolinic acid and many other metabolites are the products of Trp conversion by IDO a process critically related to tumor escape (Grohmann *et al.*, 2003).

IDO and TDO were considered the same enzyme until it was negated, when high levels of various Trp metabolites were determined in the urine of patients of diseases like bladder cancer, tuberculosis, prostate disorders, rheumatoid arthritis, hodgkins disease and leukemia. But the liver of the patients showed no elevation in TDO level (Hayaishi *et al.*, 1984; Bett, 1962). It was recommended that TDO is not the only enzyme initiating the catalysis of Trp. Second enzyme called IDO was discovered which also catalyzed the formation of kynurenine from Trp (Higuchi *et al.*, 1963).

Unlike TDO it has a wide variety of substrates. It utilizes both L- and D-tryptophan, as well as Tryptamine, 5-hydroxytryptophan, and serotonin as substrates. Molecular weight of both proteins differs from each other (Takikawa *et al.*, 1988).

IDO is not found primarily in hepatocytes like TDO, rather it is distributed ubiquitously in other organs of mammals except liver, with highest activities noticed in lung and placenta. IDO has a heme unit in the protein, it was extracted from rabbit intestine with protoheme IX its catalytic center. In vitro studies showed that, it should

be protected from H_2O_2 which is generated by the reducing system (Yamazaki *et al.*, 1985; Shimizu *et al.*, 1978).

1.2.2. Indoleamine 2, 3-dioxygenase in Human Tumors

Malignancy attains the characteristics such as resistance to inhibitory growth signals, perpetuation. It is resistive to apoptosis, angiogenesis, metastatic competencies and aggressiveness, and immune system destruction. IDO activation causes Trp catabolites elevation in the urine of cancer patients, help tumor cells to escape immune system response. The survival mechanism is expensive for the tumor cell depriving itself of a requisite amino acid i.e., Trp.

It was primarily reported by Uyttenhove (2003) that IDO is constantly expressed in most of tumor cell lines and it was further proved by IHC examination (Uyttenhove *et al.*, 2003). Subsequently, it was confirmed by studies that connection between IDO overexpression and tumor causes poor prognosis in HCC, non-small cell lung cancer (NSCLC), colon cancer, cervical and ovarian cancer (Pan *et al.*, 2008; Beutelspacher *et al.*, 2006; Ino *et al.*, 2006; Astigiano *et al.*, 2005; Okamoto *et al.*, 2005) showing activity of IDO in the malicious progression of tumors.

A study conducted on ovarian cancers showed mRNA expression of IDO and it was checked through surgical resection of tumor specimens at the stage IIIc of disease (Okamoto *et al.*, 2005). Another study carried out on stromal cells or in tumor-draining lymph nodes (TDLNs) observed IDO overexpression in them. A related survival fashion was found while assessing small cell lung cancer (SCLC) showed IDO overexpression in the eosinophils penetrating the tumors and this is associated with poor survival (Astigiano *et al.*, 2005).

A larger research work covering role of IDO in colorectal cancer, consists of 143 colorectal cancer patients and the enzyme was examined by immunohisto-chemistry (IHC). IDO was highly expressed in 39.2% tumor specimens (56/143) while IDO showed low expression in 60.8% (87/143) (Brandacher *et al.*, 2006). In TDLNs, IDO positive-staining in dendritic cells (DCs) in the melanoma patients was interrelated with poor diagnosis and often the presence of IDO positive DCs in the sentinel nodes results in melanoma formation in future (Munn *et al.*, 2004; Lee *et al.*, 2003). These observations are of immense importance in extending these results in clinically defined studies to determine where IDO may become a driver of immune escape and tumor progression. As IDO is an important agent playing role in immune tolerance of tumors, it is embraced as a new marker or target in anti-tumor treatment (Wang *et al.*, 2009).

1.2.3. IDO and Immune Response

IDO is an enzyme with evolutionarily value; it was hypothesized to work against certain microorganisms as a host-defense mechanism. Currently, it was found that T cell activation and inflammatory responses are being suppressed by IDO (Mellor and Munn, 2004; Grohmann *et al.*, 2003). It was firstly acknowledged that IDO has immunological role when it was known that the tolerance between mother and the allogeneic fetus in placenta depend on IDO (Munn *et al.*, 1998), a revolutionary work by Munn and Mellor. Two inferences were deduced from these observations that made its relevance for tumor immunology: one thing is the immune system develops a pre-existent immunosuppressive environment for the fetus so that it remains tolerant for mother immune system. Secondly, if we disturb the environment by administering

drug 1-methyl-tryptophan (1MT), an inhibitor of IDO, rejection of fetus by maternal immune system will take place. IDO do have a role in tumor immune escape as these observations impelled us to hypothecate this and tumor cell facilitate themselves by using IDO against immune system, and using 1MT against IDO favor immune response enhancement (Munn, 2006). Hence, IDO plays an important role as shown in Figure 1.1. IDO is found to express widely in most of tumor cells, DCs, macrophages, microglia, esinophils, fibroblasts and endothelial cells (Beutelspacher *et al.*, 2006; Munn *et al.*, 2004; Munn and Mellor, 2004; Odemuyiwa *et al.*, 2004; Uyttenhove *et al.*, 2003). IDO expression is controlled by cytokines e.g. Interferon-gamma (IFNγ), Interferon-alpha (IFN-α), Interferon-beta (IFN-β) and interiukin-10 (IL-10) and through Toll-like receptors (TLRs) signaling pathways by Cytotoxic T Lymphocytes Antigen-4 (CTLA4)-B7 interaction in immune cells (Puccetti and Fallarino, 2008; Agaugue *et al.*, 2006; Munn, 2006).

Deficiency in Trp inexorably resulted by overproduction of IDO. Consequently, proliferation of T cells will be destroyed then it will be unable for T cell to amplify through clonal expansion. Furthermore, it is difficult to start T cell multiplying again once it is repressed (Bauer *et al.*, 2005). Conversely, toxic Trp metabolites produced after kynurenine pathway can directly inhibit T cell function and can induce T cell apoptosis. Trp metabolites have more effect on mature activated T cells and have no significant effect on resting cells (Munn and Mellor, 2007). When tumor antigen enters the body DC suppresses T cells by inducing specific immune response against the antigen, Munn (2004) pinpointed a distinct group of DCs in TDLNs that keep on expressing IDO and helping in inhibiting T cells. Therefore, DC have important role in bringing tumor immune tolerance (Munn *et al.*, 2004).

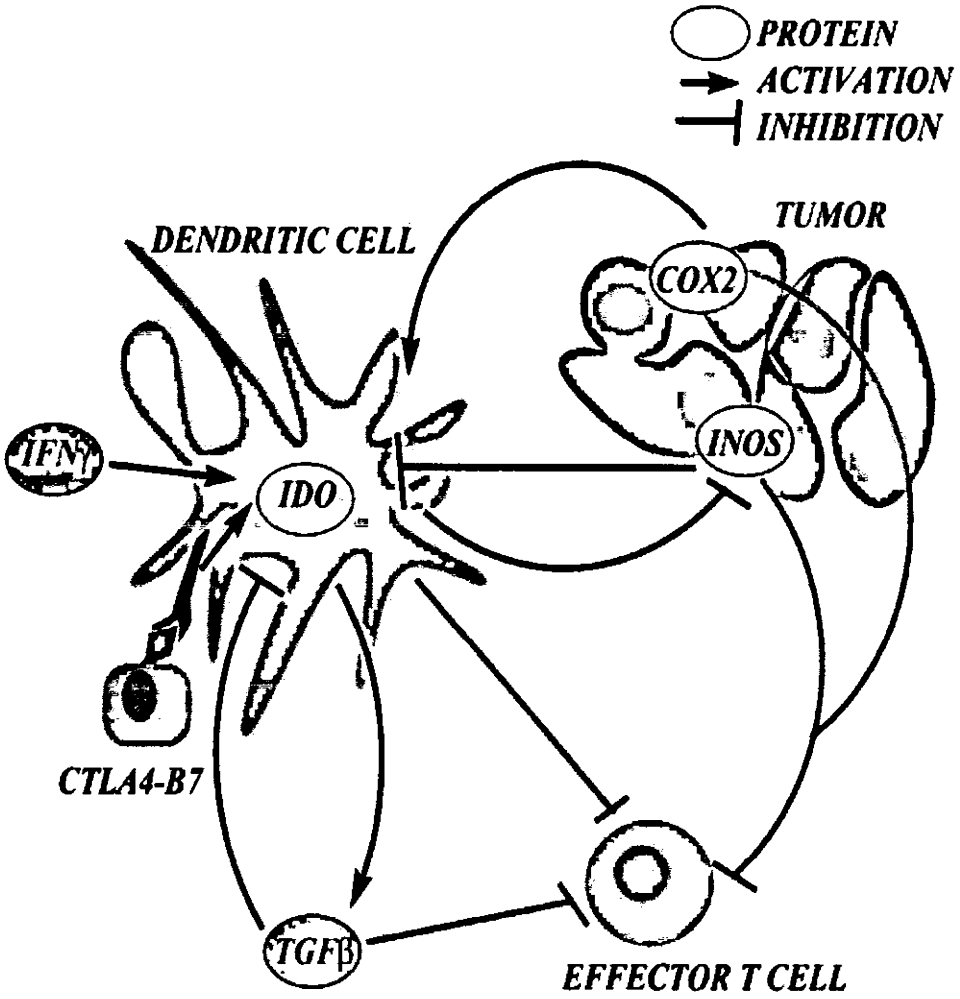


Figure 1.1: IDO inhibiting effector T cells

1.2.4. Mechanism: Controlling T cell Activity by Idoleamine 2, 3-dioxygenase

To understand the mechanisms which are the basis of the immune tolerating function of the IDO-kynurenine pathway suggests us two models:

- First is starvation and stress of immune cells through IDO-facilitated Trp depletion in the microenvironment, and consequently reducing the cell function;
- Second one is IDO-mediated gathering of cytotoxic catabolites from metabolism pathway of kynurenine (Belladoma *et al.*, 2006; Fallarino *et al.*, 2006; Terness *et al.*, 2006; Stone and Darlington, 2002).

Tryptophan (Trp)

An indispensable amino acid produced in the body, and remains in low concentration in both plasma and body tissues. In a variety of ailments and inflammation in human (Preston *et al.*, 1998), pigs (Melchoir *et al.*, 2002) and mice (Saito *et al.*, 1992), in the plasma of patients, Trp concentration drops significantly, proposing consumption of the amino acid increases in such situations. It is suspected that the Trp requirement become high when there is acute need of protein production (Preston *et al.*, 1998). Pathway of Trp catabolism in human and rodents are well established and known to have an involvement in T cell degradation in conception or even during infection (Mellor and Munn, 1999). IDO activation is the main reason of Trp degradation in kynurenine pathway. IFNγ , an inflammatory cytokine is involved in the induction of IDO (Widner *et al.*, 2000), but in the placenta, lung, epididymis and gut, its expression is constitutive (Brendan *et al.*, 2000). Kynurenine increases its

concentration in plasma due to immune activation and inflammation leading to excretion of xanthurenic acid in urine (Takikawa *et al.*, 1986). It is assumed by most that kynurenine is not the only pathway involved in trp metabolites but melatonin biosynthesis pathway is also involved where they act as antioxidants and as free radical hunters (Goda *et al.*, 1999).

Kynurenine Pathway

It starts with the induction of IDO in the cell by IFNγ as depicted in Figure 1.2. When Trp is depleted in the intracellular environment it leads to a rise in the accumulation of uncharged tryptophanyl-tRNA. General Control Nonrepressed-2 (GCN2), a member of a family of kinases called kinase, will be activated and will phosphorylate the translation initiation factor eukaryotic initiation factor 2-α and to cause anergy and/ or apoptosis through stress pathway. T cells with $GCN2^{-/-}$ will be unable to answer back to IDO activation, as it has been found in vitro study (Sharma *et al.*, 2007; Fallarino *et al.*, 2006) and some studies are carried out in vivo (Munn *et al.*, 2005).

However, in vitro T cell undergo apoptosis due to kynurenine degradation (Fallarino *et al.*, 2002, Terness *et al.*, 2002) and in vivo it suppresses the inflammation (Taher *et al.*, 2008; Bauer *et al.*, 2005; Platten *et al.*, 2005).

It has been recognized by Fallarino (2006) that in vitro immunosuppression is carried by both Trp depletion and kynurenine production (Fallarino *et al.*, 2006).

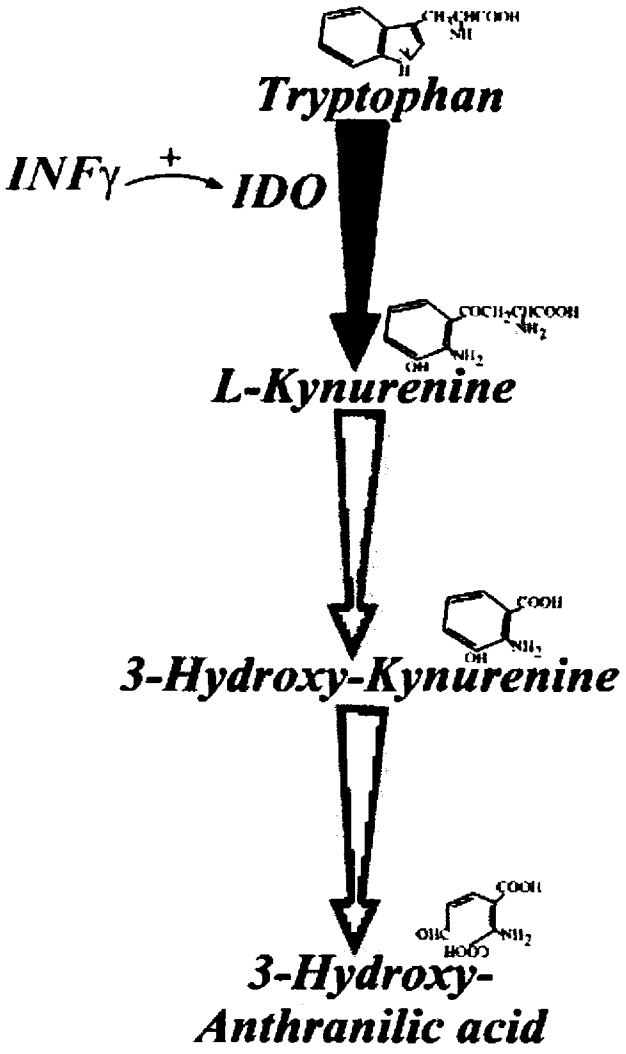


Figure 1.2: Kynurenine pathway starts when IDO facilitates degradation of Trp and it is divided into three main steps and completes at the formation of 3-Hydroxy Anthranilic acid.

1.2.5. Control of IDO by Immune Regulatory Factors

Several control mechanisms exists for IDO. It has been reviewed extensively that in human immune cells IDO activity can be strongly controlled through key regulatory proteins expression in these cells (Muller and Schrele, 2006).

If we can precisely identify the way how these regulatory proteins regulate IDO it will help greatly in understanding more deeply the human immune response. In the study good progress has been made by applying Rene Thomas formalism on these proteins network and it helps in understanding the precise molecular mechanism by which these proteins inhibit IDO in the milieu of cancer. A complex milieu of regulatory factors exist which incorporate IDO and promotes immune tolerance. Below is given a list of regulatory proteins that regulate IDO.

1.2.5.1. Interferon gamma (IFNγ)

IFNγ is a potent inducer of IDO (Orabona *et al.*, 2006). It regulates IDO both at transcriptional and post transcriptional levels (Braun *et al.*, 2005). The anti-proliferative effect of cytokine IFNγ has a connection with the induction of IDO. The anti-proliferation occurs due to the degradation of Trp in the medium, it results in lack of this essential amino acid and cell starvation (Taylor and Feng, 1991).

INDO gene encode IDO and the gene promoter region contains a sole IFNγ specific site for activation, as well as the gene region consists of two IFN-activating response elements, on which IFNα and IFNβ as well as IFNγ can bind and it can respond to all three of them. In inducing IDO expression IFNα or IFNβ has been designated as being up to 100 times less effective in activation than IFNγ (Taylor and Feng, 1991).

1.2.5.2. Inducible Nitric Oxide Synthase (iNOS)

Nitric oxide (NO) is a free-radical gas and short-living molecule. It has an important contribution in a variety of molecular biological processes comprising infectious diseases and cancer (Nathan and Xie, 1994). There are three different isoforms of NOS: inducible NOS (iNOS) endothelial NOS (eNOS) and neuronal NOS (nNOS) are produced in the body at constant rate, whereas, bacterial endotoxins and inflammatory cytokines presence lessened the iNOS production (Morris and Billiar, 1994). Only iNOS has the ability to produce NO sustainably at micro-molar level and it is the only NOS isoform involved in inflammatory courses and cancer development (Beckman *et al.*, 1990).

1.2.5.3. Transforming Growth Factor-Beta (TGFβ)

TGFβ with its signaling elements acts as determining factor of metastatic tumor cell behavior. Cancer development is influenced positively or negatively by the TGFβ's autocrine and paracrine effects exerted on tumor cells and the tumor micro-environment. TGFβ signaling pathway has been reflected to play role in both tumor suppressor as well as promoter of tumor invasion and proliferation (Derynck *et al.*, 2001). Initially TGFβ subdues the invasion of tumors, but it promotes tumor development, progression and relocation of already present tumors when TGFβ itself is present at elevated levels (Elliott and Blobe, 2005).

1.2.5.4. Cyclooxygenase-2 (COX2)

COX2 is a positive inducer of IDO activation, which favors IDO role of mediating immune suppression (Muller and Scherle, 2006). In many tumor such as NSCLC,

colon, gastric and breast tumor, COX2 is overproduced (Pereg and Lishner, 2005). COX2 influences a variety of processes of malignancy and tumor progression as it produces arachidonic-acid catabolite, prostaglandin E2 (PGE2), which results in apoptosis, immunosuppression, angiogenesis, inflammation and intrusiveness (Dannenberg *et al.*, 2001).

1.2.5.5. Cytotoxic T Lymphocyte Antigen (CTLA4) and Cluster of Differentiation 28 (CD28)

CTLA4 act as a natural decelerating machinery for immune system activation as it is a key element in T cell permissiveness. It is a main negative regulator of T cell facilitated antitumor immune reactions (Tarhini and Iqbal, 2001). T cell is activated when T cell receptor engage with major histocompatibility complex (MHC) bounded to antigen and present on the antigen presenting cell (APC) and binding of T cell surface receptor, CD28 with APC ligand of CD 80 and 86 (eg, B7-1/ CD80, B7-2/ CD86) also called B7 family member (Lenschow *et al.*, 1996).

CD28 is a homologue of CTLA4 and it has an inhibitory role and bind with co-stimulatory molecules B7 expressed on mature APC (Paterson *et al.*, 2009; Maker *et al.*, 2005; Krummel and Allison, 1995). What happen in tumor progression is that after T cells are activated, CTLA4 which is a cell-surface receptor and have competitive binding affinity relationship with CD28. In fact CTLA4 have higher binding affinity than CD28 with the B7 family, CTLA4-B7 binding is up-regulated and it effectively vie with CD28-B7 binding and send and down-regulatory signal to T cell activation, thus inhibiting T cell triggering (Linsley *et al.*, 1994). Enhancing the CD28-B7 interaction and blocking the CTLA4-B7 interaction would help in boosted

and sustained T cell initiation, as activation and concentration of IL-2, 3, 4, 5 and10 and cytokines is increased e.g, IFNγ (Alegre *et al.*, 1998; Krummel and Allison, 1995).

1.2.5.6. Suppressor of cytokine signaling-3 (SOCS3)

Cytokine facilitated processes are critically modulated by SOCS proteins (O'Shea and Murray, 2008). Up-regulation of SOCS3, by binding of CD28 with B7, inhibits the transcriptional expression of IDO driven by IFNγ (Orabona *et al.*, 2004). SOCS3 is an important regulator of IDO (Orabona *et al.*, 2008). SOCS proteins have grave role in modulating immune responses (Mellor and Munn, 2004). They possess a SOCS box and a Src homology 2 (SH2) domain. SOCS box take part in the formation of an E3 ubiquitin ligase complex and aims at several signaling proteins for proteasomal degradation (Machado *et al.*, 2008; Orr *et al.*, 2007; Wong *et al.*, 2006; Ungureanu *et al.*, 2002).

1.2.5.7. B7 Family (Cluster of Differentiation 80 and 86)

Two co-stimulatory molecules from family of B7 are B7-1 and B7-2 present on APCs. Both interact with CD28 and CTLA4 on T cells. B7-CD28 interaction favors T cell activation (Bai *et al.*, 2002), whereas binding of B7-CTLA4 heightens the destruction of T cell mediated immune response (Linsley *et al.*, 1994).

1.2.6. Biological Models of Biological Regulatory Networks (BRNs)

Two BRNs are used for applying kinetic and Hybrid modeling. The models with their brief description are given below.

1.2.6.1. IDO First BRN

Induction of IDO should be governed tightly. Homeostasis of IDO is necessary for its normal or controlled production. The potent inducer of IDO in case of tumor and infection is a cytokine, produce inflammation, IFNγ especially in APC which include macrophages and DCs (Takikawa *et al.*, 1999; Carlin *et al.*, 1989; Carlin *et al.*, 1987). IDO gene is transcribed through Janus Kinase, and Signal Transducer and Activator of Transcription (JAK/ STAT) pathway. Activation of IFNγ is done by binding of CTLA4 on B7 (CD80/ CD86) ligand present on APCs. CTLA4 and CD28, present on T cell, are the two receptors for the same ligand B7. Both the receptors are same but they function oppositely after binding with the B7 (Sharpe and Freeman, 2002; Salomon and Bluestone, 2001; Alegre *et al.*, 2001; Sansom *et al.*, 2000). CD28 takes part in promoting T cell immune response in tumor proliferation by binding to B7 but CTLA4 act to suppress immune response against tumor. It favours tumor progression by activating IDO via induction of IFNγ.

On the other hand if CD-28 binds with the B7 it activates T cell response against IDO by activating SOCS3, (Zouali, 2009). TGFβ is a selective and strong inhibitor of IDO. IFN activated transcription is repressed by TGFβ (Panek *et al.*, 1995; Devajyothi *et al.*, 1993). Natural antagonist of IFNγ is TGFβ in this case Trp metabolism is in tight immunological control (Yuan *et al.*, 1998). The model drawn in GENOTECH (Ahmad, 2009) on the basis of above information given is depicted in Figure1.3.

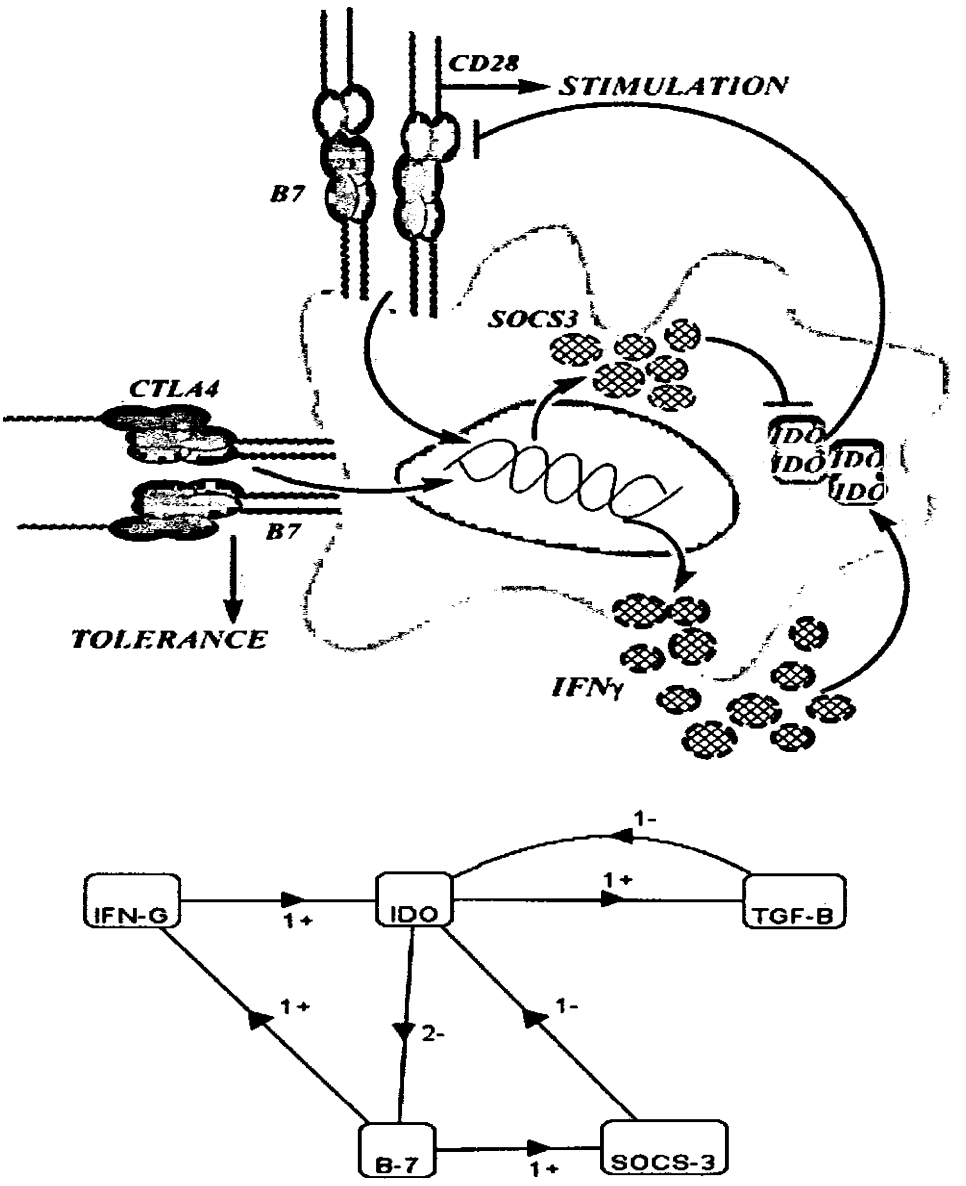


Figure 1.3: IDO First Regulatory BRN and its derived GENOTECH Model

1.2.6.2. IDO Second BRN

IDO key pathway is recruited from review done by Alexander (2006). The main pathway is given in Figure 1.4. The key players in the cancer pathway with IDO are iNOS, COX2 and TGFβ. All are immunosupresser they suppress effector T cells and cause angiogenesis, in this way favor cancer progression and proliferation. First enzyme COX2 has an important role in immune suppression regulation and generation of regulatory T cells. Second enzyme is TGFβ, a cytokine that have intense immunosuppressive effects, and cancer cells become accustomed by becoming refractory to TGFβ signaling (Muller and Schrele, 2006). The role of iNOS in tumor showed a complex picture. High-output NO production by activation of iNOS by infiltrating macrophages can induce tumor cell cytostasis and/ or cytotoxicity (Kroncke *et al.*, 1998).

The pathway extracted from phenomenon is used for modeling, and with enhancement the new model generated is given in Figure 1.4.

1.3. Biological Regulatory Network (BRN)

The life of living cells includes various highly interconnected interactions and chemically interacting small bio-molecules including, DNA, RNA, small metabolites and proteins. It gives rise to regulatory network. It is a complex process as various activities of cells are controlled. The most important molecule among different molecules types in a network is protein as it is the product of gene expression and it got importance as it regulates gene expression as shown in Figure 1.5. They contribute to linking genes to each other and then form a multiple regulatory circuit in a cell.

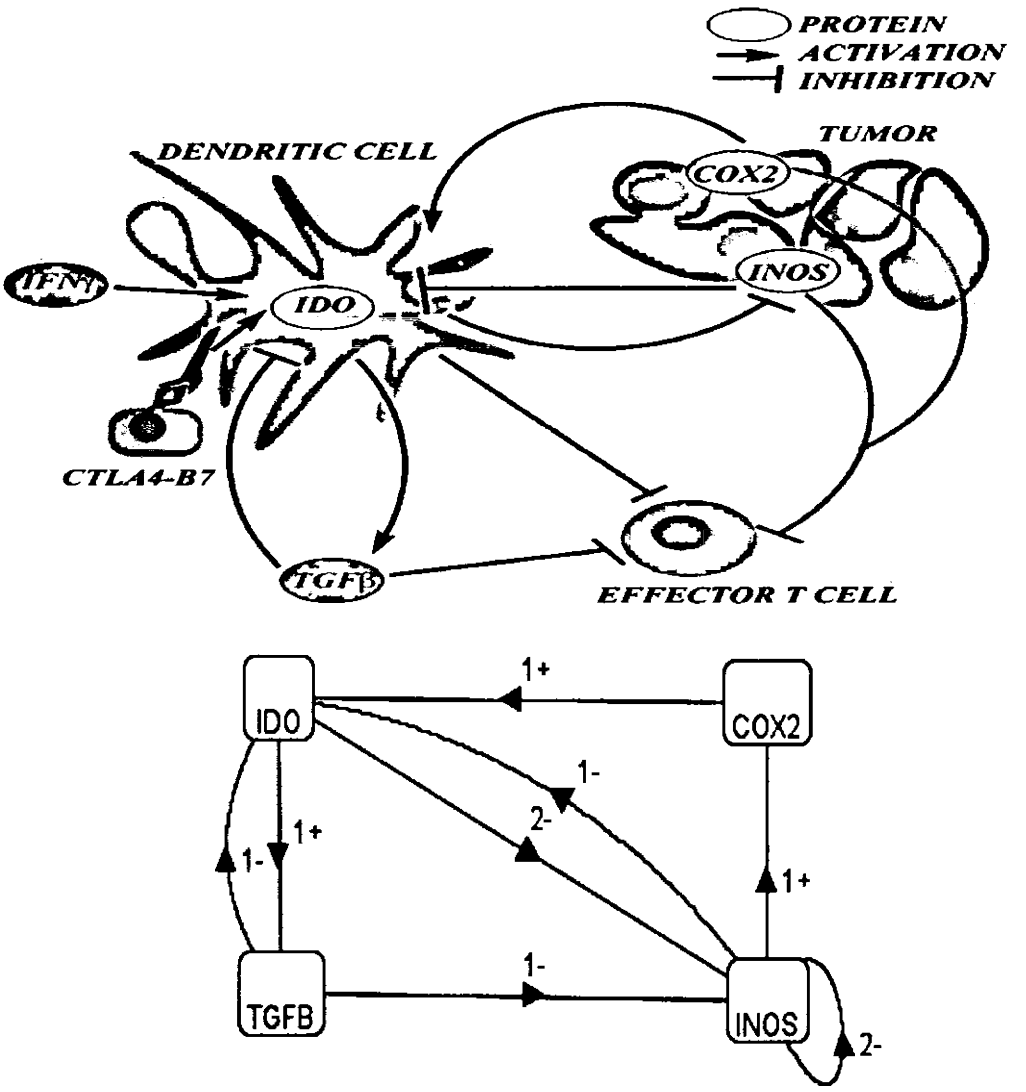


Figure 1.4: Inter-regulation of the various Cytokines and Enzymes associated with Immune suppression and below is given its Qualitative Model

The biological networks combine compound interactions in the cell and give an overall or global view of within cell activities. The relationship between molecules can be well interpreted (Junker and Schreiber, 2008). Biologists embody biological system in terms of graphs and BRNs represent interaction between genes or their products. In living organism every process has two types of important network called gene regulatory pathways and signaling pathways. Gene regulatory networks (GRNs) portray connection between genes that is based on how the expression level of one gene affect expression levels of others. Genes do not interact directly with other in its place, gene stimulation occur through proteins which are also the product of genes, it can also be affected by metabolites directly (Helms, 2008). GRNs are graphs which represent genes and regulatory products as vertices of graph and their interactions are represented with edges. These interactions are further modified by making them signed and directed. The plus '+' sign show activation and minus '-'sign inhibition (Karlebach and Shamir, 2008; Ahmad *et al.*, 2006).

1.3.1. Role of Regulatory Network

Development of body plan is maintained by large GRNs (Eric *et al.*, 2002). Analysis of biological networks is important in understanding their mechanism (Ay *et al.*, 2009; Ay *et al.*, 2008). Regulatory network acts as a skeleton.

It offers a qualitative framework on which quantitative data can be applied using qualititative modeling and simulation (Junker and Schreiber, 2008). Regulation is the main purpose of unexpressed DNA (Sneppe and Zocchi, 2005).

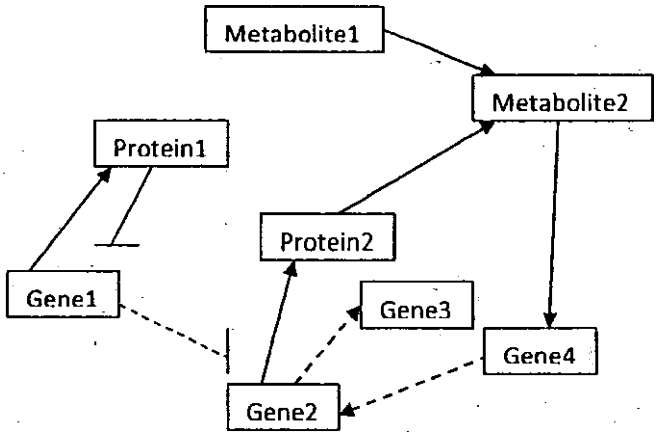


Figure 1.5: Global Biological Network Model having three important participants namely Genes, Proteins and Metabolites

Understanding, identification and study of regulatory network are the key challenges in system biology and it has potential applications in drug designing, diagnosis, therapeutically targeting and disease management (Segal *et al.*, 2004). The behaviors of biological regulatory systems are very anti-intuitive and can be solved by adequate formalism.

1.3.2. Dynamics of Regulatory Networks

Researchers now know clearly that semantics of BRNs and their interaction systems is hidden in the system's dynamics. Biologist use dynamical models using either continuous or discrete models (Ahmad *et al.*, 2006). For the last three decades GRNs are studied by pioneering modelers who have provided evidence in favor of role of dynamics in the systems. Models of network having ten genes have been formed. Either through differential equations (Gonze *et al.*, 2002; Tyson *et al.*, 1999) or discrete frame work of Boolean networks (Kauffman, 1993) or discrete automata (Thomas, 1999). They have helped a lot in better understanding of some of the major regulatory mechanisms involved in cell for example producing a broad view of dynamical phenomena responsible for epigenetic switches (Richard *et al.*, 2005; De Jong, 2002). A mechanism to control the production of proteins is the regulation of gene expression, this regulation has several forms but one of them is transcriptional regulation. A gene j is directly regulated by gene i, if protein encoded by I is transcriptional factor for gene j. This network can be defined using oriented and labeled graph and differential equations to describe the mathematical meaning of arrows on graph (Kepes, 2007).

Properties of qualitative system include the recognition of steady states or limit cycles, identification of multistable behavior and identification of oscillatory behavior (Tyson *et al.*, 2003). It also include depiction of the role of some parts of the network in terms of signal e.g. amplifiers, derivators, logic gates, and valuation of environmental changes or genetic mutations (Tyson *et al.*, 2003, Wolf and Arkin, 2003). The default option for modeling regulatory process is to use and define a differential equation. In differential models of GRNs, the gene activity is represented by a concentration of the associated RNA or proteins x_i, and the evolutions (function) of all concentrations follow a differential equation system $dx/dt = f(x)$. Biologists can take advantage from these observations leading to forming highly non-linear models with some robust threshold effects. Differential equation systems have major drawbacks. They cannot be solved analytically as they are mostly nonlinear. In addition, often the experimental data is of qualitative nature and is not suitable for evaluation of quantitative parameters for the differential model (Siebert and Bockmayr, 2006). The derivation of the dynamics from the interaction graph is not trivial as inference of lot of parameters required, this lead to modification and a tiny modification of a parameter can lead to a strong change in the dynamics. Each equation requires knowledge of one or several parameters and it is difficult to instantiate models of large networks (Ahmad *et al.*, 2006; Jong, 2002). To overcome the limitations kinetic models or discrete models are used. Piecewise linear differential equation is the part of simplified continuous framework. It is a special rate equation in which response of gene to regulatory stimuli is approximated by the use of step function (Jong, 2002). Linearity of equation is used to solve some qualitative properties such as steady states. It can be analyzed qualitatively by discretization and

recasting them into the framework of qualitative differential equation in which variable and their derivatives have discrete values. In kinetic models, modeler can discretize the concentration and can highlight the effects of threshold (i.e. use of delays). It includes Boolean networks and generalized logical networks (GLNs). The Boolean network is representation of system consisting of n variable and nodes, they take two values 0 and 1. 0 for unexpressed and 1 for expressed state, and set of logical rules together describe evolution of a system from a current state at time t to the next state at time $(t + \Delta)$ (Ahmad *et al.*, 2006). GLN is the generalization of Boolean network in which variables can have more than two values and asynchronous transitions (Thomas *et al.*, 1995). The generalized logical analysis built by Thomas and coworkers (1995) to illustrate biological network dig out the essential qualitative features of the dynamics of such systems by logical parameters. Discrete models present a qualitative description of a system dynamics and focus on the structure of the system. Yildirin and Mackey developed the theoretical model for time delay (Yildirim and Mackey, 2003). From a biological perspective, time delay in a gene regulation arises from delays caused by processes such as transcription, translation, and transport processes. Accounting this attribute is necessary to accurately capture the dynamics of the system (Altman *et al.*, 2004). Biological regulatory mechanisms include an important concept of time delay, particularly in case of transcription factors. In bio-molecular level transcription to mRNA and protein translation involves a time delay (Huang *et al.*, 2010). In kinetic logic, the on and off delays for a given gene will generally be unequal, and the delays of different gene will also be different. This shows that biological systems have asynchronous behavior, i.e., all the genes forming a system will not be transcribing at the same time.

1.3.3. Advantages of Dynamical System

Models dig out information from the data and at the same time do approximation of the parameters. The precision of mathematics in modeling enable the modeler to characterize the system fully, resulting to speculate function of the system extensively. It simulates experiments before performing them, thus saving a lot of time and money which is used in performing hit and trial experiments in wet labs. It avoids doing experiments on animals. Models can be refined by comparing experimental measurements with simulated dynamics. It provides insight on qualitative attributes of the system. The emphasis on computational methods increases due to arrival of massive amount of expression data and it surmount the difficulties of interpretation of experimental data. Huge data creates the difficulty in analysis. The variety of components and their interacting capabilities lead to cope with their complexity. This unlocks modeling new dimensions to investigate computational biological systems (Bernot *et al.*, 2007).

1.3.4. Linear Hybrid Automata (LHA)

There are two types of models quantitative and qualitative. Quantitative models have the advantage of giving detailed description of a relatively less interactions. Qualitative models integrate more interactions but kinetic details are fewer (Thakar *et al.*, 2010). A quantitative model shows how the system will work at a specified instance and also can do predictions of kinetic parameters. Qualitative models can also predict the knock-out or persistent activation of components (Thakar *et al.*, 2010). For systems having both qualitative and quantitative behavior, then LHA is a very popular modeling formalism as it has the features of both the continuous as well

as discrete system (Grosu *et al.*, 2007). It is derived from finite-state automata. The discrete states show numerous approaches of continuous dynamics of a system and transition correspond to swapping logic between these approaches. It is recently being used in modeling and analyzing biological systems such as GRNs, protein signaling pathways (PSPs), metabolic pathways etc. (Bemporad *et al.*, 2007).

Identification of regulatory network associated with IDO pathway which will further be used in discrete modeling/ Hybrid modeling formalism application on identified network. Based on the qualitative characterization of the biological regulatory pathways, the next step will be building and analysis of Qualitative Models; it will require model checker tools for the qualitative analysis of the discrete/ Hybrid models. After model generation and parameter optimization, by performing in-silico experiments and adjusting similar biological observed characters, parameters will be identified. The research will focus on behavior of the system, and for both normal pathways i.e. Oscillation and abnormal (diseased) condition i.e. dead lock state. The second half of the study is based on the development of the Hybrid model of the system using regulation Delays (Production/ Degradation delays). The results and importance of the proteins involve in the BRN will be tested by applying in-silico protein knocking out and study of stability behavior. If it can be precisely identified the way how these regulatory proteins regulate IDO it will help greatly in understanding more deeply the human immune response.

CHAPTER 2

MATERIALS AND METHODS

2. Materials and Methods

Models generated through qualitative modeling dig out information from the data and at the same time do approximation of the parameters. The precision enable the modeler to characterize the system fully, resulting to hypothesize elaborately about how the system functions. It helps in modeling and simulating experiments before execution, which benefit through saving time and money. Modeling using qualitative approach helped to generate all possible set of states showing behavior of the system. The best formalism defining the qualitative modeling is the one designed by René Thomas (Thomas and D'Ari, 1990), leading to more precise predictions. The generalized logical analysis built by R. Thomas and co-workers to illustrate biological network dig out the essential qualitative features of the dynamics of such systems by logical parameters (Thomas *et al.*, 1995; Thomas, 1981).

2.1. Logical Modeling Formalism

Logical system is a combination of a set of conjecture rules defined in systematic language. Here logic is associated with element of system by,

- A logical variable which shows level e.g., concentration.
- A logical function reflecting evolution e.g., evolution.

$$X = \theta(x, y, z, \ldots.)$$

Where θ = Logical function

$(x,y,z,\ldots.)$ = Variables

In biological system, it is most often used to deal gene expression. Dealing with gene gives gene on or off and their product present or absent.

X = 1 or 0 representing gene, 'On' or 'Off' respectively.

x = 1 or 0 representing gene-product, 'Present' or 'Absent' respectively.

Suppose a protein x is activated if and only if (iff) protein y is present and y is activated iff x is absent and y itself is present as drawn in Figure 2.1.

Mathematically,

$$x = y$$

$$y = \bar{x}y$$

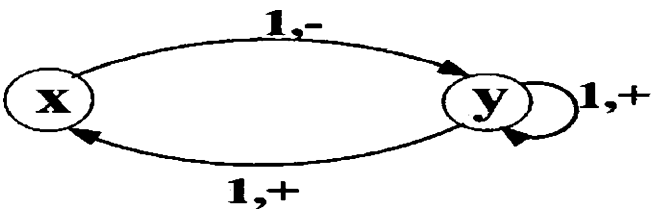
Such expressions are represented by formalism,

The formalism of qualitative modeling defined by René Thomas (Thomas and D'Ari, 1990) is a well-known formalism to construct a discrete (qualitative) model of a BRN. It is given in the form of set of definitions and explained using an example as shown in Figure 2.1.

2.1.1. Biological Regulatory Network (BRN)

A BRN is represented by a labelled oriented graph $G = (V, \varepsilon)$, where V is the set of nodes representing biological entities and ε is the set of edges representing interaction. Each vertex i of V has a boundary $\beta_i \in N$ less or equal to number of outgoing arcs (out-degree) of i. Each edge is labelled that is, $(i \rightarrow j)$ have a pair of labels (t'_{ij}, s_{ij}), $s_{ij} \in \{-, +\}$ donating sign of interaction and t'_{ij} is the threshold; its value is from 1 to β_i. The part a, of Figure 2.1 is a BRN of two proteins x and y (Thomas and D'Ari, 1990; Thomas, 1981).

a.



b.

x	y	w_x	w_y	$K_{x,wx}$	$K_{y,wy}$
0	0	{}	{x}	0	1
0	1	{y}	{x,y}	1	1
1	0	{}	{}	0	0
1	1	{y}	{y}	1	1

c.

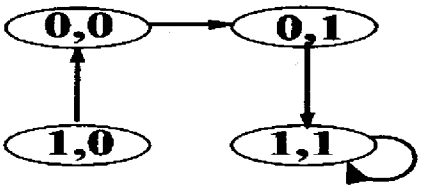


Figure 2.1: Formalism in three forms, a. a BRN graph,

b. State Table generated by BRN and c. State graph of the BRN

In the oriented graph, the information is not sufficient for explaining the dynamics of the system. For this purpose we have to first describe the qualitative states of the BRN.

2.1.2. State

Regulatory component of an oriented graph are represented in the form of a set of nodes $n = (n_1, \ldots, n_m)$ where m is the number of vertices and each n_i belongs to natural number N. Each node n_i has a positive integer max_i showing its maximum level of concentration. All possible levels of concentrations of a node are defined by the set $\{0, \ldots, max_i\}$ (Thomas and D'Ari, 1990; Thomas, 1981).

To identify that a regulator have an effect on its target element, a combination of sign and threshold is used. In defining dynamics, one should know how much a target is abstracted. Answer of this question is hidden in finding which of the regulators are effective for each state n on target i, in other words we have to know about resources of the state n.

In logical regulatory network behaviour correspond to state transition graph; nodes are signified by states and the arc joining the states are donating transition of states. $x \rightarrow y$ is showing transition from current to its descendant state as shown in Figure 2.1 (a.).

2.1.3. Resources

The set of resources $\acute{R}_i$ of an entity i in the state $n = (n_1, \ldots, n_m)$ *of the BRN* $G = (V, \varepsilon)$ is:

$$\acute{R}_i(n) = \{j \in V \mid (j \xrightarrow{(t',+)} i) \in \varepsilon \text{ and } (n_j \geq t'),$$

$$\text{or } (j \xrightarrow{(t,-)} i) \in \varepsilon \text{ and } (n_j < t')].$$

Resources represent either presence of activator or the absence of inhibitor. The level $K_{i,\acute{R}i}$ is called the focal point of i when $\acute{R}_i$ is the set of resource. Focal points values define synchronous state graph which show first dynamics of the system. Constructing a synchronous state graph require unique possible successor of state $n=(n_1, \ldots, n_m)$ which are attracted towards the state: $\acute{n}=(K_{1,\acute{R}1(n)}, K_{2,\acute{R}2(n)}, \ldots K_{m,\acute{R}m(n)})$ (Thomas and D'Ari, 1990; Thomas, 1981). Protein y is a resource for x and y is a resource of its own self and absence of x is a resource of y as shown in state table of Figure 2.1(b.).

There are two drawbacks in the definition:

Two or more variables can change at a time, while *in-vivo* probability of several variables to reach threshold is negligible. It is not clear which one will reach threshold first.

An abstract expression level should evolve gradually in reality, in case of synchronous, a variable passes two or more thresholds, and it is not realistic (Ahmad *et al.*, 2007).

This can be overcome by replacing synchronous with asynchronous state graph. Collection of transitions which modify only one transition at a time is used to replace each diagonal transition of the synchronous state graph (Ahmad *et al.*, 2007).

An evolution operator $\Rightarrow$: *for* $x, K \in N$ is defined as follows. $x \Rightarrow K$ is equal to

- $x-1$ *if* $x > K$,
- $x+1$ *if* $x < K$ and
- x *if* $x = K$.

2.1.4. State Graph

An asynchronous state graph, $(S, \rightarrow)$ of a biological regulatory graph $G = (V, \varepsilon)$, is defined as:

- The set of vertices are represented by the set of states $\Pi_{i \in V}\{0, ..., \beta_i\}$.
- The state $n = (n_1, \ldots, n_m)$ has a transition to the state $p = (p_1, ..., p_m)$ *iff*
- i is a unique state that is $i \in \{1, ..., m\}$ such that $p_i \neq n_i$, and $p_i = (n_i \Rightarrow K_{i, R_i(n)})$ or
- $n_i = p_i$ *and* $\forall i \in \{1, ..., m\}$ $n_i = K_{i, R_i(n)}$ (Thomas and D'Ari, 1990; Thomas, 1981).

The part c. of Figure 2.1, is a state graph.

2.2. Qualitative Modeling of IDO associated BRNs

BRNs are graphs which represent genes and regulatory products (proteins) as vertices and their interactions are represented with edges. These interactions are further customized by making them signed and directed. The plus '+' sign show activation and minus '-' sign inhibition (Karlebach and Shamir, 2008; Ahmad *et al.*, 2006). The interactions in a BRN may lead to regulatory circuits. The circuits are of two types: positive or negative circuit. A circuit is said to be negative if it has odd number of negative interactions otherwise it is positive (Thomas and D'Ari, 1990). Occurrence of a positive circuit in a pathway represents epigenetics or multistationarity. Negative circuits symbolize homeostasis or stable fluctuation. Both are necessary and sufficient conditions in a qualitative model (Kaufman *et al.*, 2007; Thomas, 1981). It is verified

to be useful and well-suited for the qualitative modeling of BRNs. In Figure, 2.2 and 2.3, modeled IDO BRNs are presented.

2.2.1. Modeling of IDO First BRN

The first IDO BRN has one positive loop; spanning around IDO, B7 and SOCS3 and having even numbers (2) of negative interactions as shown in IDO BRN in Figure 2.2. Three negative loops: one from IDO to TGFβ, second enclosing IFNγ, IDO and B7 third one enclosing IDO, TGFβ, IDO, B7 and SOCS3. The regulatory network of IDO is generated using GENOTECH (Ahmad, 2009). One stable steady state is generated which is showing diseased state where IDO is over expressed and its inhibitors are absent leading to uncontrolled tumour proliferation and growth.

The main inducer of IDO is IFNγ which is activated when CTLA4 is attached with B7 ligand. The main suppressor of IDO is TGFβ which has both the immunosuppressive and tumor suppression roles. Overexpression of IDO leads to disease condition in which tumor progress without any hindrance as a result immune response is totally damaged by IDO. Table 2.1, lists all the regulators of the BRN of the Figure 2.2, where column 1 is showing proteins and column 2 represents activator and 3rd column is showing inhibitor and last one is the associated list of the parameters. These parameters were used to the results of present study.

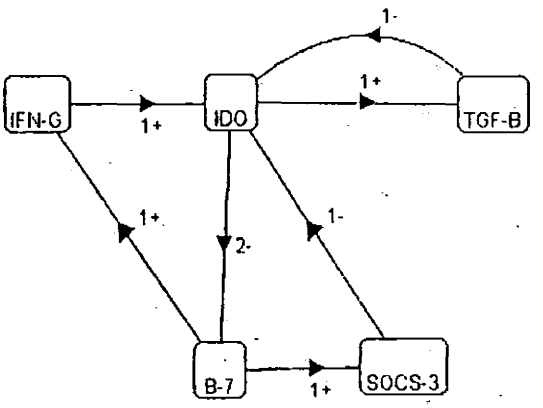


Figure 2.2: IDO first BRN constructed using R. Thomas logic

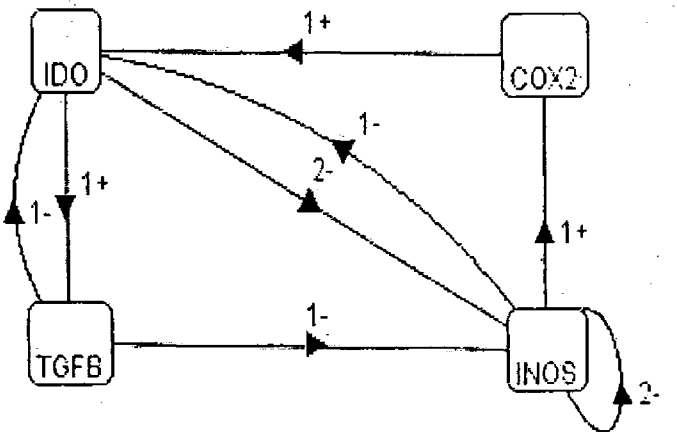


Figure 2.3: IDO Second Model: drawn in GENOTECH

2.2.2. Modeling of IDO Second BRN

There are two positive loops in IDO second BRN; first spanning around IDO and iNOS, and second covering IDO, TGFβ and iNOS having even numbers (4) of negative interactions as shown in IDO BRN in Figure 2.3. Four negative loops: one from IDO to TGFβ, second enclosing IDO, iNOS and COX2 third one enclosing IDO, TGFβ, iNOS and COX2 and the last one is on iNOS as shown in Figure 2.3.

The regulatory network of IDO is generated using GENOTECH. Two stable steady states are generated.

The main inducer of IDO is IFNγ which is activated when tumor is appeared in the body the second main inducer is COX2, which itself is an immunosupressor activated by iNOS. The main suppressor of IDO is TGFβ which has both the immunosuppressive and tumor suppression roles the second suppressor is iNOS. There is an interesting relationship between IDO and iNOS as both are inhibiting each other. Table 2.2, enlists all the regulators of the IDO and other proteins involved in the second IDO BRN, Figure 2.3, where column 1 is showing proteins and column 2 represents activator and 3rd column is showing inhibitor and last one is the associated list of the parameters. These parameters were used in the results of present study.

Table 2.1: Parameters Optimization of IDO First BRN

Index	Proteins	Activtors	Inhibitors	Parameters
1.	IFN-γ	B-7	nil	K(IFN-γ, {})= 0 K(IFN-γ, {B-7})=1
2.	B-7	Tumor Signal	IDO	K(B-7, {})= 0 K(B-7, {IDO})=1
3.	TGF-β	IDO	nil	K(TGF-β, {})= 0 K(TGF-β, {IDO})=1
4.	SOCS-3	B-7	nil	K(SOCS-3, {})= 0 K(SOCS-3, {B-7})=1
5.	IDO	IFN-γ	TGF-β SOCS-3	K(IDO, {})= 0 K(IDO, {IFN-γ})=2 K(IDO, {TGF-β})= 2 K(IDO, {SOCS-3})=2 K(IDO, {TGF-β, IFN-γ})= 2 K(IDO, {SOCS-3, IFN-γ})= 2 K(IDO, {TGF-β, SOCS-3})=2 K(IDO, {TGF-β, IFN-γ, SOCS-3})= 2

Table 2.2: Parameter Optimization of IDO Second BRN

Index	Proteins	Activtors	Inhibitors	Parameters
1.	iNOS	Tumor Signal	TGF-β IDO	K(iNOS, {})= 0 K(iNOS, {TGF-β})=2 K(iNOS, {IDO})= 2 K(iNOS, {IDO,TGF-β})=2 K(iNOS, {IDO,TGF-β, iNOS})=2
2.	TGF-β	IDO	nil	K(TGF-β, {})= 0 K(TGF-β, {IDO})=1
3.	COX-2	iNOS	nil	K(COX-2, {})= 0 K(COX-2, {iNOS})=1
4.	IDO	COX-2	TGF-β iNOS	K(IDO, {})= 0 K(IDO, {COX-2})=2 K(IDO, {TGF-β})= 2 K(IDO, {iNOS})=2 K(IDO, {TGF-β, COX-2})= 2 K(IDO, {COX-2, iNOS})= 2 K(IDO, {TGF-β, iNOS})=2 K(IDO, {TGF-β, iNOS, COX-2})= 2

2.3. Qualitative Modeling Tool for Parameter Optimization

GENOTECH (Ahmad, 2009) tool is used to construct and analyze qualitative modeling of the BRN through generating steady states. The tool aid and implement the discrete modeling formalism of a BRN. Apart from the discrete modeling, this tool also facilitate in the analysis of stead state behaviors (cycles and stable states). Homeostasis and epigenetics are the two types of behaviors studied using the tool. Positive loops are the necessary and sufficient conditions in a BRN to study epigenetics or stable states and for homeostasis it is the necessary and sufficient to have negative loops as represented in the BRNs of Figure 2.2 and 2.3. The steady state behavior is observed by doing fine tuning of parameters (Table 2.1 and 2.2).

2.4. Hybrid Modeling

After fine tuning of parameters and analysis of both IDO BRNs, the next step comes that is real time modeling also called Hybrid modeling, Combination of discrete system with continuous differential equation leads to formation of hybrid systems, it cover the switching of real life behavior observed inside the cell, using hybrid automaton (Alur *et al.*, 2001).

A BRN model is very valuable theoretically when there are many feedback loops of protein exist in the model. In these models identification and study of vital loops, which lead to observed phenotype, is difficult without the aid of computer programs. Computer assistance is helpful, in a way biologists can perform simulation which will be helpful in exploring possible behaviors. Without computer aided simulations, the

properties of BRN and validation of hypothesis remain inadequate and deficient. First it is necessary to define the BRN formally as done in Bernot (2007), defining BRN help express BRN in logical formulae which will able computer to perform model checking. The enhancement of the above formalism in the form of temporal networks which take into account delays of product production and/ or degradation (Bernot *et al.*, 2007).

Regulation defined by Kaufman and Thomas (2007), it is the process that control rate of production and degradation of a protein in the BRN according to the system requirement and with respect to environmental limits. They used the asynchronous approach. One main advantage of asynchronous approach of Thomas is that formalized biological hypothesis can be encoded into logical temporal properties and can be validated automatically using model checking tools (Kaufman *et al.*, 2007).

2.4.1. Hybrid Automaton (HA)

A famous modeling formalism exhibiting both discrete and continuous behavior is called Hybrid automaton (HA) (Alur *et al.*, 1995; Lynch *et al.*, 2003). It is a modified version of finite state automaton in which discrete states are matched/ linked to continuous dynamics of a system. In a HA, the discrete and continuous, both types of variables were used to represent the state of the system. In a dynamical system the continuous variables evolve when it reaches a certain threshold level at the same time then the discrete variable shift to new value. Then in the new state, the continuous variable starts to evolve again (Antoniotti *et al.*, 2003). Embedded systems are modeled using HA which includes automated highway systems, air traffic management, embedded automotive controllers, robotics and real time circuits. HA is

being used recently to model and analyze BRN such as bio-molecular networks (Alur *et al.*, 2001), metabolic processes (Hespanha and Singh, 2005; Lincoln and Tiwari, 2004), PSPs (Ghosh and Tomlin, 2004) and immune response (Tieri *et al.*, 2005). BRNs are fundamentally hybrid in nature: biochemical concentrations continuously vary in nature it is possible they can be discretized by using distinct states.

LHA is a finite sequence of 6 terms (ℓ, ℓ_0, X, P, E, Inv, pif) where,

- A fixed set of locations denoted by ℓ
- ℓ_0 is a part of ℓ representing initial location
- X is a fixed number of clocks
- Delay constraints in a finite set are denoted by P
- Finite set of edges $\varepsilon = (\ell, g, R, \ell') \in E$, it represents an edge has a guard g and it transmit from ℓ to ℓ' and the set reset $R \subseteq X$
- Inv: work is to allocate immutable to a location
- pif: $\ell \times X \rightarrow \{-1,0,1\}$, mapping of evolution rate with each pair (ℓ, n)

2.4.2. Clocks and Delays

A protein evolves in a continuous and in a non-linear function. In discrete logical formalism of Thomas (Thomas, 1991) there is a discrete level involves in the protein evolution, this fact is overlooked in this formalism. Modeling BRN require addition of temporal constraints (Siebert and Bockmayr, 2008; Siebert and Bockmayr, 2006; Bernot *et al.*, 2004) to show continuous evolution. To resolve the issue hybrid

modeling (Ahmad *et al.*, 2007) method have been projected which introduced time interval and clocks, since it is accepted that protein need delay to evolve.

The protein x and y has clock h_x and h_y respectively, represents that the proteins are synchronously evolving with time thus making discrete to continuous system. The clocks are acting as guards controlling the transition of the system. The clocks act as transition guards. The clocks have boolean values and the rate of clock is set to zero when it cover the complete transition between two states (Ahmad *et al.*, 2009). The clock calculates the most recent change occurs in the system and current value represents the time passed after the fresh transition or change is occurring in the protein discrete state space. For the protein x, LHA place over a hypercube representing the delay. The delay for any protein is of two types a positive delay showing rate of synthesis or activation denoted by δ^+ ($\delta_x{}^+ > 0$), similarly when the protein is being inhibited or degrading it is represented by δ^- ($\delta_x{}^- < 0$).

2.4.3. Invariance Kernel

A set in which all the points starting the trajectory keep the points within the set is called invariant set and the largest of this set is called invariance kernel. Invariance kernel gives information about the behavior of the cycle. It is a set of states which generate a trajectory by primordial permissible command and then remain in it by satisfying the constraints forever.

In a BRN, let all the temporal state space is given by a subset $\mathcal{K}$. When $x \in \mathcal{K}$, then set of $\mathcal{K}$ is immutable it says that every trajectory or pathway starting in x is feasible and executable in $\mathcal{K}$. The largest unalterable subset of $\mathcal{K}$ is known as invariance kernel.

If the system comes outside the kernel then it will move in divergence trajectories leading to stable steady states.

2.4.4. Period

The results obtained from HyTech (Henzinger *et al.*, 1997) analysis require definition of full Period (denoted by π (p)). It is the sum of all the delays (once at each expression level) that a gene goes through sequentially (Ahmad, 2009). The gene's original round can be greater than the full time period as there can be lazy phases in gene expression (with no increase or decrease) (Ahmad, 2009). The results obtained from Hytech are expressed as constraints, showing the nature of the cycle.

2.5. Tool for Hybrid Modeling

An appropriate model checker tool is necessary for implementing and analyzing LHA, HyTech (Henzinger *et al.*, 1997) model checker is the best option as it comes with a set of convenient manipulation commands and it is mainly develop and designed for the authentication of real time systems. HyTech is given preference over other tools as other limit the LHA to a restricted class of timed automaton (Bengtsson *et al.*, 1998) and second problem is that other tools have insufficient commands for analyzing the system (Frehse, 2005). It is used to find delays to trajectories leading to stable steady state and also invariance kernel.

Analysis of existing behavior and satisfying the essential conditions related to behavior can be managed using this model checker as it synthesize constraint with respect to parameters.

CHAPTER 3

RESULTS AND DISCUSSIONS

3. Results and Discussion

Even after surgical amputation HCC diagnosis is very meager with a low survival rate of 25–39% (Pan *et al.*, 2008). Tumor progression is elucidated by two molecular mechanisms, one covering immune tolerance by tumor antigen and second relating to immunosuppression in tumor microenvironment favoring immune escape (Zou, 2005; Mapara and Sykes, 2004). IDO is involved in both the tumor progression mechanisms. There is a fragile equilibrium exist between IDO silencing and overexpression. In case of IDO absence, it will result in rejection of allogenic fetal as tested in mice model (Munn *et al.*, 1998) in the same manner uncontrolled activation of IDO favors tumor proliferation as depicted by diseased states of the both IDO BRNs.

3.1. Qualitative Modeling of First BRN

3.1.1. State Table

After accomplishing the parameter optimization as shown in Table 2.1 following state Table 3.1 is readily generated. It gives details of all input states given in first column of Table 3.1 and their respective output states, called transition states in last column of same table. It also contains two middle columns, in which former depicts weight (w) of input state and latter represents the final and maximum a protein can attain after applying the w. According to biological knowledge the system have an initial state which is represented in blue colour and the system also have an epigenetic state, dead lock state, represented in red colour.

Table 3.1: State Table for First IDO Model

IFNG,IDO,B7,SOCS3,TGFβ	wIFNγ, wIDO, wB7, wSOCS3, wTGFβ	kIFNγ, kIDO, kB7, kSOCS3, kTGFβ	Transition states
0 0 0 0 0	{},{SOCS-3, TGF-B},{IDO},{},{}	0 2 1 0 0	[0, 1, 0, 0, 0] [0, 0, 1, 0, 0]
0 0 0 0 1	{},{SOCS-3},{IDO},{},{}	0 2 1 0 0	[0, 1, 0, 0, 1] [0, 0, 1, 0, 1] [0, 0, 0, 0, 0]
0 0 0 1 0	{},{TGF-B},{IDO},{},{}	0 2 1 0 0	[0, 1, 0, 1, 0] [0, 0, 1, 1, 0] [0, 0, 0, 0, 0]
0 0 0 1 1	{},{},{IDO},{},{}	0 0 1 0 0	[0, 0, 1, 1, 1] [0, 0, 0, 0, 1] [0, 0, 0, 1, 0]
0 0 1 0 0	**{B-7},{SOCS-3, TGF-B},{IDO}, {B-7},{}**	**1 2 1 1 0**	**[1, 0, 1, 0, 0]** **[0, 1, 1, 0, 0]** **[0, 0, 1, 1, 0]**
0 0 1 0 1	{B-7},{SOCS-3},{IDO},{B-7},{}	1 2 1 1 0	[1, 0, 1, 0, 1] [0, 1, 1, 0, 1] [0, 0, 1, 1, 1] [0, 0, 1, 0, 0]
0 0 1 1 0	{B-7},{TGF-B},{IDO},{B-7},{}	1 2 1 1 0	[1, 0, 1, 1, 0] [0, 1, 1, 1, 0]
0 0 1 1 1	{B-7},{},{IDO},{B-7},{}	1 0 1 1 0	[1, 0, 1, 1, 1] [0, 0, 1, 1, 0]
0 1 0 0 0	{},{SOS-3, TGF-B},{IDO},{},{IDO}	0 2 1 0 1	[0, 2, 0, 0, 0] [0, 1, 1, 0, 0] [0, 1, 0, 0, 1]
0 1 0 0 1	{},{SOCS-3},{IDO},{},{IDO}	0 2 1 0 1	[0, 2, 0, 0, 1] [0, 1, 1, 0, 1]
0 1 0 1 0	{},{TGF-B},{IDO},{},{IDO}	0 2 1 0 1	[0, 2, 0, 1, 0] [0, 1, 1, 1, 0] [0, 1, 0, 0, 0] [0, 1, 0, 1, 1]
0 1 0 1 1	{},{},{IDO},{},{IDO}	0 0 1 0 1	[0, 0, 0, 1, 1] [0, 1, 1, 1, 1] [0, 1, 0, 0, 1]
0 1 1 0 0	{B-7},{SOCS-3, TGF-B},{IDO}, {B-7},{IDO}	1 2 1 1 1	[1, 1, 1, 0, 0] [0, 2, 1, 0, 0] [0, 1, 1, 1, 0] [0, 1, 1, 0, 1]
0 1 1 0 1	{B-7},{SOCS-3},{IDO}, {B-7},{IDO}	1 2 1 1 1	[1, 1, 1, 0, 1] [0, 2, 1, 0, 1] [0, 1, 1, 1, 1]
0 1 1 1 0	{B-7},{TGF-B},{IDO},{B-7},{IDO}	1 2 1 1 1	[1, 1, 1, 1, 0] [0, 2, 1, 1, 0]

										[0, 1, 1, 1, 1]
0	1	1	1	1	{B-7},{},{IDO},{B-7},{IDO}	1	0	1	1	1	[1, 1, 1, 1, 1] [0, 0, 1, 1, 1]
0	2	0	0	0	{},{SOCS-3, TGF-B},{},{},{IDO}	0	2	0	0	1	[0, 2, 0, 0, 1]
0	**2**	**0**	**0**	**1**	**{},{SOCS-3},{},{},{IDO}**	**0**	**2**	**0**	**0**	**1**	[]
0	2	0	1	0	{},{TGF-B},{},{},{IDO}	0	2	0	0	1	[0, 2, 0, 0, 0] [0, 2, 0, 1, 1]
0	2	0	1	1	{},{},{},{},{IDO}	0	0	0	0	1	[0, 1, 0, 1, 1] [0, 2, 0, 0, 1]
0	2	1	0	0	{B-7},{SOCS-3, TGF-B},{},{B-7},{IDO}	1	2	0	1	1	[1, 2, 1, 0, 0] [0, 2, 0, 0, 0] [0, 2, 1, 1, 0] [0, 2, 1, 0, 1]
0	2	1	0	1	{B-7},{SOCS-3},{},{B-7},{IDO}	1	2	0	1	1	[1, 2, 1, 0, 1] [0, 2, 0, 0, 1] [0, 2, 1, 1, 1]
0	2	1	1	0	{B-7},{TGF-B},{},{B-7},{IDO}	1	2	0	1	1	[1, 2, 1, 1, 0] [0, 2, 0, 1, 0] [0, 2, 1, 1, 1]
0	2	1	1	1	{B-7},{},{},{B-7},{IDO}	1	0	0	1	1	[1, 2, 1, 1, 1] [0, 1, 1, 1, 1] [0, 2, 0, 1, 1]
1	0	0	0	0	{},{IFN-G,SOCS-3, TGF-B},{IDO},{},{}	0	2	1	0	0	[0, 0, 0, 0, 0] [1, 1, 0, 0, 0] [1, 0, 1, 0, 0]
1	0	0	0	1	{},{IFN-γ, SOCS-3},{IDO},{},{}	0	2	1	0	0	[0, 0, 0, 0, 1] [1, 1, 0, 0, 1] [1, 0, 1, 0, 1] [1, 0, 0, 0, 0]
1	0	0	1	0	{},{IFN-γ, TGF-B},{IDO},{},{}	0	2	1	0	0	[0, 0, 0, 1, 0] [1, 1, 0, 1, 0] [1, 0, 1, 1, 0] [1, 0, 0, 0, 0]
1	0	0	1	1	{},{IFN-γ},{IDO},{},{}	0	2	1	0	0	[0, 0, 0, 1, 1] [1, 1, 0, 1, 1] [1, 0, 1, 1, 1] [1, 0, 0, 0, 1] [1, 0, 0, 1, 0]
1	0	1	0	0	{B-7},{IFN-γ, SOCS-3, TGF-B},{IDO},{B-7},{}	1	2	1	1	0	[1, 1, 1, 0, 0] [1, 0, 1, 1, 0]
1	0	1	0	1	{B-7},{IFN-γ, SOCS-3},{IDO},{B-7},{}	1	2	1	1	0	[1, 1, 1, 0, 1] [1, 0, 1, 1, 1] [1, 0, 1, 0, 0]
1	0	1	1	0	{B-7},{IFN-γ, TGF-B},{IDO},{B-7},{}	1	2	1	1	0	[1, 1, 1, 1, 0]

1	0	1	1	1	{B-7}, {IFN-γ},{IDO},{B-7},{}	1	2	1	1	0	[1, 1, 1, 1, 1] [1, 0, 1, 1, 0]
1	1	0	0	0	{},{IFN-γ, SOCS-3, TGF-B}, {IDO},{},{IDO}	0	2	1	0	1	[0, 1, 0, 0, 0] [1, 2, 0, 0, 0] [1, 1, 1, 0, 0] [1, 1, 0, 0, 1]
1	1	0	0	1	{},{IFN-γ, SOCS-3},{IDO},{}, {IDO}	0	2	1	0	1	[0, 1, 0, 0, 1] [1, 2, 0, 0, 1] [1, 1, 1, 0, 1]
1	1	0	1	0	{},{IFN-γ, TGF-B}, {IDO},{},{IDO}	0	2	1	0	1	[0, 1, 0, 1, 0] [1, 2, 0, 1, 0] [1, 1, 1, 1, 0] [1, 1, 0, 0, 0] [1, 1, 0, 1, 1]
1	1	0	1	1	{},{IFN-γ},{IDO},{},{IDO}	0	2	1	0	1	[0, 1, 0, 1, 1] [1, 2, 0, 1, 1] [1, 1, 1, 1, 1] [1, 1, 0, 0, 1]
1	1	1	0	0	{B-7},{IFN-γ, SOCS-3, TGF-B}, {IDO},{B-7},{IDO}	1	2	1	1	1	[1, 2, 1, 0, 0] [1, 1, 1, 1, 0] [1, 1, 1, 0, 1]
1	1	1	0	1	{B-7},{IFN-γ, SOCS-3},{IDO}, {B-7},{IDO}	1	2	1	1	1	[1, 2, 1, 0, 1] [1, 1, 1, 1, 1]
1	1	1	1	0	{B-7},{IFN-γ, TGF-B},{IDO}, {B-7},{IDO}	1	2	1	1	1	[1, 2, 1, 1, 0] [1, 1, 1, 1, 1]
1	1	1	1	1	{B-7},{IFN-γ},{IDO},{B-7},{IDO}	1	2	1	1	1	[1, 2, 1, 1, 1]
1	2	0	0	0	{},{IFN-γ, SOCS-3, TGF-B},{},{}, {IDO}	0	2	0	0	1	[0, 2, 0, 0, 0] [1, 2, 0, 0, 1]
1	2	0	0	1	{},{IFN-γ, SOCS-3},{},{},{IDO}	0	2	0	0	1	[0, 2, 0, 0, 1]
1	2	0	1	0	{},{IFN-γ, TGF-B},{},{},{IDO}	0	2	0	0	1	[0, 2, 0, 1, 0] [1, 2, 0, 0, 0] [1, 2, 0, 1, 1]
1	2	0	1	1	{},{IFN-γ},{},{},{IDO}	0	2	0	0	1	[0, 2, 0, 1, 1] [1, 2, 0, 0, 1]
1	2	1	0	0	{B-7},{IFN-γ, SOCS-3, TGF-B},{}, {B-7},{IDO}	1	2	0	1	1	[1, 2, 0, 0, 0] [1, 2, 1, 1, 0] [1, 2, 1, 0, 1]
1	2	1	0	1	{B-7},{IFN-γ, SOCS-3},{},{B-7}, {IDO}	1	2	0	1	1	[1, 2, 0, 0, 1] [1, 2, 1, 1, 1]
1	2	1	1	0	{B-7,{IFN-γ, TGF-B},{}, {B-7},{IDO}	1	2	0	1	1	[1, 2, 0, 1, 0] [1, 2, 1, 1, 1]
1	2	1	1	1	{B-7},{IFN-γ},{},{B-7},{IDO}	1	2	0	1	1	[1, 2, 0, 1, 1]

An input state can have more than one final state it depends on target (K), set in parameterization formula. Generally a state can have (n-1) output states where 'n' is the number of element (proteins) involved in the BRN of the system.

The initial state of the BRN is [0,0,1,0,0] in which after tumour invasion an antigen is attached on B7. This binding activates three possible states arises from it as shown in Figure 3.1 and Table 3.1. First one is, [0,0,1,1,0], in which after T cell activation SOCS3 inhibitor of IDO is expressed. Next is [1,0,1,0,0], here after T cell activation inflammation is caused by IFNγ expressed and in the last case [0,1,1,0,0], IDO is expressed with T cell activation. But the main transition is activation of IFNγ [1,0,1,1,0] then leading to activation of IDO [1,1,1,1,0].

3.1.2. State Graph

A state graph Figure 3.1 was generated after applying parameters of Table 2.1 on the first qualitative model of IDO. The Figure 3.1 has a key shown in green coloured oval which represent order of proteins in a state which starts from IFNγ (IFN-G) and last one is TGFβ (TGF-B). The graph presented draw all possible state transitions a state can have, after following the parameterization formula. All the information is hidden in the graph of Figure 3.1; the graph is used for further analysis. The information is hidden and scattered in the Figure 3.1. The red coloured steady state is a stable steady state, as it has no outgoing but only incoming, from that state system cannot move further ahead. It is a diseased condition in IDO BRN the state made the system stuck and restricting it to move further ahead so that's why it is called a deadlock state.

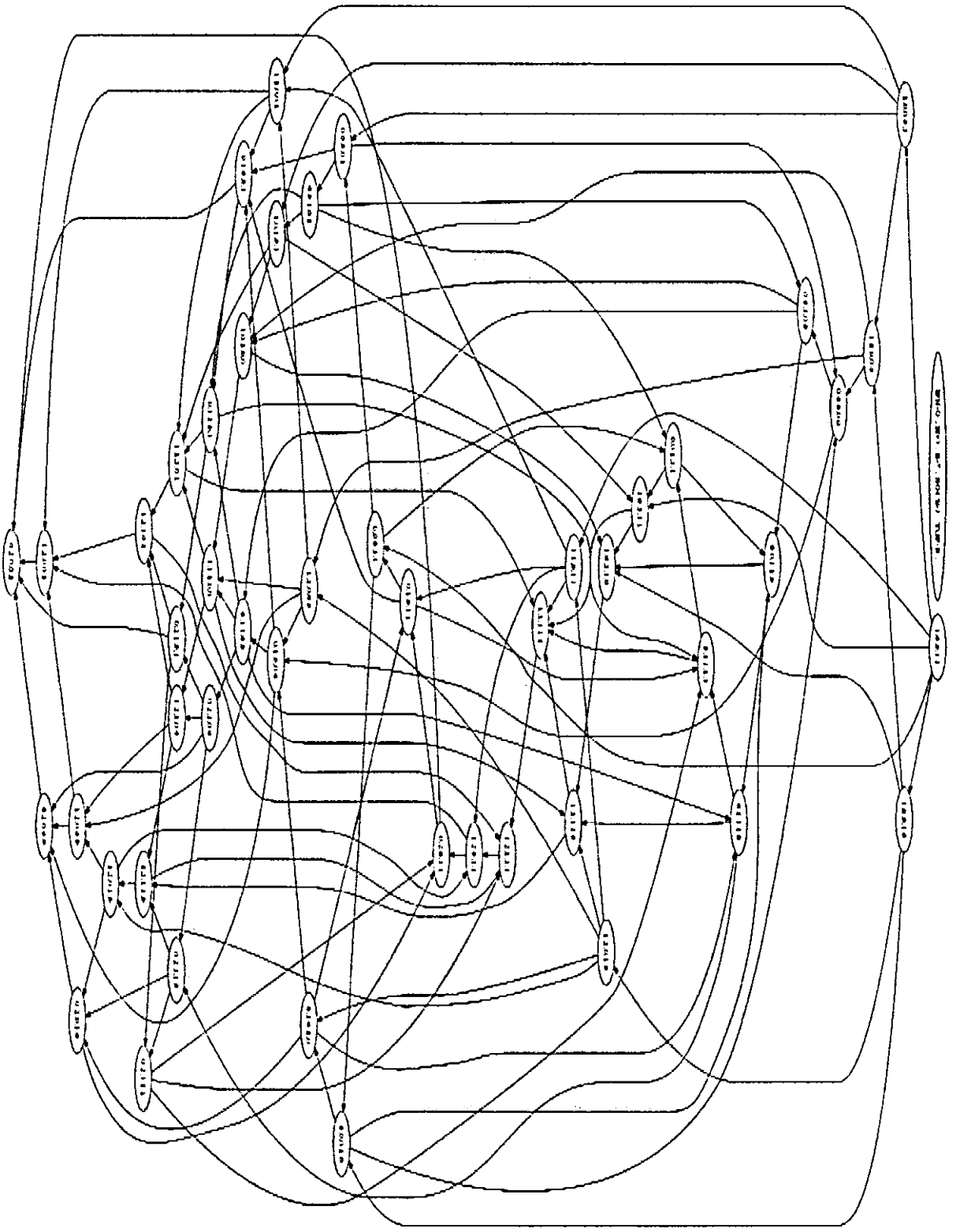


Figure 3.1: State Graph of First IDO BRN

3.1.3. Homeostasis of IDO BRN

The IDO BRN show normal and healthy function when it is regulated in a cyclic form. In Figure 3.2 three cyclic behavior are shown which differ in number of states. The system moved in a cyclic behavior when both B7 and SOCS3 are in active form. Further in a number of events, shown by each state, the system go through to come back to initial state. The cycles varies from each other on the basis of size, if favorable states are added the life span of cycle increased and small cycles have an opportunity to evolve into larger cycles. The cycles varies from each other at state [0,1,0,1,1], this state can be called an evolving state. It is an important state as it can have three output states and all leads to a new cyclic state. It could be an important state in clinical trials for drug targets.

3.1.4. Channel towards Stable Steady State

Many transitions exist at each state. There are many pathways exists moving from initial state toward dead lock state. A list of some pathways is shown in Table 3.2.

In Table 3.2, a list of some pathways is given moving from initial state to deadlock state.

The states mention in the blue are the states representing the cyclic behaviour and the blue state turning to black is showing deviation of cyclic behaviour to dead lock state or stable steady state. First two indices are exhibiting the shortest pathway the BRN can adopt to reach the output state. First index show the transition state behaviour towards the output state which is achieved without entering into the whole cyclic behaviour. This is the most lethal one as there is only one chance in the pathway to enter into the cycle where there is 50 % chance to be or out in homeostasis.

3.1.3. Homeostasis of IDO BRN

The IDO BRN show normal and healthy function when it is regulated in a cyclic form. In Figure 3.2 three cyclic behavior are shown which differ in number of states. The system moved in a cyclic behavior when both B7 and SOCS3 are in active form. Further in a number of events, shown by each state, the system go through to come back to initial state. The cycles varies from each other on the basis of size, if favorable states are added the life span of cycle increased and small cycles have an opportunity to evolve into larger cycles. The cycles varies from each other at state [0,1,0,1,1], this state can be called an evolving state. It is an important state as it can have three output states and all leads to a new cyclic state. It could be an important state in clinical trials for drug targets.

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Table 3.2: Possible Pathways Leading toward Stable Steady States

Index	Pathway to Stable Steady State
1.	00100, 10100, 11100, 11110, 11111, 12111, 12011, 12001, **02001**
2.	**00100**,00110, 10110, 11110, 12110, 12010, 02010, 02011, **02001**
3.	00100,00110, 10110, 11110, **11111**, 12111, 12011, 02011, **02001**
4.	**00100**,00110, 10110, 11110, 12110, **12111**, **12011**, 02011, **02001**
5.	00100, 10100, 10110, 11110, 12110, 12111, 12011, 02011, **02001**
6.	**00100**,00110, 10110, 11110, 12110, 12010, 02010, **02000**, **02001**
7.	00100,00110, 10110, 11110, 12110, 12010, 12000, 12001, **02001**
8.	**00100**,00110, 10110, 11110, **11111**, **12111**, **12011**, 02011, 01011, 01001, **02001**
9.	00100,00110, 10110, 11110, 12110, **12111**, **12011**, 02011, 01011, 01001, **02001**
10.	00100,00110, 10110, 11110, 12110, 12010, 02010, 02011, 01011, 01001, **02001**
11.	**00100**,00110, 10110, 11110, 12110, **12111**, **12011**, 02011, 01011, 01001, **01101**, 02101, **02001**
12.	00100,00110, 10110, 11110, 12110, 12010, 02010, 02011, 01011, 01001, 01101, 02101, **02001**
13.	00100,00110, 10110, 11110, 12110, 12111, 12011, 02011, 01011, 01111, 11111, 12111, 12011, 02011, **02001**

14.	00100,00110, 10110, 11110, 12110, 12111, 12011, 02011, 01011, 01001, 01101, 01111, 11111, 12111, 12011, 02011,**02001**
15.	00100,00110, 10110, 11110, 12110, 12010, 02010, 02011, 01011, 01001, 01101, 01111, 11111, 12111, 12011, 12011, 02011, **02001**
16.	00100,00110, 10110, 11110, 12110, 12111, 12011, 02011, **01011, 00011**, 00111, 10111, 11111, 12111, 12011, 12011, 02011, **02001**
17.	00100,00110, 10110, 11110, 12110, 12010, 02010, 02011, 01011, 01001, 01101, 01111, 00111, 00110, 10110, 11110, 12110, 12010, 02010, 02011, **02001**

The second pathway enters into homeostasis after passing through initial state, and then it diverges directly from homeostasis toward epigenetics.

The last index is showing one possible longest pathway the system can cover to move towards the stable steady state. It covers the whole one cycle then diverges from it as depicted in Figure 3.1.

3.2. Qualitative & Hybrid Modeling of Second IDO BRN

Second IDO BRN is derived from work done by Muller (2006). All the proteins involved in the BRN are immunosuppressors and they all regulate each other and maintain normal body functions through homeostasis. They lead to disease and tumor progression state when all are overexpressed and all are behaving as immunosuppressors.

3.2.1. State Table of Second IDO BRN

The state table (Table 3.3), generated after parameterization done in Table 2.2 is representing all possible states and transition of input state to output state. The initial state is in blue color i.e. 0000. It shows when system is in normal or dormant state. It has two output transition states as shown in last column of Table 3.3. There are two stable steady states bolded in red i.e. 2001 and 2111. The former epigenetic condition was the same which was generated in first IDO BRN as shown in Table 3.1. It is further verifying the role of IDO and TGFβ in cancer progression and making them suitable and favorable targets for drug designing.

Table 3.3: State Table of Second IDO BRN

IDO,COX2,INOS,TGFβ				wIDO, wCOX2, wINOS, wTGFB	kIDO, kCOX2, kINOS, kTGFB				Transitions state
0	0	0	0	{INOS, TGFB},{},{IDO, INOS, TGFB},{}	2	0	2	0	[1, 0, 0, 0] [0, 0, 1, 0]
0	0	0	1	{INOS},{},{IDO, INOS},{}	2	0	0	0	[1, 0, 0, 1] [0, 0, 0, 0]
0	0	1	0	{TGFB},{INOS},{IDO,INOS,TGF},{}	2	1	2	0	[1, 0, 1, 0] [0, 1, 1, 0] [0, 0, 2, 0]
0	0	1	1	{},{INOS},{IDO, INOS},{}	0	1	1	0	[0, 1, 1, 1] [0, 0, 1, 0]
0	0	2	0	{TGFB},{INOS},{IDO, TGFB},{}	2	1	2	0	[1, 0, 2, 0] [0, 1, 2, 0]
0	0	2	1	{},{INOS},{IDO},{}	0	1	2	0	[0, 1, 2, 1] [0, 0, 2, 0]
0	1	0	0	{COX2, INOS, TGFB},{},{IDO, INOS, TGFB},{}	2	0	2	0	[1, 1, 0, 0] [0, 0, 0, 0] [0, 1, 1, 0]
0	1	0	1	{COX2, INOS},{},{IDO, INOS},{}	2	0	0	0	[1, 1, 0, 1] [0, 0, 0, 1] [0, 1, 0, 0]
0	1	1	0	{COX2, TGFB},{INOS},{IDO, INOS, TGFB},{}	2	1	2	0	[1, 1, 1, 0] [0, 1, 2, 0]
0	1	1	1	{COX2},{INOS},{IDO, INOS},{}	2	1	1	0	[1, 1, 1, 1] [0, 1, 1, 0]
0	1	2	0	{COX2, TGFB},{INOS},{IDO, TGFB},{}	2	1	2	0	[1, 1, 2, 0]
0	1	2	1	{COX2},{INOS},{IDO},{}	2	1	2	0	[1, 1, 2, 1] [0, 1, 2, 0]
1	0	0	0	{INOS, TGFB},{},{IDO, INOS, TGFB},{IDO}	2	0	2	1	[2, 0, 0, 0] [1, 0, 1, 0] [1, 0, 0, 1]
1	0	0	1	{INOS},{},{IDO, INOS},{IDO}	2	0	0	1	[2, 0, 0, 1]
1	0	1	0	{TGFB},{INOS},{IDO, INOS, TGFB},{IDO}	2	1	2	1	[2, 0, 1, 0] [1, 1, 1, 0] [1, 0, 2, 0] [1, 0, 1, 1]
1	0	1	1	{},{INOS},{IDO, INOS},{IDO}	0	1	1	1	[0, 0, 1, 1] [1, 1, 1, 1]
1	0	2	0	{TGFB},{INOS},{IDO, TGFB},{IDO}	2	1	2	1	[2, 0, 2, 0] [1, 1, 2, 0] [1, 0, 2, 1]
1	0	2	1	{},{INOS},{IDO},{IDO}	0	1	2	1	[0, 0, 2, 1] [1, 1, 2, 1]
1	1	0	0	{COX2, INOS, TGFB},{},{IDO, INOS, TGFB},{IDO}	2	0	2	1	[2, 1, 0, 0] [1, 0, 0, 0] [1, 1, 1, 0]

								[1, 1, 0, 1]
1	1	0	1	{COX2, INOS},{},{IDO, INOS},{IDO}	2	0	0	1	[2, 1, 0, 1] [1, 0, 0, 1]
1	1	1	0	{COX2, TGFB},{INOS},{IDO, INOS, TGFB},{IDO}	2	1	2	1	[2, 1, 1, 0] [1, 1, 2, 0] [1, 1, 1, 1]
1	1	1	1	{COX2},{INOS},{IDO, INOS},{IDO}	2	1	1	1	[2, 1, 1, 1]
1	1	2	0	{COX2, TGFB},{INOS},{IDO, TGFB},{IDO}	2	1	2	1	[2, 1, 2, 0] [1, 1, 2, 1]
1	1	2	1	{COX2},{INOS},{IDO},{IDO}	2	1	2	1	[2, 1, 2, 1]
2	0	0	0	{INOS, TGFB},{},{INOS, TGFB},{IDO}	2	0	0	1	[2, 0, 0, 1]
2	0	0	1	{INOS},{},{INOS},{IDO}	2	0	0	1	[]
2	0	1	0	{TGFB},{INOS},{INOS, TGFB},{IDO}	2	1	1	1	[2, 1, 1, 0] [2, 0, 1, 1]
2	0	1	1	{},{INOS},{INOS},{IDO}	0	1	1	1	[1, 0, 1, 1] [2, 1, 1, 1]
2	0	2	0	{TGFB},{INOS},{TGFB},{IDO}	2	1	2	1	[2, 1, 2, 0] [2, 0, 2, 1]
2	0	2	1	{},{INOS},{},{IDO}	0	1	0	1	[1, 0, 2, 1] [2, 1, 2, 1] [2, 0, 1, 1]
2	1	0	0	{COX2, INOS, TGFB},{},{INOS, TGFB},{IDO}	2	0	0	1	[2, 0, 0, 0] [2, 1, 0, 1]
2	1	0	1	{COX2, INOS},{},{INOS},{IDO}	2	0	0	1	[2, 0, 0, 1]
2	1	1	0	{COX2, TGFB},{INOS},{INOS, TGFB},{IDO}	2	1	1	1	[2, 1, 1, 1]
2	1	1	1	{COX2},{INOS},{INOS},{IDO}	2	1	1	1	[]
2	1	2	0	{COX2, TGFB},{INOS},{TGFB},{IDO}	2	1	2	1	[2, 1, 2, 1]
2	1	2	1	{COX2},{INOS},{},{IDO}	2	1	0	1	[2, 1, 1, 1]

3.2.2. State Graph of Second IDO BRN

The state table as shown (Table 3.3) generated the state graph of the IDO BRN which is shown in Figure 3.3. It depicted all the states represented in Table 3.3, interacting with all other states. It has hidden homeostasis. The epigenetic conditions are shown in red. It also has a key showing the order of proteins ordered in a state space.

3.2.3. Cycles Generated by Second IDO BRN

The homeostatic behaviour is shown in the form of cycles. The list of cycles generated are shown in Table 3.4,

In Table 3.4, first three cycles started with 1 concentration of iNOS and in last three iNOS have concentration of 2. Both cycles follow the same pattern. Initially cycle starts with the activation of iNOS then IDO is produced which is controlled by both TGFβ and iNOS. COX2 remain silent if it is activated, start expressing, homeostasis is disturbed and it will move towards epigenetics. First three are three different forms of the same cycle with little alterations. Last three cycles start with same initial state i.e. 0020. First and fourth cycles are of normal lengths. Third and last are the shortest cycles and second and fifth are the longest cycles showing the control on IDO concentration. The longest cycle have one advantage and a disadvantage.

Advantage is that it can control the IDO concentration at late level too and it is a mixture of both short and normal length cycles. Whereas it has the disadvantage that it has more states and as the number of states increases it has high probability of being vulnerable to diversions towards epigenetics.

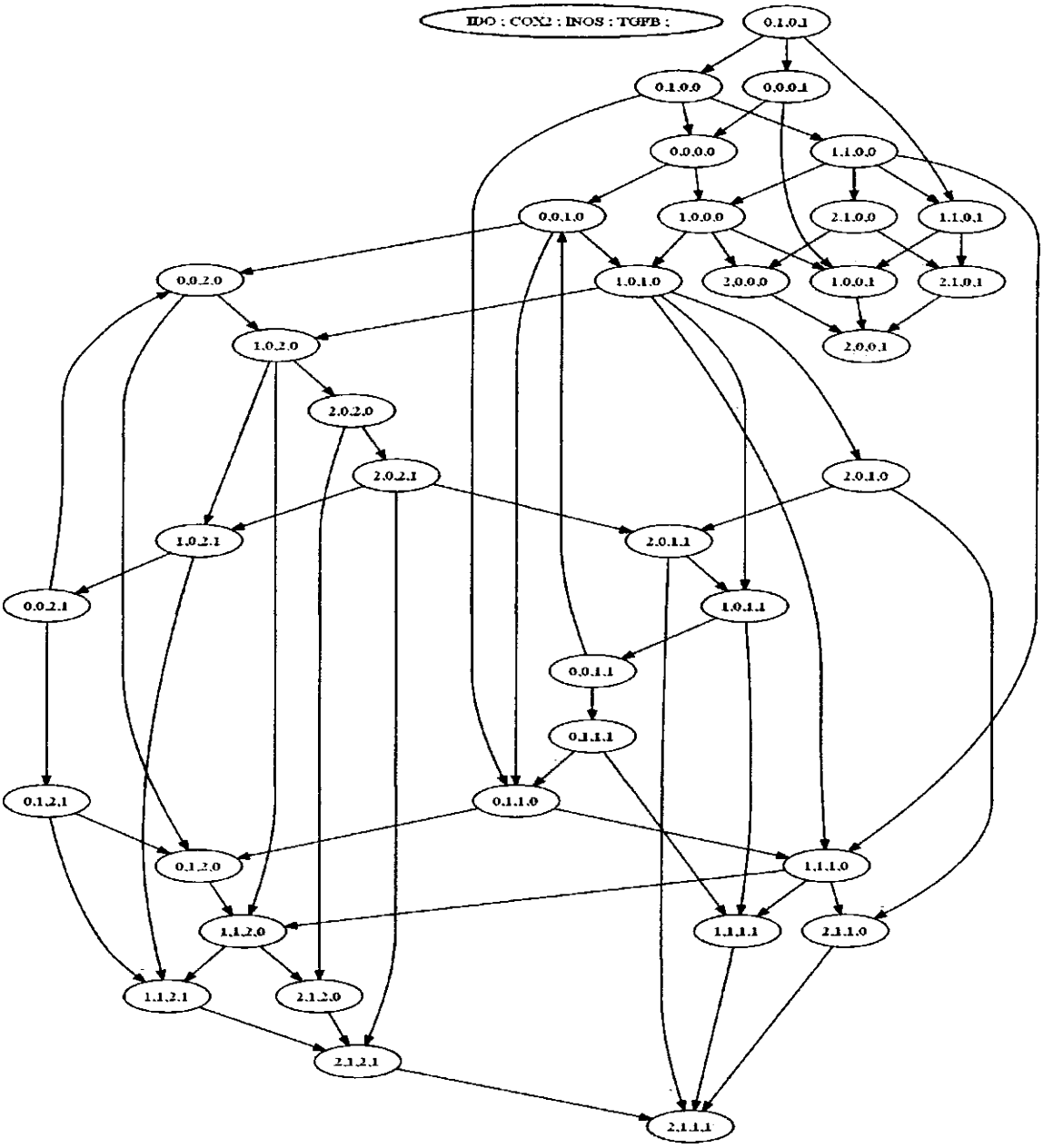

Figure 3.3: State Graph of Second IDO BRN

Table 3.4: Cyclic Behaviors Shown by Second IDO BRN

Index	Cycles
1.	0010 → 1010 → 2010 → 2011 → 1011 → 0011 → 0010
2.	0010 → 1010 → 1020 → 2020 → 2021 → 2011 → 1011 → 0011 → 0010
3.	0010 → 1010 → 1011 → 0011 → 0010
4.	0020 → 1020 → 2020 → 2021 → 1021 → 0021 → 0020
5.	0020 → 1020 → 2020 → 2021 → 2011 → 1011 → 0011 → 0010 → 0020
6.	0020 → 1020 → 1021 → 0021 → 0020

Only first pathway is selected for further analysis. Invariance kernels are derived for each cycle using Hybrid modelling tools. It provides the scientist with temporal delay constraint to keep the system in a regulatory state for increasing the life expectancy of patients.

3.2.4. Channel towards Stable Steady State

One or more than one transitions exist at each state as shown in Figure 3.3, On the basis of these varieties at each state there are many channels or pathways exist moving from initial state toward dead lock state. A list of some pathways is depicted in Table 3.5.

First two pathways are leading towards state 2001, it is the most dangerous state as if IDO is initiated before iNOS, the pathway readily moves towards critical deadlock state. There is no homeostasis in this pathway for recovery. All other pathways are leading to state 2111 where all the proteins are acting as immunosuppressors. This state can be achieved by both types of pathways, with or without going into the cycles. The trajectory leading to deadlock state 2111 have a number of same length smallest pathways. They vary on the basis of transitions state the greater the number of options in transitions toward the deadlock state the greater the viable it would be. The pathway indexed at 13 is the largest trajectory towards disease state. It provides greater opportunities in controlling it from driving towards disease state by applying temporal delays. These pathways are further used for real time modeling, for generating and studying time constraints. It will help in controlling the proteins within time medium. Figure 3.4 depicting a pictoral view of states of cycle diverging towards deadlock state.

Table 3.5: Pathways Leading to Deadlock States

Index	Pathway towards Deadlock states
1.	0000, 1000, 1001, **2001**
2.	0000, 1000, 2000, **2001**
3.	0000, 1000, 1010, 2010, 2110, **2111**
4.	0000, 1000, 1010, 2010, 2011, **2111**
5.	0000, 1000, 1010, 1110, 2110, **2111**
6.	0000, 1000, 1010, 1011, 1111, **2111**
7.	0000, 1000, 1010, 1011, 0011, 0111, 1111, **2111**
8.	0000, 1000, 1010, 1110, 2110, **2111**
9.	0000, 1000, 1010, 1110, 1111, **2111**
10.	0000, 1000, 1010, 1110, 1120, 2120, 2121, **2111**
11.	0000, 1000, 1010, 1110, 1120, 1121, 2121, **2111**
12.	0000, 0010, 1010, 1011, 0011, 0010, 0110, 1110, 2110, **2111**
13.	0000, 1000, 1010, 1011, 0011, 0010, 0110, 0120, 1120, 2120, 2121, **2111**
14.	0000, 1000, 1010, 1011, 0011, 0010,1010, 2010, 2110, **2111**
15.	0000, 1000, 1010, 1011, 0011, 0010, 1010, 1110, 2110, **2111**
16.	0000, 0010, 0110, 1110, 2110, **2111**
17.	0000, 0010, 0110, 1110, 1120, 1121, 2121, **2111**
18.	0000, 0010, 1010, 1110, 1120, 1121, 2121, **2111**

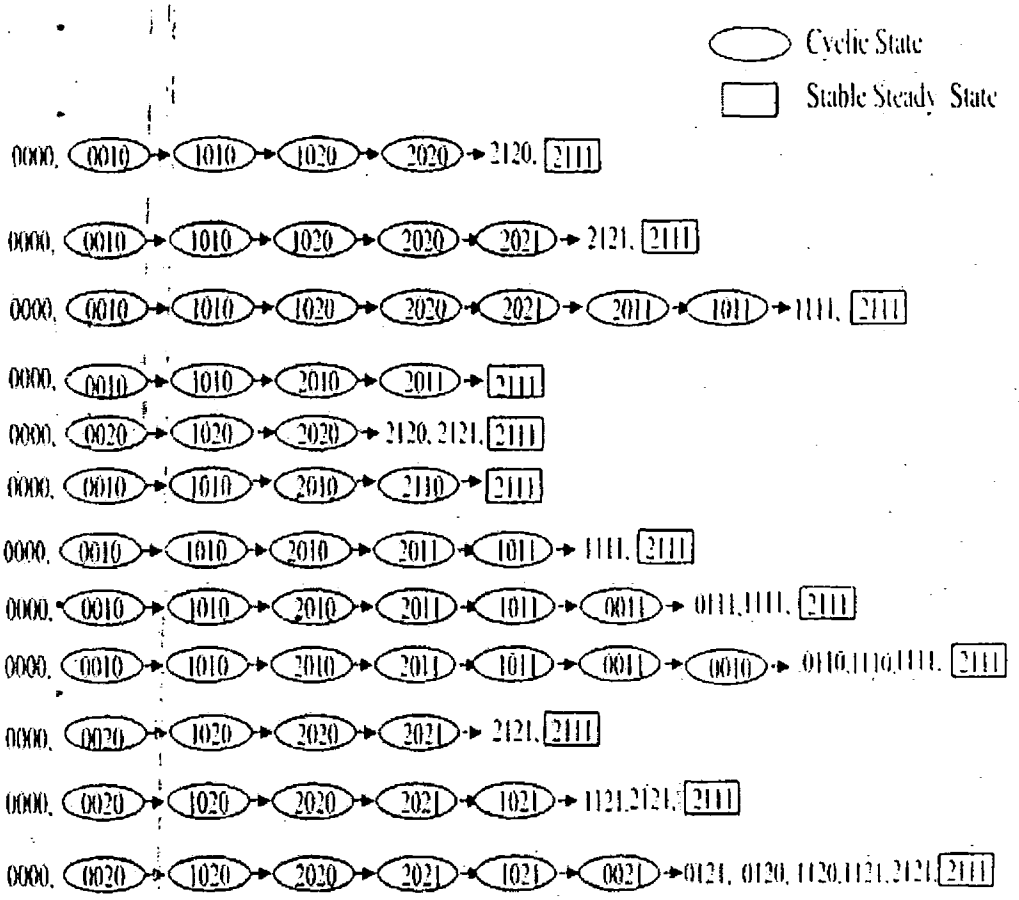

Figure 3.4: States diverting from Cycle toward Stable Steady State

3.2.5. Hybrid Modeling of IDO BRN

Hybrid modeling achieved using HyTech tool. It has given results in the form of time delays for tracking deadlock states and invariance kernels for maintaining system in homeostasis. Both are very important in controlling clinical trials and deriving important results by following and altering delay constraints.

3.2.5.1. Time Delays of Pathways leading to Stable Steady State

The time constraint of each state towards the output state is shown in Table 3.6. Not all the states are enlisted here as some of the states are neither involved in the path of cycle nor in the path leading towards deadlock state as traverse from the one initial state. The delays are given in the form of equations in which δ represent change of, '+' or '-' sign represent activation and degradation respectively and base value of δ represents the element and its location from where it start evolving. The delay of first index shows that,

$$0000 \rightarrow 0010 \quad = \quad \delta^{+}_{INOS0000} \leq \delta^{+}_{IDO0000}$$

The state moves towards state 0000 to 0010, the system is activated and iNOS concentration start to buildup. It will start when the positive time delay $\delta^{+}_{INOS0000}$ of iNOS i.e. production of iNOS protein, is less than or equal to positive time delay $\delta^{+}_{IDO0000}$ of IDO. The delay for 0000 to 1000 is opposite to the first delay.

$$0000 \rightarrow 1000 \quad = \quad \delta^{+}_{IDO0000} \leq \delta^{+}_{INOS0000}$$

Table 3.6: Time Delays of Each State of IDO BRN

Transition	Time Delay
0000 → 0010	$\delta^+_{INOS0000} \leq \delta^+_{IDO0000}$
0000 → 1000	$\delta^+_{IDO0000} \leq \delta^+_{INOS0000}$
1000 → 2000	$\delta^+_{IDO1000} + \delta^+_{IDO0000} \leq \delta^+_{INOS1000}$ $\delta^+_{IDO1000} + \delta^+_{IDO0000} \leq \delta^+_{TGF\beta1000}$
1000 → 1001	$\delta^+_{TGF\beta1000} \leq \delta^+_{IDO1000} + \delta^+_{IDO0000}$ $\delta^+_{TGF\beta1000} \leq \delta^+_{INOS1000}$
1000 → 1010	$\delta^+_{INOS1000} \leq \delta^+_{IDO1000} + \delta^+_{IDO0000}$ $\delta^+_{TGF\beta1000} \leq \delta^+_{INOS1000}$
2000 → 2001	$\delta^+_{TGF\beta1000} \leq \delta^+_{IDO1000} + \delta^+_{IDO0000}$
1001 → 2001	$\delta^+_{IDO1001} \leq \delta^+_{TGF\beta1000} + \lvert\delta^+_{IDO0000}\rvert$ $\delta^+_{IDO1000} + \delta^+_{IDO0000} \leq \delta^+_{INOS1000}$
1010 → 2010	$\delta^+_{IDO0010} \leq \delta^+_{INOS1000} + \delta^+_{INOS0000}$ $\delta^+_{IDO1010} \leq \delta^+_{IDO1000}$
1010 → 1110	$\delta^+_{COX21010} \leq \delta^+_{INOS1010} + \delta^+_{INOS1000}$ $\delta^+_{COX21010} \leq \delta^+_{TGF\beta1010}$
1010 → 1011	$\delta^+_{TGF\beta1010} \leq \delta^+_{INOS1010} + \delta^+_{INOS1000}$ $\delta^+_{TGF\beta1010} \leq \delta^+_{COX21010}$
1010 → 1020	$\delta^+_{INOS1010} + \delta^+_{INOS1000} \leq \delta^+_{COX21010}$ $\delta^+_{INOS1010} + \delta^+_{INOS1000} \leq \delta^+_{TGF\beta1010}$
0010 → 1010	$\delta^+_{IDO0010} \leq \delta^+_{INOS0010} + \delta^+_{INOS0000}$ $\delta^+_{IDO0010} \leq \delta^+_{COX20010}$
0010 → 0110	$\delta^+_{COX20010} \leq \delta^+_{INOS0010} + \delta^+_{INOS0000}$ $\delta^+_{COX20010} \leq \delta^+_{IDO0010}$
0010 → 0020	$\delta^+_{INOS0010} + \delta^+_{INOS0000} \leq \delta^+_{COX20010}$ $\delta^+_{INOS0010} + \delta^+_{INOS0000} \leq \delta^+_{IDO0010}$

$0110 \rightarrow 0120$	$\delta^{+}_{INOS0110} + \delta^{+}_{INOS0000} \leq \delta^{+}_{IDO0110}$
$0120 \rightarrow 1120$	$\delta^{+}_{IDOS0120} \leq \delta^{+}_{TGF\beta 0120}$
$0110 \rightarrow 1110$	$\delta^{+}_{IDO0110} \leq \delta^{+}_{INOS0110} + \delta^{+}_{INOS0000}$
$0111 \rightarrow 1111$	$\delta^{+}_{TGF\beta 1010} + \delta^{+}_{IDO0111} \leq \delta^{+}_{INOS0111} + \delta^{+}_{INOS1000}$
$0111 \rightarrow 0110$	$\delta^{-}_{TGF\beta 0111} + \delta^{+}_{COX20011} \leq \delta^{+}_{TGF\beta 1010}$
$2010 \rightarrow 2110$	$\delta^{+}_{COX22010} \leq \delta^{+}_{TGF\beta 2010}$
$2010 \rightarrow 2011$	$\delta^{+}_{TGF\beta 2010} \leq \delta^{+}_{COX22010}$
$1110 \rightarrow 2110$	$\delta^{+}_{IDO1110} + \delta^{+}_{IDO0010} \leq \delta^{+}_{INOS1110} + \delta^{+}_{INOS1000}$ $\delta^{+}_{IDO1110} + \delta^{+}_{IDO0010} \leq \delta^{+}_{TGF\beta 1110}$
$1110 \rightarrow 1111$	$\delta^{+}_{TGF\beta 1110} \leq \delta^{+}_{IDO1110} + \delta^{+}_{IDO0010}$ $\delta^{+}_{TGF\beta 1110} \leq \delta^{+}_{INOS1110} + \delta^{+}_{INOS1000}$
$1111 \rightarrow 2111$	$\delta^{+}_{IDO1111} + \delta^{+}_{COX21011} \leq \lvert\delta^{-}_{IDO2011}\rvert$
$1110 \rightarrow 1120$	$\delta^{+}_{INOS1110} + \delta^{+}_{INOS1000} \leq \delta^{+}_{IDO1110} + \delta^{+}_{IDO0010}$ $\delta^{+}_{INOS1110} + \delta^{+}_{INOS1000} \leq \delta^{+}_{TGF\beta 1110}$
$2011 \rightarrow 2111$	$\delta^{+}_{COX22011} \leq \lvert\delta^{-}_{IDO2011}\rvert$
$1020 \rightarrow 1120$	$\delta^{+}_{COX21020} \leq \delta^{+}_{TGF\beta 1020}$ $\delta^{+}_{COX21020} \leq \delta^{+}_{IDO1020} + \delta^{+}_{IDO0010}$
$1020 \rightarrow 2020$	$\delta^{-}_{IDO1020} + \delta^{+}_{IDO0010} \leq \delta^{+}_{TGF\beta 1020}$ $\delta^{-}_{IDO1020} + \delta^{+}_{IDO0010} \leq \delta^{+}_{COX21020}$
$1020 \rightarrow 1021$	$\delta^{+}_{TGF\beta 1020} \leq \delta^{+}_{COX21020}$ $\delta^{+}_{TGF\beta 1020} \leq \delta^{+}_{IDO1020} + \delta^{+}_{IDO0010}$
$1011 \rightarrow 0011$	$\delta^{-}_{IDO1011} + \delta^{+}_{IDO0010} \leq \delta^{+}_{TGF\beta 1010}$
$1011 \rightarrow 1111$	$\delta^{+}_{COX21011} + \delta^{+}_{IDO0010} \leq \lvert\delta^{-}_{IDO1011}\rvert + 2\delta^{+}_{TGF\beta 1010}$
$0011 \rightarrow 0111$	$\delta^{+}_{COX20011} + \delta^{+}_{IDO0000} \leq \lvert\delta^{-}_{IDO1011}\rvert + 2\delta^{+}_{TGF\beta 1010}$

$0011 \rightarrow 0010$	$\delta^{+}_{TGF\beta 1010} + \delta^{-}_{TGF\beta 0011} \leq \delta^{-}_{IDO1011} + \delta^{+}_{IDO0000}$
$0020 \rightarrow 1020$	$\delta^{+}_{IDO0010} \leq \delta^{+}_{COX20020}$
$0020 \rightarrow 0120$	$\delta^{+}_{COX20010} \leq \delta^{+}_{IDO0020}$
$2020 \rightarrow 2120$	$\delta^{+}_{COX22020} \leq \delta^{+}_{TGF\beta 2020}$
$2020 \rightarrow 2021$	$\delta^{+}_{TGF\beta 2020} \leq \delta^{+}_{COX22020}$
$2021 \rightarrow 2011$	$\delta^{-}_{INOS2021} + \delta^{+}_{TGF\beta 2020} \leq \delta^{-}_{IDO1020}$
$2021 \rightarrow 2121$	$\delta^{+}_{COX22021} \leq \lvert\delta^{-}_{INOS2021}\rvert + \delta^{-}_{IDO1020}$
$2021 \rightarrow 1021$	$\delta^{-}_{IDO2021} + \delta^{+}_{TGF\beta 2020} \leq \delta^{-}_{IDO1020}$
$2110 \rightarrow 2111$	$\delta^{+}_{TGF\beta 2110} \leq \delta^{+}_{INOS2110} + \delta^{+}_{INOS1000}$
$1120 \rightarrow 2120$	$\delta^{+}_{IDO0120} + \delta^{+}_{IDO1120} \leq \delta^{+}_{TGF\beta 1120}$
$1120 \rightarrow 1121$	$\delta^{+}_{TGF\beta 1120} \leq \delta^{+}_{IDO0120} + \delta^{+}_{IDO1120}$
$1021 \rightarrow 1121$	$\delta^{+}_{IDO1021} + \delta^{+}_{IDO0010} + \delta^{+}_{COX21021} \leq 2\delta^{+}_{TGF\beta 1020}$
$1021 \rightarrow 0021$	$2\delta^{+}_{TGF\beta 1020} \leq \delta^{+}_{IDO1021} + \delta^{+}_{IDO0010} + \delta^{+}_{COX21021}$
$2120 \rightarrow 2121$	$\delta^{-}_{INOS2121} + \delta^{+}_{TGF\beta 2120} \leq \delta^{+}_{IDO1120}$
$1121 \rightarrow 2121$	$\delta^{+}_{IDO1120} \leq \delta^{+}_{IDO1121}$
$2121 \rightarrow 2111$	$\delta^{+}_{COX22021} + \delta^{-}_{INOS2121} \leq \delta^{-}_{IDO1020}$
$2011 \rightarrow 2111$	$\delta^{+}_{COX22011} \leq \lvert\delta^{-}_{IDO2011}\rvert$
$2011 \rightarrow 1011$	$\delta^{-}_{IDO2011} + \delta^{+}_{TGF\beta 2010} \leq \delta^{+}_{IDO0010}$

The delays can also be in a complex form, e.g.

$$1000 \rightarrow 2000 \quad = \quad \delta^{+}_{IDO1000} + \delta^{+}_{IDO0000} \leq \delta^{+}_{INOS1000}$$

It shows that activation rate not only depends on input state 1000 but its concentration build up starts at previous state 0000 to reach highest concentration. The delay of a protein can depend on two different proteins. There are also delays for controlling rate of degradation of a protein e.g.

$$2021 \rightarrow 1021 \quad = \quad \delta^{-}_{IDO2021} + \delta^{+}_{TGF\beta 2020} \leq \delta^{-}_{IDO1020}$$

It shows degradation delay of IDO brings activation of TGFβ, its inhibitor with it. Both should combine and their delays should be less.

On the basis of the information given in Table 3.6 state transitions trajectories can be designed for favorable pathways and it will also give the source for controlling the desired state transition using these constraints.

3.2.5.2. Time Delays for the whole pathway

Table 3.7 is identical to Table 3.5, it has addition of one more feature i.e. delay to each transition and has state transition as well.

$$0000 \xrightarrow{\delta^{+}_{IDO0000} \leq \delta^{+}_{INOS0000}} 1000 \xrightarrow{\delta^{+}_{TGF\beta 1000} \leq \delta^{+}_{IDO1000} + \delta^{+}_{IDO0000}} 1001 \xrightarrow{\delta^{+}_{IDO1001} \leq \delta^{+}_{TGF\beta 1000} + |\delta^{+}_{IDO0000}|}$$

2001

The above trajectory shows that delay of IDO should be less than iNOS and next says $\delta^{+}_{TGF\beta 1000}$ is less than delay of IDO at 1000 and 0000. The last is the combination of previous two i.e. $\delta^{+}_{IDO1001}$ is less than both previous delays.

Table 3.7: Pathways with Time Delays Moving towards Deadlock State

Index	Pathway towards Deadlock states
1.	$0000 \xrightarrow{\delta^+_{IDO0000} \le \delta^+_{INOS0000}} 1000 \xrightarrow{\delta^+_{TGF\beta 1000} \le \delta^+_{IDO1000} + \delta^+_{IDO0000}} 1001$ $\xrightarrow{\delta^+_{IDO1001} \le \delta^+_{TGF\beta 1000} + \lvert\delta^+_{IDO0000}\rvert} \mathbf{2001}$
2.	$0000 \xrightarrow{\delta^+_{IDO0000} \le \delta^+_{INOS0000}} 1000 \xrightarrow{\delta^+_{IDO1000} + \delta^+_{IDO0000} \le \delta^+_{INOS1000}} 2000$ $\xrightarrow{\delta^+_{TGF\beta 1000} \le \delta^+_{IDO1000} + \delta^+_{IDO0000}} \mathbf{2001}$
3.	$0000 \xrightarrow{\delta^+_{IDO0000} \le \delta^+_{INOS0000}} 1000 \xrightarrow{\delta^+_{INOS1000} \le \delta^+_{IDO1000} + \delta^+_{IDO0000}} 1010$ $\xrightarrow{\delta^+_{IDO1010} \le \delta^+_{IDO1000}} 2010 \xrightarrow{\delta^+_{COX22010} \le \delta^+_{TGF\beta 2010}} 2110 \xrightarrow{\delta^+_{TGF\beta 2110} \le \delta^+_{INOS2110} + \delta^+_{INOS1000}}$ **2111**
4.	$0000 \xrightarrow{\delta^+_{IDO0000} \le \delta^+_{INOS0000}} 1000 \xrightarrow{\delta^+_{INOS1000} \le \delta^+_{IDO1000} + \delta^+_{IDO0000}} 1010 \xrightarrow{\delta^+_{IDO1010} \le \delta^+_{IDO1000}}$ $2010 \xrightarrow{\delta^+_{TGF\beta 2010} \le \delta^+_{COX22010}} 2011 \xrightarrow{\delta^+_{COX22011} \le \lvert\delta^-_{IDO2011}\rvert} \mathbf{2111}$
5.	$0000 \xrightarrow{\delta^+_{IDO0000} \le \delta^+_{INOS0000}}$ $1000 \xrightarrow{\delta^+_{INOS1000} \le \delta^+_{IDO1000} + \delta^+_{IDO0000}} 1010 \xrightarrow{\delta^+_{COX21010} \le \delta^+_{INOS1010} + \delta^+_{INOS1000}}$ $1110 \xrightarrow{\delta^+_{IDO1110} + \delta^+_{IDO0010} \le \delta^+_{TGF\beta 1110}} 2110 \xrightarrow{\delta^+_{TGF\beta 2110} \le \delta^+_{INOS2110} + \delta^+_{INOS1000}} \mathbf{2111}$
6.	$0000 \xrightarrow{\delta^+_{IDO0000} \le \delta^+_{INOS0000}}$ $1000 \xrightarrow{\delta^+_{INOS1000} \le \delta^+_{IDO1000} + \delta^+_{IDO0000}} 1010 \xrightarrow{\delta^+_{TGF\beta 1010} \le \delta^+_{COX21010}}$ $1011 \xrightarrow{\delta^+_{COX21011} + \delta^+_{IDO0010} \le \lvert\delta^-_{IDO1011}\rvert + 2\delta^+_{TGF\beta 1010}} 1111$ $\xrightarrow{\delta^+_{IDO1111} + \delta^+_{COX21011} \le \lvert\delta^-_{IDO2011}\rvert} \mathbf{2111}$
7.	$0000 \xrightarrow{\delta^+_{IDO0000} \le \delta^+_{INOS0000}}$ $1000 \xrightarrow{\delta^+_{INOS1000} \le \delta^+_{IDO1000} + \delta^+_{IDO0000}} 1010 \xrightarrow{\delta^+_{TGF\beta 1010} \le \delta^+_{COX21010}} 1011$ $\xrightarrow{\delta^-_{IDO1011} + \delta^+_{IDO0010} \le \delta^+_{TGF\beta 1010}} 0011 \xrightarrow{\delta^+_{COX20011} + \delta^+_{IDO0000} \le \lvert\delta^-_{IDO1011}\rvert + 2\delta^+_{TGF\beta 1010}}$ $0111 \xrightarrow{\delta^+_{TGF\beta 1010} + \delta^+_{IDO0111} \le \delta^+_{INOS0111} + \delta^+_{INOS1000}}$ $1111 \xrightarrow{\delta^+_{IDO1111} + \delta^+_{COX21011} \le \lvert\delta^-_{IDO2011}\rvert} \mathbf{2111}$

8.	$0000 \xrightarrow{\delta^+_{IDO0000} \le \delta^+_{INOS0000}} 1000 \xrightarrow{\delta^+_{INOS1000} \le \delta^+_{IDO1000} + \delta^+_{IDO0000}} 1010$ $\xrightarrow{\delta^+_{COX21010} \le \delta^+_{INOS1010} + \delta^+_{INOS1000}} 1110 \xrightarrow{\delta^+_{IDO1110} + \delta^+_{IDO0010} \le \delta^+_{TGF\beta1110}}$ $2110 \xrightarrow{\delta^+_{TGF\beta2110} \le \delta^+_{INOS2110} + \delta^+_{INOS1000}} \mathbf{2111}$
9.	$0000 \xrightarrow{\delta^+_{IDO0000} \le \delta^+_{INOS0000}} 1000 \xrightarrow{\delta^+_{INOS1000} \le \delta^+_{IDO1000} + \delta^+_{IDO0000}} 1010$ $\xrightarrow{\delta^+_{COX21010} \le \delta^+_{TGF\beta1010}} 1110 \xrightarrow{\delta^+_{TGF\beta1110} \le \delta^+_{IDO1110} + \delta^+_{IDO0010}}$ $1111 \xrightarrow{\delta^+_{IDO1111} + \delta^+_{COX21011} \le \lvert\delta^-_{IDO2011}\rvert} \mathbf{2111}$
10.	$0000 \xrightarrow{\delta^+_{IDO0000} \le \delta^+_{INOS0000}} 1000 \xrightarrow{\delta^+_{INOS1000} \le \delta^+_{IDO1000} + \delta^+_{IDO0000}} 1010$ $\xrightarrow{\delta^+_{COX21010} \le \delta^+_{INOS1010} + \delta^+_{INOS1000}} 1110$ $\xrightarrow{\delta^+_{INOS1110} + \delta^+_{INOS1000} \le \delta^+_{IDO1110} + \delta^+_{IDO0010}} 1120$ $\xrightarrow{\delta^+_{IDO0120} + \delta^+_{IDO1120} \le \delta^+_{TGF\beta1120}} 2120 \xrightarrow{\delta^-_{INOS2121} + \delta^+_{TGF\beta2120} \le \delta^+_{IDO1120}}$ $2121 \xrightarrow{\delta^+_{COX22021} + \delta^-_{INOS2121} \le \delta^-_{IDO1020}} \mathbf{2111}$
11.	$0000 \xrightarrow{\delta^+_{IDO0000} \le \delta^+_{INOS0000}} 1000 \xrightarrow{\delta^+_{INOS1000} \le \delta^+_{IDO1000} + \delta^+_{IDO0000}} 1010$ $\xrightarrow{\delta^+_{COX21010} \le \delta^+_{INOS1010} + \delta^+_{INOS1000}} 1110 \xrightarrow{\delta^+_{INOS1110} + \delta^+_{INOS1000} \le \delta^+_{TGF\beta1110}}$ $1120 \xrightarrow{\delta^+_{TGF\beta1120} \le \delta^+_{IDO0120} + \delta^+_{IDO1120}} 1121 \xrightarrow{\delta^+_{IDO1120} \le \delta^+_{IDO1121}}$ $2121 \xrightarrow{\delta^+_{COX22021} + \delta^-_{INOS2121} \le \delta^-_{IDO1020}} \mathbf{2111}$
12.	$0000 \xrightarrow{\delta^+_{INOS0000} \le \delta^+_{IDO0000}} 0010 \xrightarrow{\delta^+_{IDO0010} \le \delta^+_{INOS0010} + \delta^+_{INOS0000}} 1010$ $\xrightarrow{\delta^+_{TGF\beta1010} \le \delta^+_{INOS1010} + \delta^+_{INOS1000}} 1011 \xrightarrow{\delta^-_{IDO1011} + \delta^+_{IDO0010} \le \delta^+_{TGF\beta1010}}$ $0011 \xrightarrow{\delta^+_{TGF\beta1010} + \delta^-_{TGF\beta0011} \le \delta^-_{IDO1011} + \delta^+_{IDO0000}} 0010 \xrightarrow{\delta^+_{COX20010} \le \delta^+_{IDO0010}}$ $0110 \xrightarrow{\delta^+_{IDO0110} \le \delta^+_{INOS0110} + \delta^+_{INOS0000}} 1110 \xrightarrow{\delta^+_{IDO1110} + \delta^+_{IDO0010} \le \delta^+_{TGF\beta1110}}$ $2110 \xrightarrow{\delta^+_{TGF\beta2110} \le \delta^+_{INOS2110} + \delta^+_{INOS1000}} \mathbf{2111}$
13.	$0000 \xrightarrow{\delta^+_{IDO0000} \le \delta^+_{INOS0000}} 1000 \xrightarrow{\delta^+_{INOS1000} \le \delta^+_{IDO1000} + \delta^+_{IDO0000}} 1010$ $\xrightarrow{\delta^+_{TGF\beta1010} \le \delta^+_{INOS1010} + \delta^+_{INOS1000}} 1011 \xrightarrow{\delta^-_{IDO1011} + \delta^+_{IDO0010} \le \delta^+_{TGF\beta1010}}$ $0011 \xrightarrow{\delta^+_{TGF\beta1010} + \delta^-_{TGF\beta0011} \le \delta^-_{IDO1011} + \delta^+_{IDO0000}} 0010 \xrightarrow{\delta^+_{COX20010} \le \delta^+_{INOS0010} + \delta^+_{INOS0000}}$ $0110 \xrightarrow{\delta^+_{INOS0110} + \delta^+_{INOS0000} \le \delta^+_{IDO0110}} 0120 \xrightarrow{\delta^+_{IDOS0120} \le \delta^+_{TGF\beta0120}}$

	$1120 \xrightarrow{\delta^+_{IDO0120}+\delta^+_{IDO1120}\leq\delta^+_{TGF\beta1120}} 2120 \xrightarrow{\delta^-_{INOS2121}+\delta^+_{TGF\beta2120}\leq\delta^+_{IDO1120}}$ $2121 \xrightarrow{\delta^+_{COX22021}+\delta^-_{INOS2121}\leq\delta^-_{IDO1020}} \mathbf{2111}$
14.	$0000 \xrightarrow{\delta^+_{IDO0000}\leq\delta^+_{INOS0000}} 1000 \xrightarrow{\delta^+_{INOS1000}\leq\delta^+_{IDO1000}+\delta^+_{IDO0000}} 1010$ $\xrightarrow{\delta^+_{TGF\beta1010}\leq\delta^+_{INOS1010}+\delta^+_{INOS1000}} 1011 \xrightarrow{\delta^-_{IDO1011}+\delta^+_{IDO0010}\leq\delta^+_{TGF\beta1010}}$ $0011 \xrightarrow{\delta^+_{TGF\beta1010}+\delta^-_{TGF\beta0011}\leq\delta^-_{IDO1011}+\delta^+_{IDO0000}} 0010 \xrightarrow{\delta^+_{IDO0010}\leq\delta^+_{INOS0010}+\delta^+_{INOS0000}}$ $1010 \xrightarrow{\delta^+_{IDOS0120}\leq\delta^+_{TGF\beta0120}} 2010 \xrightarrow{\delta^+_{COX22010}\leq\delta^+_{TGF\beta2010}}$ $2110 \xrightarrow{\delta^+_{TGF\beta2110}\leq\delta^+_{INOS2110}+\delta^+_{INOS1000}} \mathbf{2111}$
15.	$0000 \xrightarrow{\delta^+_{IDO0000}\leq\delta^+_{INOS0000}} 1000 \xrightarrow{\delta^+_{INOS1000}\leq\delta^+_{IDO1000}+\delta^+_{IDO0000}} 1010$ $\xrightarrow{\delta^+_{TGF\beta1010}\leq\delta^+_{INOS1010}+\delta^+_{INOS1000}} 1011 \xrightarrow{\delta^-_{IDO1011}+\delta^+_{IDO0010}\leq\delta^+_{TGF\beta1010}}$ $0011 \xrightarrow{\delta^+_{TGF\beta1010}+\delta^-_{TGF\beta0011}\leq\delta^-_{IDO1011}+\delta^+_{IDO0000}} 0010$ $\xrightarrow{\delta^+_{COX21010}\leq\delta^+_{INOS1010}+\delta^+_{INOS1000}} 1110 \xrightarrow{\delta^+_{IDO1110}+\delta^+_{IDO0010}\leq\delta^+_{TGF\beta1110}}$ $2110 \xrightarrow{\delta^+_{TGF\beta2110}\leq\delta^+_{INOS2110}+\delta^+_{INOS1000}} \mathbf{2111}$
16.	$0000 \xrightarrow{\delta^+_{INOS0000}\leq\delta^+_{IDO0000}} 0010 \xrightarrow{\delta^+_{COX20010}\leq\delta^+_{IDO0010}}$ $0110 \xrightarrow{\delta^+_{IDO0110}\leq\delta^+_{INOS0110}+\delta^+_{INOS0000}} 1110 \xrightarrow{\delta^+_{IDO1110}+\delta^+_{IDO0010}\leq\delta^+_{TGF\beta1110}}$ $2110 \xrightarrow{\delta^+_{TGF\beta2110}\leq\delta^+_{INOS2110}+\delta^+_{INOS1000}} \mathbf{2111}$
17.	$0000 \xrightarrow{\delta^+_{INOS0000}\leq\delta^+_{IDO0000}} 0010 \xrightarrow{\delta^+_{COX20010}\leq\delta^+_{IDO0010}}$ $0110 \xrightarrow{\delta^+_{IDO0110}\leq\delta^+_{INOS0110}+\delta^+_{INOS0000}} 1110 \xrightarrow{\delta^+_{INOS1110}+\delta^+_{INOS1000}\leq\delta^+_{TGF\beta1110}} 1120$ $\xrightarrow{\delta^+_{TGF\beta1120}\leq\delta^+_{IDO0120}+\delta^+_{IDO1120}} 1121 \xrightarrow{\delta^+_{IDO1120}\leq\delta^+_{IDO1121}}$ $2121 \xrightarrow{\delta^+_{COX22021}+\delta^-_{INOS2121}\leq\delta^-_{IDO1020}} \mathbf{2111}$
18.	$0000 \xrightarrow{\delta^+_{INOS0000}\leq\delta^+_{IDO0000}} 0010 \xrightarrow{\delta^+_{IDO0010}\leq\delta^+_{INOS0010}+\delta^+_{INOS0000}} 1010$ $\xrightarrow{\delta^+_{COX21010}\leq\delta^+_{INOS1010}+\delta^+_{INOS1000}} 1110 \xrightarrow{\delta^+_{INOS1110}+\delta^+_{INOS1000}\leq\delta^+_{TGF\beta1110}} 1120$ $\xrightarrow{\delta^+_{TGF\beta1120}\leq\delta^+_{IDO0120}+\delta^+_{IDO1120}} 1121 \xrightarrow{\delta^+_{IDO1120}\leq\delta^+_{IDO1121}}$ $2121 \xrightarrow{\delta^+_{COX22021}+\delta^-_{INOS2121}\leq\delta^-_{IDO1020}} \mathbf{2111}$

These delays are helpful in designing experiments on IDO in case of tumor as it is giving temporal delays for the states involved in a pathway moving towards stable steady state.

3.2.5.3. Invariance Kernel Results

The Hybrid modeling results of the first homeostatic cycle, mentioned in Table 3.4 and Figure 3.3. It is shown in Figure 3.5.

$$\delta^{+}_{IDO0010} \leq \delta^{+}_{INOS0010} \quad \ldots\ldots\ldots(i)$$

The equation (i) of delay constraints represents that rate of activation of IDO is faster and less at location 0010 than iNOS, as shown in Figure 3.6a., so that it can move to the next state in cycle i.e. 1010, it is the condition that should be followed for cyclic behavior or homeostasis from location 0010 to 1010.

If this condition is negated or overruled that is, rate of activation of iNOS at location 0010 is faster and less than IDO at location 0010 than iNOS will reach its maximum concentration 0020, which make it deviate from cycle and move all the way to steady state 2111, a deadlock state. To follow normal behavior this constraint should remain true.

$$|\delta^{-}_{IDO1011}| + \delta^{+}_{IDO0010} \geq |\delta^{-}_{TGF\beta0011}| + \delta^{+}_{TGF\beta0010} \quad \ldots\ldots\ldots(ii)$$

It can be rewritten as, $\pi IDO \geq \pi TGF\beta$

π represents the period $\delta^{+} + \delta^{-}$ makes a period.

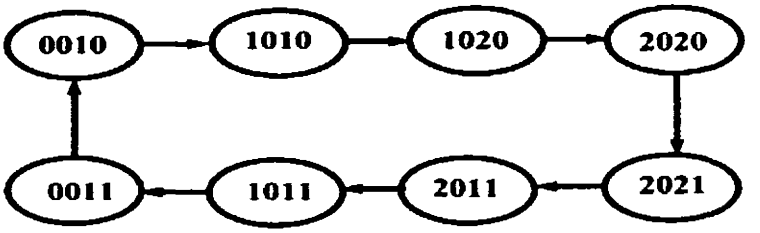

Figure 3.5: Homeostasis used in Invariance Kernel

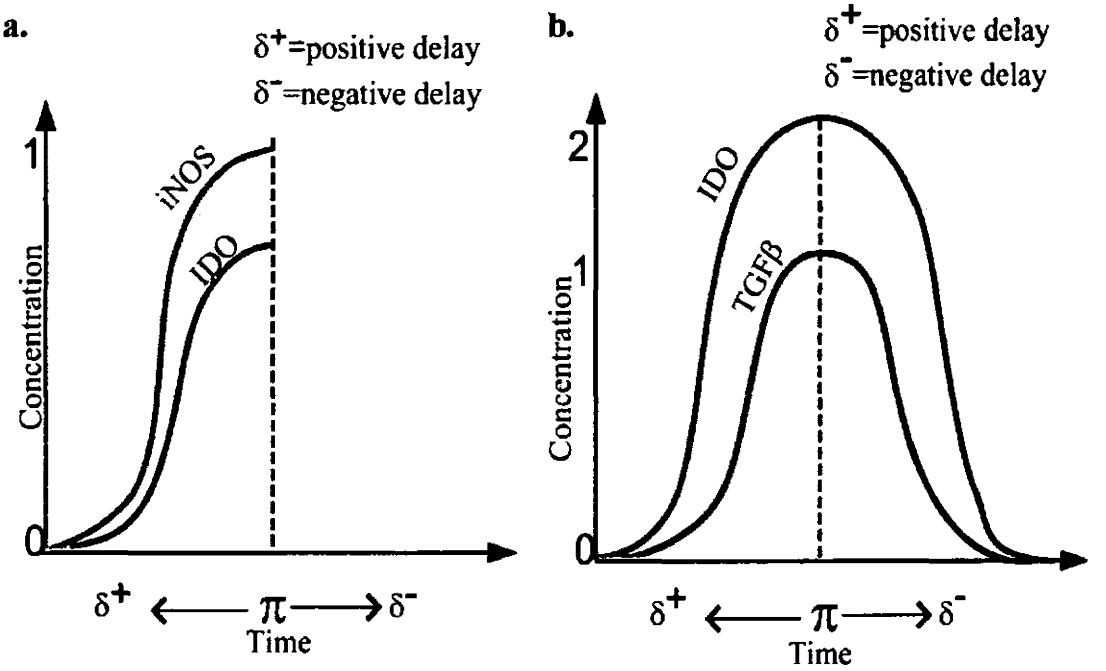

Figure 3.6: Time Constraints of equations (i) and (ii),

a. δ^+ of IDO is less than iNOS, **b.** π of TGFβ should be less than IDO

As shown in the equation (ii) and Figure 3.6 b., the activation and degradation delays of IDO are slower than TGFβ's activation and degradation delays in the IDO BRN. The TGFβ activation is faster than IDO as it is necessary that TGFβ gene should start activating faster than IDO at location 0010 so that it can control IDO concentration levels in incoming states. TGFβ degradation delay is also rapid than IDO because prolonged production of TGFβ at location 0011 will halt the cycle as it will hinder the normal pathway transition that is moving toward state 0010, start the cyclic behavior, with IDO its existence is also detrimental as it can lead to diseased state and both together will favor the tumor progression.

$$|\delta^{-}_{IDO2021}| + \delta^{+}_{IDO0010} \leq |\delta^{-}_{INOS2021}| + \delta^{+}_{INOS2010} \quad \ldots\ldots\ldots \text{(iii)}$$

The equation (iii), shows relationship between IDO and iNOS both regulate each other. In the absence of IDO iNOS is activated as IDO control its transcription factor same is the case with iNOS it also inhibit IDO activator. Both also have same potent inducer, IFNγ. Here period of IDO is smaller and faster than period of iNOS. As shown below and in Figure 3.7a.

$$\pi IDO \leq \pi INOS$$

Production rate of IDO is rapid at 0010 location so that the normal cycle continues as the state transit to 1010. It can deviate if rate of synthesis of iNOS is faster than IDO at the location 2010, iNOS will move to the higher concentration 2020, it can lead to production of COX2 as shown in Figure 3.3, which is harmful for the body and cause fast tumor progression in the body. Like activation delay degradation delay is also important if iNOS is not degraded at location 2021, this state is crucial stage if iNOS

sustains its production with IDO and TGFβ it will then activate COX2, which further enhance the IDO activity by strengthening its concentration as well as all the imunosuppressors are being activated at next stage and they will raise a destructive attack on the body's immune system and favors the tumor with T cells being destroyed fully. To help control this situation rate of degradation of IDO should be greater than iNOS so that IDO concentration remain under safe level as shown in Figure 3.7a.

$$\delta^{+}_{TGF\beta 0011} \leq \delta^{+}_{IDO1011} \quad \ldots\ldots\ldots\text{(iv)}$$

Degradation delay of TGFβ should be less than degradation rate of IDO according to equation (iv). If TGFβ degradation rate take time it will divert from 0010 state to 0111 which will eventually lead to 2111 state, which is a diseased state. IDO should move to state 0011 else it can move to state 1111, here all immunosuppressor are activated and then IDO will attain its sustained position from where system is unable to maintain homeostasis. The delay constraint is also shown in Figure 3.7b.

$$|\delta^{+}_{IDO1011}| + \delta^{+}_{IDO0011} \leq |\delta^{-}_{TGF\beta 0011}| \quad \ldots\ldots\ldots\text{(v)}$$

The equation (v) shows that the whole period of IDO, πIDO, i.e. activation and inhibition should be less than the degradation time of TGFβ as shown in Figure 3.7c., at location 0011.

This is in accordance with nature that the inhibitor, TGFβ, of IDO should degrade it and also take it to extreme low level that it should start its building up from scratch and help the body control IDO from reaching to dangerous level.

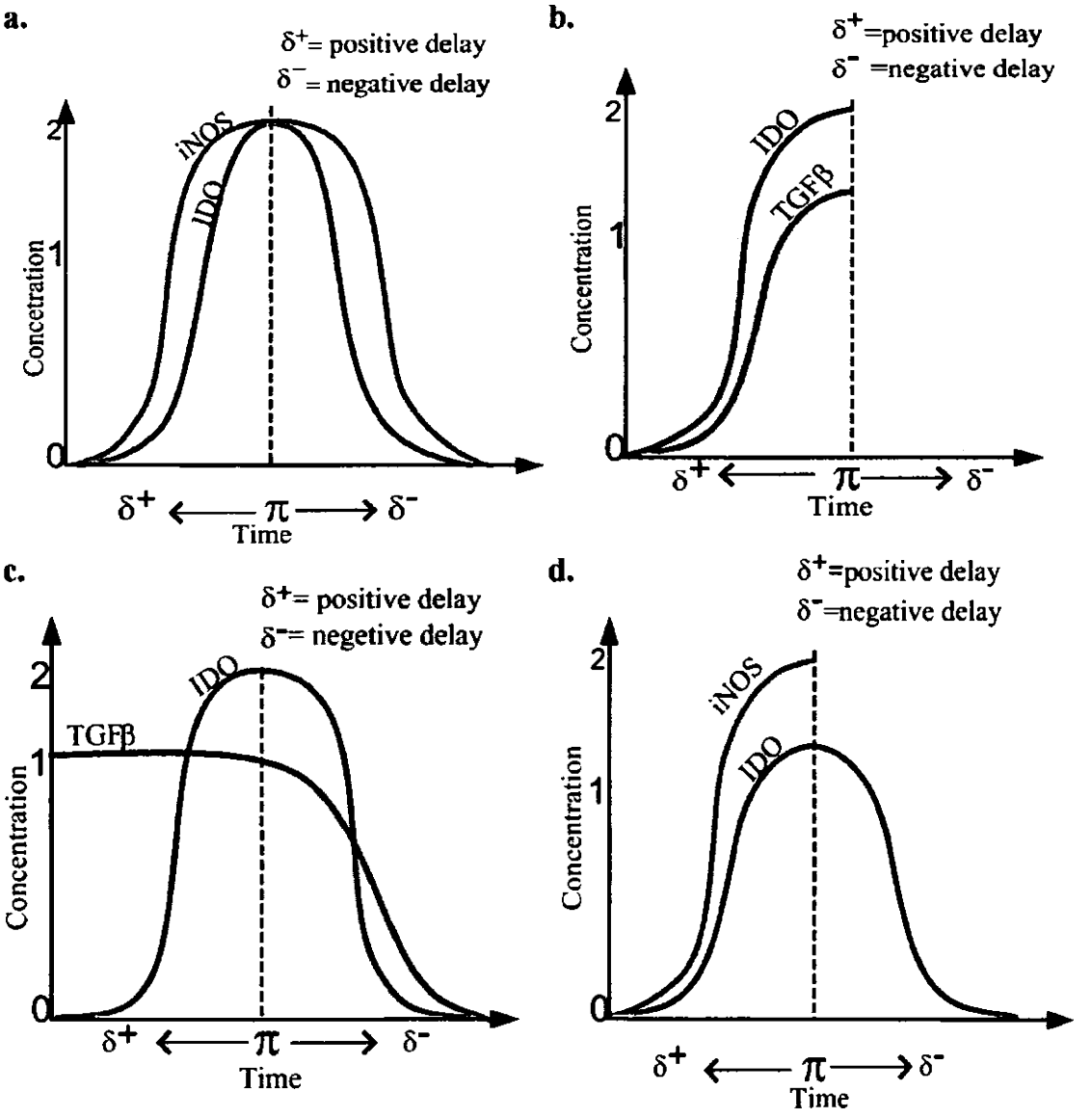

Figure 3.7: **a.** iNOS full life cycle should be greater than period of IDO, **b.** and **c.** Activation delay of TGFβ is less than that of activation delay and period of IDO, and **d.** Activation delay of iNOS is less than whole period of IDO.

The activation delay of IDO is also rapid at 0011 as it degrade at previous stage 1011, it start struggling to reach threshold level.

$$|\delta^{-}_{IDO2011}| + \delta^{+}_{IDO0010} \leq |\delta^{-}_{INOS2021}| + \delta^{+}_{INOS2010} \quad \ldots\ldots\ldots (vi)$$

$$\pi IDO \leq \pi INOS$$

The period of IDO is less than iNOS, according to the equation (vi) and Figure 3.7b. IDO reaches its maximum concentration that is before iNOS which too can have a maximum level of 2. IDO starts its activation after iNOS is activated an it has reached its first level i.e. 1 but IDO is activated after iNOS and it reaches its maximum level before iNOS as shown at location 2010, after IDO reaches its maximum level than iNOS start building again to achieve its maximum level. This is because both regulate each other and both having negative effect on each other, IDO suppresses iNOS from reaching maximum level. After iNOS have attains maximum level, it helps in fast degradation of IDO with the help of TGFβ, that's why cycle of IDO is faster than iNOS. As iNOS is at maximum level and TGFβ is activated IDO degradation starts, iNOS also start degrading due to TGFβ and IDO it declines to level 1 but IDO diminishes to zero 0, faster than iNOS as iNOS maintains its level to 1.

$$|\delta^{-}_{IDO2010}| + \delta^{+}_{IDO0010} \leq \delta^{+}_{INOS2010} \quad \ldots\ldots\ldots (vii)$$

Period of IDO is also less than activation delay of iNOS at 2010 as shown in Figure 3.7 d., and in equation (vii). It is because that iNOS should reach its maximum level slowly if it reaches fastly or with IDO both will have their immunosuppressive roles more strong then their normal working and both at their higher levels in the absence of TGFβ is dangerous than the system will move easily is without any hindrance to

diseased state. Slower activation time of iNOS and as well as less time required for IDO to complete its cycle is necessary and sufficient conditions for normal behavior of the system. It will help maintain homeostasis.

$$\delta^{+}_{IDO0010} \leq \delta^{+}_{INOS1010} \quad \ldots\ldots\ldots(viii)$$

$$\delta^{+}_{IDO0010} \leq \delta^{+}_{INOS0010} \quad \ldots\ldots\ldots(ix)$$

$$\delta^{+}_{IDO0010} \leq \delta^{+}_{INOS2010} \quad \ldots\ldots\ldots(x)$$

The equation (viii), show that IDO start activating at 0010 before iNOS where it is going to level 2 (two) from location 1010. IDO should be at level 1 before iNOS to be at level 2 and the time period for IDO should be less than iNOS, as if iNOS is rapidly building up then it will reach the maximum level and will deviate from cycle (homeostasis), it will move to state 1020.

In the equation (ix), there is a competition exist at location 0010 between both iNOS and IDO as both can inhibit each other so if IDO is suppressed by iNOS then IDO will not be able to produce to perform its normal function and a condition will occur where iNOS is high and the cycle will jump to next cycle and there are chances in between where the system can easily move towards tumor state.

After second state analysis in the cycle, as discussed in the above paragraph, the relationship between IDO and iNOS continues to exist that started between IDO and iNOS at location 0010. The time delay constraint of IDO with iNOS move to state 1010 from 0010 then finally to 2010 but location of IDO delay remains the same as shown in equation (x), and Figure 3.6a. If this delay constraint is overruled then the normal cycle transition to 2020 will be replaced by 2011 or 2110 these are the most

dangerous states that directly move to dead lock state i.e. **2111**. It can be seen by graph generated in Figure 3.3.

$$|\delta^{-}_{TGF\beta 0011}| \geq |\delta^{-}_{IDO1011}| + \delta^{+}_{IDO0010} \quad \ldots\ldots\ldots(xi)$$

The degradation delay of TGFβ at steady state 0011 is greater than to both the degradation delay of IDO at state 1011 and production delay at 0010, showed in equation (xi) and in Figure 3.7c. The TGFβ is main suppressor of IDO so to control IDO it should degrade after IDO concentration level is adjusted to normal or safe level. IDO is degraded one state before TGFβ. If degradation time of IDO prolong it will move out of cycle and iNOS will activate COX-2 which will further enhance IDO concentration and move to state 1011 to 1111 and then to 2111, diseased state, as shown in graph Figure 3.3.

3.3. Protein Knockout Studies

Protein knocking out was performed using Genotech model a protein was silenced in the model. Three proteins were knocked out i.e., TGFβ, COX2 and iNOS. It helped in understanding the changes occurred. When a protein was knock out, what effects were lost with protein? What were the hidden benefits a protein presence can give? All can be studied with protein knock out.

3.3.1. Model of TGFβ Knockout

The first protein knocked out from the model was TGFβ. The resultant model and its state graph are given below in Figure 3.8. There exited two stable steady states both were representing diseased conditions and first one was 2000, it occurred at the beginning as there was no control over IDO protein and TGFβ was not enough alone to control IDO. In the presence and absence of TGFβ IDO was moving to dead state, another inhibitor was required e.g. SOCS3 as shown by IDO first BRN homeostasis and iNOS as depicted by second IDO BRN homeostasis. The graph was split into two types of transition pathways and both stable steady states were not linked to same initial condition. As TGFβ was necessary to link both the states in the IDO BRN to form one graph and it form a negative feedback loop in the system which helps in regulating IDO in the body with iNOS. The homeostasis was destroyed as negative feedback loop was diminished due to TGFβ knockout.

3.3.2. COX2 Knock Out

The knockout of COX2 was shown in Figure 3.9, model. COX2 knockout haven't disturbed the cyclic behavior of the main IDO model as shown in Figure 1.4. COX2 was at dormant state, in homeostasis, in second BRN of IDO. So the same cycles or homeostasis were generated after COX2 knock out. One diseased state was reduced due to absence of COX2. It concluded that controlling COX2 in tumor helped in controlling tumor progression.

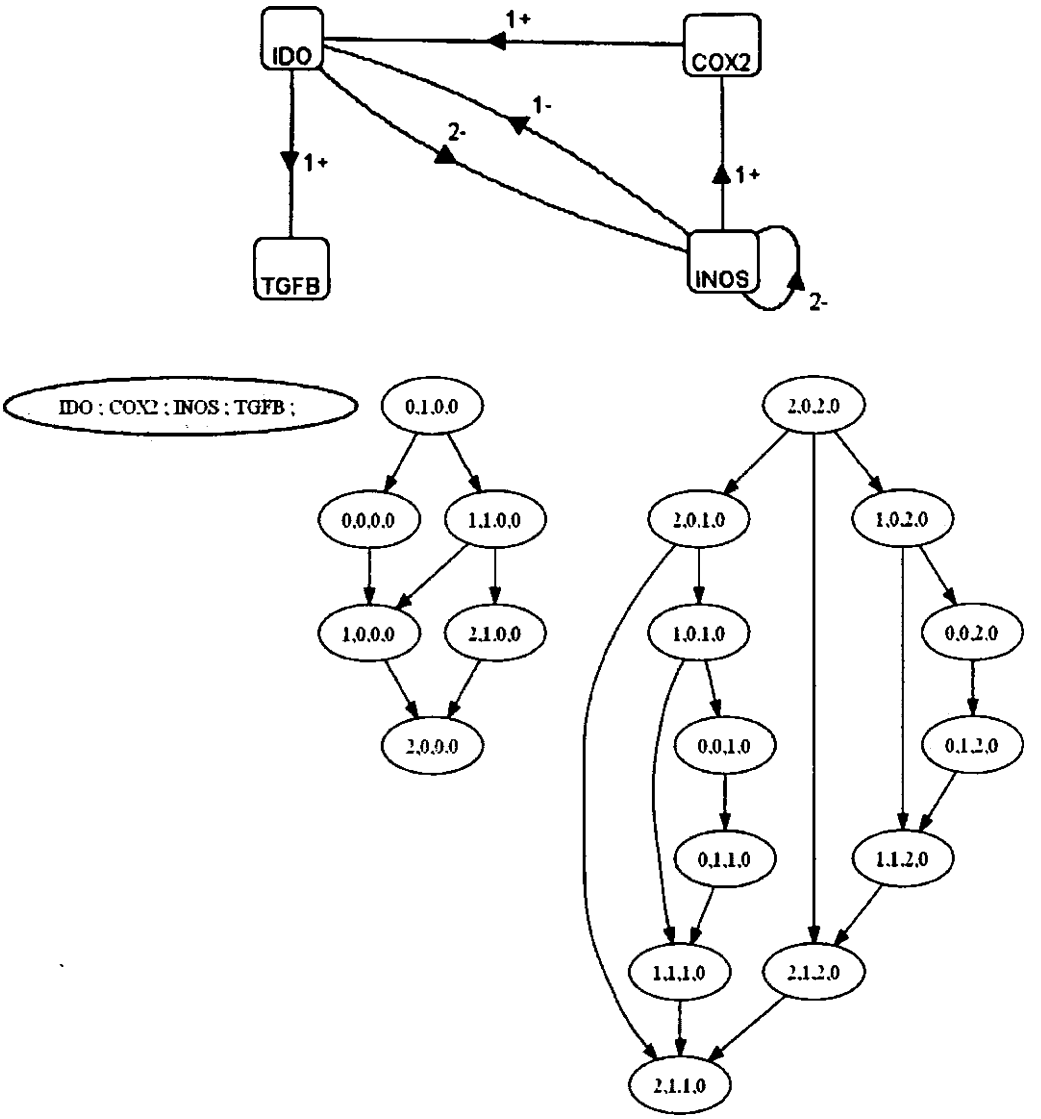

Figure 3.8: Knock-out model and State Graph of TGFβ

The state graph was also mentioned in Figure 3.9. The cycles / homeostasis generated were listed below.

- Cycle n°0 :[[0, 0, 1, 0], [1, 0, 1, 0], [2, 0, 1, 0], [2, 0, 1, 1], [1, 0, 1, 1], [0, 0, 1, 1], [0, 0, 1, 0]]
- Cycle n°1 :[[0, 0, 1, 0], [1, 0, 1, 0], [1, 0, 2, 0], [2, 0, 2, 0], [2, 0, 2, 1], [2, 0, 1, 1], [1, 0, 1, 1], [0, 0, 1, 1], [0, 0, 1, 0]]
- Cycle n°2 :[[0, 0, 1, 0], [1, 0, 1, 0], [1, 0, 1, 1], [0, 0, 1, 1], [0, 0, 1, 0]]
- Cycle n°3 :[[0, 0, 2, 0], [1, 0, 2, 0], [2, 0, 2, 0], [2, 0, 2, 1], [1, 0, 2, 1], [0, 0, 2, 1], [0, 0, 2, 0]]
- Cycle n°4 :[[0, 0, 2, 0], [1, 0, 2, 0], [2, 0, 2, 0], [2, 0, 2, 1], [2, 0, 1, 1], [1, 0, 1, 1], [0, 0, 1, 1], [0, 0, 1, 0], [0, 0, 2, 0]]
- Cycle n°5 :[[0, 0, 2, 0], [1, 0, 2, 0], [1, 0, 2, 1], [0, 0, 2, 1], [0, 0, 2, 0]]

Two types of cycles appeared due to higher level of iNOS. Both INOS and TGFβ were activated and controlling the IDO. There was no positive effect of COX2 presence in IDO second BRN.

3.3.3. iNOS Knock out Model

Knock out of iNOS was shown in Figure 3.10 with its state graph. It showed that the system remain in the homeostasis. The knockout of iNOS was very important. As it was silenced; COX2 become inactive it was shown that iNOS was the main inducer of COX2 in the BRN.

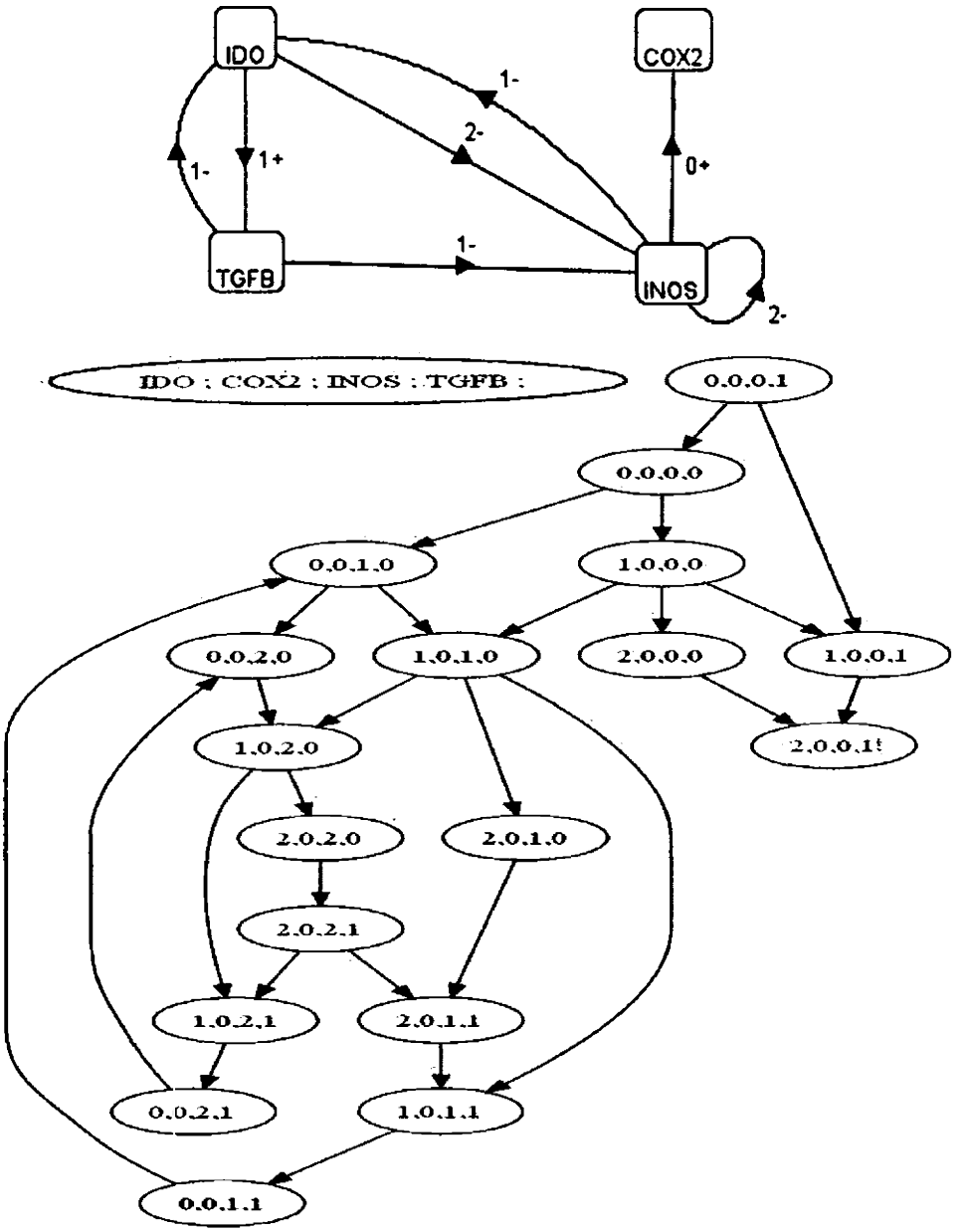

Figure 3.9: Model and State Graph Generated after COX2 Knockout

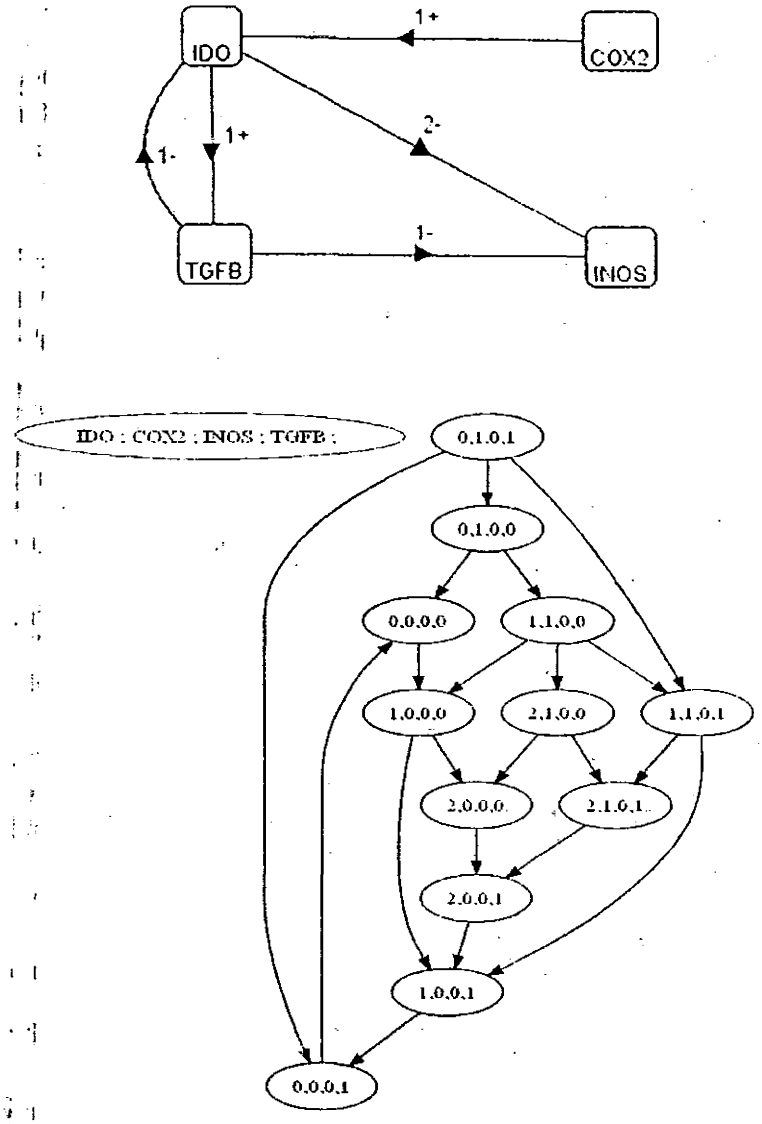

Figure 3.10: iNOS Knock out Model and State Graph

Two cycles were generated as shown below in which only TGFβ and IDO were regulating each other.

- Cycle n°0 :[[0, 0, 0, 0], [1, 0, 0, 0], [2, 0, 0, 0], [2, 0, 0, 1], [1, 0, 0, 1], [0, 0, 0, 1], [0, 0, 0, 0]]
- Cycle n°1 :[[0, 0, 0, 0], [1, 0, 0, 0], [1, 0, 0, 1], [0, 0, 0, 1], [0, 0, 0, 0]]

The cycles were implementable when there were no potent inhibitors for IDO as well as no main activator for it. It was also possible at initial states of tumor progression when tumor was not in metastatic stage as TGFβ was not yet converted into immunosuppresor due to physiological changes occurred in the cellular environment.

The research conducted on qualitative and Hybrid modeling of IDO second BRN was summarized in the form of Figure 3.11. It revealed all the information gathered after the qualitative modeling. It was showing both types of pathways the one leading toward homeostasis and the other deviating towards stable steady states. The blue colored transition were between homeostatic states and the deviation from these states were shown with dotted transition states and the dotted red states were the most crucial states as they lead the system directly towards the diseased state.

Both the steady stable states i.e. 20001 in first IDO BRN and 2111 and 2001 in second model were in accordance with the fact that over expression of IDO is detrimental and lead to reduced or low probability of recovery from cancer (Ino *et al.*, 2006; Muller *et al.*, 2005, Astigiano *et al.*, 2005). In HCC studies high concentration of IDO is also a cause of rapid tumor progression (Pan *et al.*, 2006).

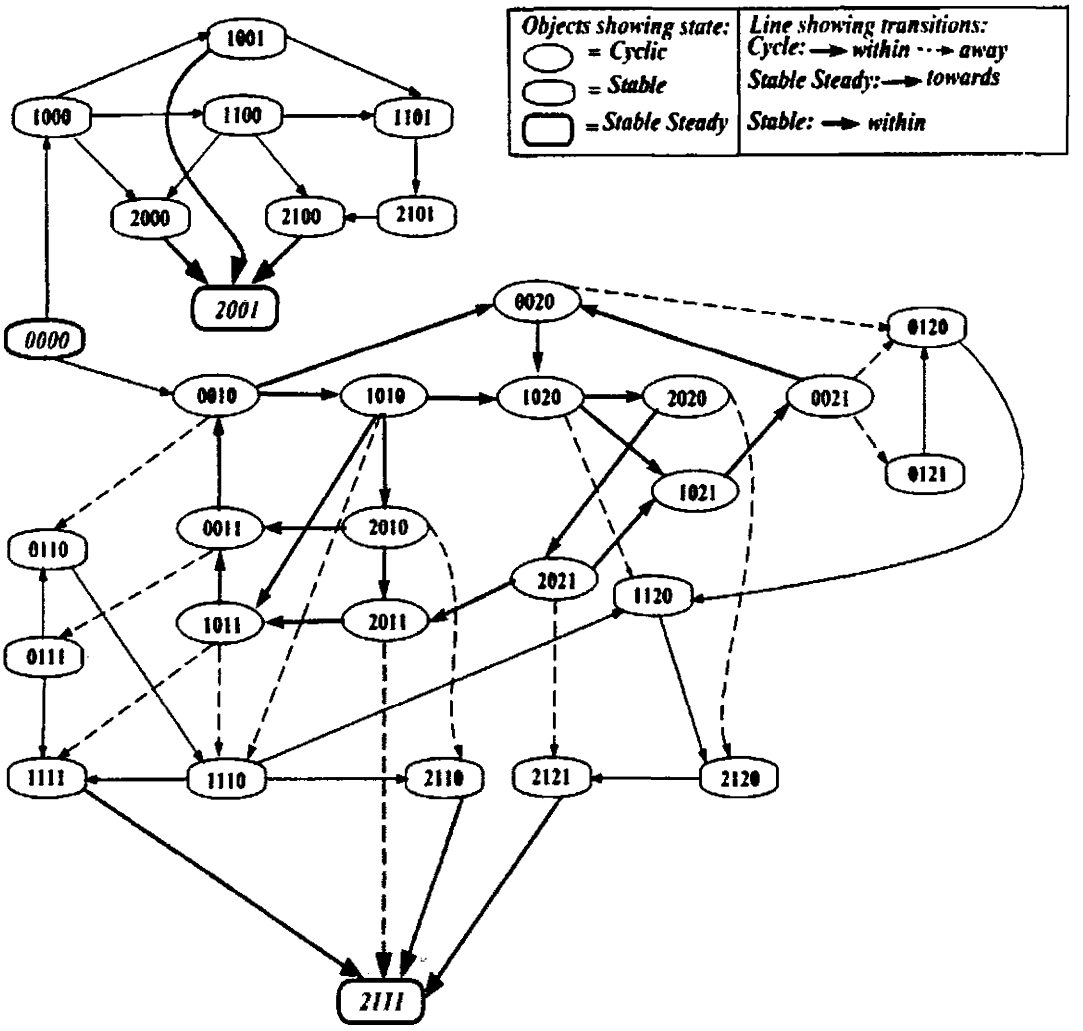

Figure 3.11: Transition Graph Summarizing Qualitative Modeling Results

The objective of the research is to help in the cure of tumor at each state. This is accomplished by a state graph in Figure 3.1, 3.3 and 3.11. As it show all the possible states that can be generated in the BRN and it shows how a tumor progresses in the body in the presence of immunosuppressors especially in case of IDO, it shows all types and all possible trajectories followed by a tumor. This will help understanding the tumor progression in an elaborative manner and help in clinical trials as each type and stage of patients can be diagnosed and treated.

Like state transitions in Figure 3.11, the state table is also contributive as it provide information about each steady state i.e. how it changes to new state and which element is controlled by which element and in which states it can transit. It is a competent tool and it will be efficacious in controlling disease states and aid in moulding the states towards desirable states.

The IDO protein is ubiquitously present in the cell. It is regulated in the body; homeostasis is maintained to keep cell normal function. TGFβ is a two edged sword as it can be a tumor suppressor as well as immunosuppresor by promoting tumor development (Wakefield and Roberts, 2002). It offer multiple functionalities one of which is homeostasis maintenance of immune system (Blobe *et al.*, 2000) by regulating IDO in a cyclic fashion, represented by cycles of Figure 3.4, 3.11 and Table 3.4. It is immunosuppresor as shown in stable steady states behavior of first IDO model i.e. 20001 and in stable steady states of second IDO model i.e. 2001 and 2111.

It is proved that as COX2 is activated in the BRN IDO reaches high concentration, it will result in deviation from homeostasis and lead to diseased state depicted in state graph Figure 3.11 and Table 3.3 , COX2 produces PGE2, in human DC gene expression profiling, it greatly influence up-regulation of IDO expression (Popov and Schultze, 2008).

Hybrid Modeling generate time delay feature as shown in Table 3.6 for steady stable steady states trajectories and homeostasis. It will help maintaining system in specified trajectory by altering these delays and lengthening of the specific constraints to keep the system in desirable steady state. It provides us the pathway followed by trajectories along each dimension and these delays are Hybrid constraints of dynamical system (Halasz *et al.*, 2007). The Table 3.7, is labeling whole pathways, derived from state graph Figure 3.3 and 3.11, can be used further for designing controlled medium for tumor control and providing guidelines for clinical experiments designing (Comet *et al.*, 2010). The IDO BRN is based on body proteins as artificial inhibitors introduced in the body have limitations. Using protein present in the cell as inhibitors by applying qualitative modeling saves the body cells from the side effects of artificial inhibitors and provides natural mechanism for controlling disease without introducing foreign substances into the body. This is the advantage of system biology and its qualitative modeling. In the studies only one protein is dealt at a time but in this paper four immunosuppressors are working with each other and controlling each other here their homeostasis maintenance is important. This will help in chemotherapeutic studies of cancer and will aid chemotherapy procedure if done with it.

Most of the proteins, producing immunological resistance, used in the IDO BRNs including CTLA-4, inhibitors of IDO and COX2 are being used in experimental studies on chemotherapy and their combinations are experimented in clinical trials (Meric *et al.*, 2006; Muller *et al.*, 2005; Mokyr *et al.*, 1998). These two BRNs act as a tool on the basis of it scientist and doctors can step forward in immunotherapy techniques in antitumor immunity.

The knock out model of TGFβ in Figure 3.8, is showing antitumor role of it. Both the deadlock states are due to lack of its antitumor effect and homeostasis, normal body functioning in combating disease is totally lost due to TGFβ silencing. TGFβ activated after malignancy, first inhibits tumor development through blocking and destroying tumor proliferating cells (Yingling *et al.*, 2004). However, as time passes and tumor cells evolve, they become rebellious to TGFβ inhibition and the antitumor ability of TGFβ will eventually change into tumor favoring feature, as shown in the second deadlock state of IDO second BRN, Figure 3.3, when the system deviate from homeostasis to deadlock state, TGFβ is immunosuppressive at late stage of tumor (Yingling *et al.*, 2004; Dumont and Arteaga, 2003).

Knock out model of COX2, Figure 3.9, illustrates that cyclic behavior remains undisturbed as COX2 has no role in homeostasis and one epigenetic state is removed. The system can be maintained in healthy state as homeostasis is maintained and deviation to diseased state is not possible. One stable steady state is eliminated, that depend on immunosuppressors, out of two that are generated in second IDO BRN. A study carried out on lung carcinoma model confirmed that silencing of COX2 using selective inhibitors give positive results increasing survival rate and reducing tumor

progression, controlling COX2 means that IDO is controlled as it greatly influence up-regulation of IDO expression (Popov and Schultze, 2008; Stolina *et al.*, 2000).

iNOS inhibition provided fruitful results as shown in Figure 3.10, inhibition of iNOS accompanied silencing of COX2 as well as it is a main inducer of COX2 in the IDO BRN. All diseased states are eliminated and a homeostasis is maintained in the BRN. iNOS produce NO and catabolize arginine and produce COX2 which further cause high tumoricidal action and tumor progression (Lorsbach *et al.*, 1993).

3.4. Conclusion

IDO BRNs are important tools for providing the best and possible drug designing target as the modeling of the BRN provide a deep insight into the behavior of the system, providing all trajectories towards homeostasis and deadlock states their threshold values for each state transition. It is further embellished by the introduction of time delay constraints in the BRN; they are providing the checkpoints for controlling protein activation and inhibition with respect to time context. The modeling formalism provide natural cancer controlling therapeutic targets as the BRN include all immunosuppresors regulating each other rather dealing each protein as a single target for inhibition and designing single inhibitors for each and every protein the qualitative and Hybrid modeling and analysis are dealing with whole phenomenon as a single entity. The results are helpful and have futuristic prospects as on the basis of this clinical trials and experiments for immunotherapy can be designed and it will save time and money. It can bring fruitful results in tumor control and in increasing life expectancy after tumor invasion.

CHAPTER 4

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REFERENCES

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ANNEXURE

```
var
hIDO,hCOX2,hINOS,hTGFB:analog;
k,n:discrete;
dpIDO0020,dpTGFB0020,dpCOX20020,dpIDO0021,dnTGFB0021,dpCOX20021,dpTGFB0
120,dpIDO0120,dpIDO0121,dnTGFB0121,dpIDO1010,dpINOS1010,dpINOS0110,dpIDO10
01,dpIDO1000,dpIDO1101,dpIDO1020,dpCOX21020,dpTGFB1020,dpIDO1100,dpIDO111
0,dpINOS1110,dpIDO1111,dpIDO1120,dpIDO1121,dnINOS1121,dpCOX22000,dpINOS200
0,dpTGFB2000,dnIDO2010,dpCOX22010,dpINOS2010,dpTGFB2010,dnIDO2011,dpCOX2
2011,dnIDO2020,dpCOX22020,dnINOS2020,dpTGFB2020,dnIDO2021,dpCOX22021,dnIN
OS2021,dpTGFB2100,dpINOS2100,dnCOX22100,dnCOX22101,dpINOS2110,dpTGFB211
0,dnINOS2120,dpTGFB2120,dnINOS2121,dpTGFB1120,dpIDO,dnIDO1011,dnIDO,dpCOX
2,dnCOX2,dpINOS,dnINOS,dpTGFB,dnTGFB,dnCOX21111,dnINOS1111,dnINOS1110,dp
TGFB1110,dnCOX21101,dnCOX21100,dpINOS1100,dpTGFB1100,dpCOX21011,dnINOS1
011,dnTGFB1011,dnIDO1010,dpCOX21010,dnINOS1010,dpTGFB1010,dpCOX21000,dpIN
OS1000,dpTGFB1000,dpIDO0111,dnINOS0111,dnTGFB0111,dpIDO0110,dpTGFB0110,dp
IDO0101,dnCOX20101,dpINOS0101,dnTGFB0101,dpIDO0100,dnCOX20100,dpINOS0100,
dpTGFB0100,dpIDO0011,dpCOX20011,dnTGFB0011,dpIDO0010,dpCOX20010,dpTGFB0
010,dpIDO0001,dpCOX20001,dpINOS0001,dnTGFB0001,dpIDO0000,dpCOX20000,dpINO
S0000,dpTGFB0000,dpINOS0010,dpINOS0011,dpINOS0111,dnIDO1020,dnIDO1021,dpC
OX21021,dnTGFB1021: parameter;
automaton auto
synclabs: ;
initially loc_0000;
-- pour la configuration 0,0,0,0
loc loc_0000: while hIDO <= dpIDO0000 & hCOX2 <= dpCOX20000 & hINOS <=
dpINOS0000 & hTGFB <= dpTGFB0000 wait
{dhIDO=1,dhCOX2=1,dhINOS=1,dhTGFB=1}
when hIDO=dpIDO0000  do {hIDO'=0, k'=k+1} goto loc_1000;
--when hCOX2=dpCOX20000 do {hCOX2'=0,k'=k+1} goto loc_0100;
when hINOS=dpINOS0000 do {hINOS'=0,k'=k+1} goto loc_0010;
--when hTGFB=dpTGFB0000 do {hTGFB'=0,k'=k+1} goto loc_0001;

-- pour la configuration 0,0,0,1
loc loc_0001: while hIDO <= dpIDO0001 &  hINOS <= dpINOS0001 &  hTGFB >=
dnTGFB0001  wait {dhIDO=1,dhCOX2=0,dhINOS=1,dhTGFB=-1}
when hIDO=dpIDO0001 do {hIDO'=0,k'=k+1} goto loc_1001;
--when hINOS=dpINOS0001 do {hINOS'=0,k'=k+1} goto loc_0011;
when hTGFB=dnTGFB0001 do {hTGFB'=0,k'=k+1} goto loc_0000;

-- pour la configuration 0,0,1,0
loc loc_0010: while hIDO <= dpIDO0010 & hCOX2 <= dpCOX20010 & hINOS <=
dpINOS0010 & hTGFB <= dpTGFB0010  wait
{dhIDO=1,dhCOX2=1,dhINOS=1,dhTGFB=1}
when hIDO=dpIDO0010 do {hIDO'=0,k'=k+1} goto loc_1010;
when hCOX2=dpCOX20010 do {hCOX2'=0,k'=k+1} goto loc_0110;
when hINOS=dpINOS0010 do {hINOS'=0,k'=k+1} goto loc_0020;
--when hTGFB=dpTGFB0010 do {hTGFB'=0,k'=k+1} goto loc_0011;

-- pour la configuration 0,0,1,1
--loc loc_0011: while hIDO <= dpIDO0011 & hCOX2 <= dpCOX20011 & hINOS <=
dpINOS0011 & hTGFB >= dnTGFB0011 wait {dhIDO=1,dhCOX2=1,dhINOS=1,dhTGFB=-
1}
```

```
loc loc_0011: while  hCOX2 <= dpCOX20011 & hTGFB >= dnTGFB0011 wait
{dhIDO=0,dhCOX2=1,dhINOS=0,dhTGFB=-1}
--when hIDO=dpIDO0011 do {hIDO'=0,k'=k+1} goto loc_1011;
when hCOX2=dpCOX20011 do {hCOX2'=0,k'=k+1} goto loc_0111;
--when hINOS=dpINOS0011 do {hINOS'=0,k'=k+1} goto loc_0021;
when hTGFB=dnTGFB0011 do {hTGFB'=0,k'=k+1} goto loc_0010;

-- pour la configuration 0,0,2,0
loc loc_0020: while hIDO <= dpIDO0020 & hCOX2 <= dpCOX20020  &  hTGFB <=
dpTGFB0020 wait {dhIDO=1,dhCOX2=1,dhINOS=0,dhTGFB=1}
when hIDO=dpIDO0010 do {hIDO'=0,k'=k+1} goto loc_1020;
when hCOX2=dpCOX20010 do {hCOX2'=0,k'=k+1} goto loc_0120;
--when hTGFB=dpTGFB0010 do {hTGFB'=0,k'=k+1} goto loc_0021;
-- pour la configuration 0,0,2,1
loc loc_0021: while hIDO <= dpIDO0021 & hCOX2 <= dpCOX20021 & hTGFB >=
dnTGFB0021 wait {dhIDO=1,dhCOX2=1,dhINOS=0,dhTGFB=-1}
--when hIDO = dpIDO0021 do {hIDO'=0,k'=k+1} goto loc_1021;
when hCOX2=dpCOX20021 do {hIDO'=0,k'=k+1} goto loc_0121;
when hTGFB=dnTGFB0021 do {hIDO'=0,k'=k+1} goto loc_0020;

-- pour la configuration 0,1,0,0
loc loc_0100: while hIDO <= dpIDO0100 & hCOX2 >= dnCOX20100 & hINOS <=
dpINOS0100 &  hTGFB <= dpTGFB0100  wait {dhIDO=1,dhCOX2=
1,dhINOS=1,dhTGFB=1}
when hIDO=dpIDO0100 do {hIDO'=0,k'=k+1} goto loc_1100;
when hCOX2=dnCOX20100 do {hCOX2'=0,k'=k+1} goto loc_0000;
when hINOS=dpINOS0100 do {hINOS'=0,k'=k+1} goto loc_0110;
--when hTGFB=dpTGFB0100 do {hTGFB'=0,k'=k+1} goto loc_0101;

-- pour la configuration 0,1,0,1
loc loc_0101: while hIDO <= dpIDO0101 & hCOX2 >= dnCOX20101 & hINOS <=
dpINOS0101 &  hTGFB >= dnTGFB0101 wait {dhIDO=1,dhCOX2=-
1,dhINOS=1,dhTGFB=-1}
when hIDO=dpIDO0101 do {hIDO'=0,k'=k+1} goto loc_1101;
when hCOX2=dnCOX20101 do {hCOX2'=0,k'=k+1} goto loc_0001;
--4when hINOS=dpINOS0101 do {hINOS'=0,k'=k+1}  goto loc_0111;
when hTGFB=dnTGFB0101 do {hTGFB'=0,k'=k+1} goto loc_0100;

-- pour la configuration 0,1,1,0
loc loc_0110: while hIDO <= dpIDO0110 & hINOS <= dpINOS0110 & hTGFB <=
dpTGFB0110 wait {dhIDO=1,dhCOX2=0,dhINOS=1,dhTGFB=1}
when hIDO=dpIDO0110 do {hIDO'=0,k'=k+1} goto loc_1110;
when hINOS=dpINOS0110 do {hINOS'=0,k'=k+1} goto loc_0120;
--2when hTGFB=dpTGFB0110 do {hTGFB'=0,k'=k+1} goto loc_0111;

-- pour la configuration 0,1,1,1
loc loc_0111: while hIDO <= dpIDO0111 & hINOS <= dpINOS0111 &  hTGFB >=
dnTGFB0111 wait {dhIDO=1,dhCOX2=0,dhINOS=1,dhTGFB=-1}
when hIDO=dpIDO0111 do {hIDO'=0,k'=k+1} goto loc_1111;
--2when hINOS=dpINOS0111 do {hINOS'=0,k'=k+1} goto loc_0121;
when hTGFB=dnTGFB0111 do {hTGFB'=0,k'=k+1} goto loc_0110;
```

```
-- pour la configuration 0,1,2,0
loc loc_0120: while hIDO <= dpIDO0120 & hTGFB <= dpTGFB0120 wait
{dhIDO=1,dhCOX2=0,dhINOS=0,dhTGFB=1}
when hIDO=dpIDO0120 do {hIDO'=0,k'=k+1} goto loc_1120;
--2when hTGFB=dpTGFB0120 do {hTGFB'=0,k'=k+1} goto loc_0121;

-- pour la configuration 0,1,2,1
loc loc_0121: while hIDO <= dpIDO0121 & hTGFB >= dnTGFB0121 wait
{dhIDO=1,dhCOX2=0,dhINOS=0,dhTGFB=-1}
when hIDO=dpIDO0121 do {hIDO'=0,k'=k+1} goto loc_1121;
when hTGFB=dnTGFB0121 do {hTGFB'=0,k'=k+1} goto loc_0120;

-- pour la configuration 1,0,0,0
loc loc_1000: while hIDO <= dpIDO1000 & hCOX2 <= dpCOX21000 & hINOS <=
dpINOS1000 & hTGFB <= dpTGFB1000 wait
{dhIDO=1,dhCOX2=1,dhINOS=1,dhTGFB=1}
when hIDO = dpIDO1000 do {hIDO'=0,k'=k+1} goto loc_2000;
--4when hCOX2=dpCOX21000 do {hCOX2'=0,k'=k+1} goto loc_1100;
when hINOS=dpINOS1000 do {hINOS'=0,k'=k+1} goto loc_1010;
when hTGFB=dpTGFB1000 do {hTGFB'=0,k'=k+1} goto loc_1001;

-- pour la configuration 1,0,0,1
loc loc_1001: while hIDO <= dpIDO1001 wait
{dhIDO=1,dhCOX2=0,dhINOS=0,dhTGFB=0}
when hIDO = dpIDO1001 do {hIDO'=0,k'=k+1} goto loc_2001;

-- pour la configuration 1,0,1,0
loc loc_1010: while hIDO >= dpIDO1010 & hCOX2 <= dpCOX21010 & hINOS <=
dpINOS1010 &  hTGFB <= dpTGFB1010 wait
{dhIDO=1,dhCOX2=1,dhINOS=1,dhTGFB=1}
when hIDO=dpIDO1010 do {hIDO'=0,k'=k+1} goto loc_2010;
when hCOX2=dpCOX21010 do {hCOX2'=0,k'=k+1} goto loc_1110;
when hINOS=dpINOS1010 do {hINOS'=0,k'=k+1} goto loc_1020;
when hTGFB=dpTGFB1010 do {hTGFB'=0,k'=k+1} goto loc_1011;

-- pour la configuration 1,0,1,1
loc loc_1011: while hIDO >= dnIDO1011 & hCOX2 <= dpCOX21011 & hTGFB <=
dnTGFB1011 wait {dhIDO=-1,dhCOX2=1,dhINOS=0,dhTGFB=-1}
when hIDO=dnIDO1011 do {hIDO'=0,k'=k+1} goto loc_0011;
when hCOX2=dpCOX21011 do {hCOX2'=0,k'=k+1} goto loc_1111;
--3when hTGFB=dnTGFB1011 do {hTGFB'=0,k'=k+1} goto loc_1010;

-- pour la configuration 1,0,2,0
loc loc_1020: while hIDO <= dpIDO1020 & hCOX2 <= dpCOX21020 &  hTGFB <=
dpTGFB1020 wait {dhIDO=1,dhCOX2=1,dhINOS=0,dhTGFB=1}
when hIDO = dnIDO1020 do {hIDO'=0,k'=k+1} goto loc_2020;
when hCOX2=dpCOX21020 do {hCOX2'=0,k'=k+1} goto loc_1120;
when hTGFB=dpTGFB1020 do {hTGFB'=0,k'=k+1} goto loc_1021;

-- pour la configuration 1,0,2,1
```

```
loc loc_1021: while hIDO >= dnIDO1021 & hCOX2 <= dpCOX21021 & hTGFB >=
dnTGFB1021 wait {dhIDO=-1,dhCOX2=1,dhINOS=0,dhTGFB=-1}
when hIDO=dnIDO1021 do {hIDO'=0,k'=k+1} goto loc_0021;
when hCOX2=dpCOX21021 do {hCOX2'=0,k'=k+1} goto loc_1121;
--3when hTGFB=dnTGFB1021 do {hINOS'=0,k'=k+1} goto loc_1020;

-- pour la configuration 1,1,0,0
loc loc_1100: while hIDO <= dpIDO1100 & hCOX2 >= dnCOX21100 & hINOS <=
dpINOS1100 &  hTGFB <= dpTGFB1100 wait {dhIDO=1,dhCOX2=-
1,dhINOS=1,dhTGFB=1}
when hIDO = dpIDO1100 do {hIDO'=0,k'=k+1} goto loc_2100;
when hCOX2=dnCOX21100 do {hCOX2'=0,k'=k+1} goto loc_1000;
when hINOS=dpINOS1100 do {hINOS'=0,k'=k+1} goto loc_1110;
when hTGFB=dpTGFB1100 do {hTGFB'=0,k'=k+1} goto loc_1101;

-- pour la configuration 1,1,0,1
loc loc_1101: while hIDO <= dpIDO1101 & hCOX2 >= dnCOX21101 wait
{dhIDO=1,dhCOX2=-1,dhINOS=0,dhTGFB=0}
when hIDO = dpIDO1101 do {hIDO'=0,k'=k+1} goto loc_2101;
when hCOX2=dnCOX21101 do {hCOX2'=0,k'=k+1} goto loc_1001;

-- pour la configuration 1,1,1,0
loc loc_1110: while hIDO <= dpIDO1110 & hINOS <= dpINOS1110 &  hTGFB <=
dpTGFB1110 wait {dhIDO=1,dhCOX2=0,dhINOS=1,dhTGFB=1}
when hIDO = dpIDO1110 do {hIDO'=0,k'=k+1}  goto loc_2110;
when hINOS=dpINOS1110 do {hINOS'=0,k'=k+1} goto loc_1120;
when hTGFB=dpTGFB1110 do {hTGFB'=0,k'=k+1} goto loc_1111;

-- pour la configuration 1,1,1,1
loc loc_1111: while hIDO <= dpIDO1111 wait
{dhIDO=1,dhCOX2=0,dhINOS=0,dhTGFB=0}
when hIDO = dpIDO1111 do {hIDO'=0,k'=k+1} goto loc_2111;

-- pour la configuration 1,1,2,0
loc loc_1120: while hIDO <= dpIDO1120 & hTGFB <= dpTGFB1120  wait
{dhIDO=1,dhCOX2=0,dhINOS=0,dhTGFB=1}
when hIDO = dpIDO1120 do {hIDO'=0,k'=k+1}  goto loc_2120;
when hTGFB=dpTGFB1120 do {hTGFB'=0,k'=k+1} goto loc_1121;

-- pour la configuration 1,1,2,1
loc loc_1121: while hIDO <= dpIDO1121 & hINOS <= dnINOS1121  wait
{dhIDO=1,dhCOX2=0,dhINOS=-1,dhTGFB=0}
when hIDO = dpIDO1120 do {hIDO'=0,k'=k+1}  goto loc_2121;
--2when hINOS=dnINOS1110 do {hINOS'=0,k'=k+1}  goto loc_1111;

-- pour la configuration 2,0,0,0
loc loc_2000: while hCOX2 <= dpCOX22000 & hINOS <= dpINOS2000 &  hTGFB <=
dpTGFB2000 wait {dhIDO=0,dhCOX2=1,dhINOS=1,dhTGFB=1}
--5when hCOX2=dnCOX21100 do {hCOX2'=0,k'=k+1} goto loc_2100;
--when hINOS=dpINOS1100 do {hINOS'=0,k'=k+1} goto loc_2010;
when hTGFB=dpTGFB1100 do {hTGFB'=0,k'=k+1} goto loc_2001;
```

```
-- pour la configuration 2,0,0,1
loc loc_2001: while True wait {dhIDO=0,dhCOX2=0,dhINOS=0,dhTGFB=0}
-- pour la configuration 2,0,1,0
loc loc_2010: while hIDO >= dnIDO2010 & hCOX2 <= dpCOX22010 & hINOS <=
dpINOS2010 &  hTGFB <= dpTGFB2010 wait {dhIDO=-
1,dhCOX2=1,dhINOS=1,dhTGFB=1}
--2when hIDO = dnIDO2010 do {hIDO'=0,k'=k+1} goto loc_1010;
when hCOX2=dpCOX22010 do {hCOX2'=0,k'=k+1} goto loc_2110;
--when hINOS=dpINOS2010 do {hINOS'=0,k'=k+1} goto loc_2020;
when hTGFB=dpTGFB2010 do {hTGFB'=0,k'=k+1} goto loc_2011;

-- pour la configuration 2,0,1,1
loc loc_2011: while hIDO >= dnIDO2011 & hCOX2 <= dpCOX22011 wait {dhIDO=-
1,dhCOX2=1,dhINOS=0,dhTGFB=0}
when hIDO = dnIDO2011 do {hIDO'=0,k'=k+1} goto  loc_1011;
when hCOX2=dpCOX22011 do {hCOX2'=0,k'=k+1} goto loc_2111;

-- pour la configuration 2,0,2,0
loc loc_2020: while hIDO >= dnIDO2020 & hCOX2 <= dpCOX22020 & hINOS >=
dnINOS2020 &  hTGFB <= dpTGFB2020 wait {dhIDO=-1,dhCOX2=1,dhINOS=-
1,dhTGFB=1}
--when hIDO = dnIDO2020 do {hIDO'=0,k'=k+1}  goto loc_1020;
when hCOX2=dpCOX22020 do {hCOX2'=0,k'=k+1} goto loc_2120;
--when hINOS=dnINOS2020 do {hINOS'=0,k'=k+1} goto loc_2010;
when hTGFB=dpTGFB2020 do {hTGFB'=0,k'=k+1} goto loc_2021;

-- pour la configuration 2,0,2,1
loc loc_2021: while hIDO >= dnIDO2021 & hCOX2 <= dpCOX22021 & hINOS >=
dnINOS2021 wait {dhIDO=-1,dhCOX2=1,dhINOS=-1,dhTGFB=0}
when hIDO = dnIDO2021 do {hIDO'=0,k'=k+1}  goto loc_1021;
when hCOX2=dpCOX22021 do {hCOX2'=0,k'=k+1} goto loc_2121;
when hINOS=dnINOS2021 do {hINOS'=0,k'=k+1} goto loc_2011;

-- pour la configuration 2,1,0,0
loc loc_2100: while hCOX2 >= dnCOX22100 & hINOS <= dpINOS2100 &  hTGFB <=
dpTGFB2100 wait {dhIDO=0,dhCOX2=-1,dhINOS=1,dhTGFB=1}
when hCOX2=dnCOX22100 do {hCOX2'=0,k'=k+1} goto loc_2000;
--when hINOS=dpINOS2100 do {hINOS'=0,k'=k+1} goto loc_2110;
when hTGFB=dpTGFB2100 do {hTGFB'=0,k'=k+1} goto loc_2101;

-- pour la configuration 2,1,0,1
loc loc_2101: while hCOX2 >= dnCOX22101 wait {dhIDO=0,dhCOX2=-
1,dhINOS=0,dhTGFB=0}
when hCOX2=dnCOX22101 do {hCOX2'=0,k'=k+1} goto loc_2001;

-- pour la configuration 2,1,1,0
loc loc_2110: while hINOS <= dpINOS2110 &  hTGFB <= dpTGFB2110 wait
{dhIDO=0,dhCOX2=0,dhINOS=1,dhTGFB=1}
--when hINOS=dpINOS2110 do {hINOS'=0,k'=k+1} goto loc_2120;
when hTGFB=dpTGFB2110 do {hTGFB'=0,k'=k+1} goto loc_2111;
```

```
-- pour la configuration 2,1,1,1
loc loc_2111: while True wait {dhIDO=0,dhCOX2=0,dhINOS=0,dhTGFB=0}

-- pour la configuration 2,1,2,0
loc loc_2120: while hINOS >= dnINOS2120 &  hTGFB <= dpTGFB2120 wait
{dhIDO=0,dhCOX2=0,dhINOS=-1,dhTGFB=1}
--2when hINOS=dnINOS2120 do {hINOS'=0,k'=k+1} goto loc_2110;
when hTGFB=dpTGFB2120  do {hTGFB'=0,k'=k+1} goto loc_2121;

-- pour la configuration 2,1,2,1
loc loc_2121: while hINOS >= dnINOS2121 wait {dhIDO=0,dhCOX2=0,dhINOS=-
1,dhTGFB=0}
when hINOS=dnINOS2121 do {hINOS'=0,k'=k+1} goto loc_2111;
end
var
 init_reg, acces : region;
init_reg := loc[auto] = loc_0000 & hINOS=0 & hIDO=0  & hCOX2=0 &  hTGFB =0;

acces:= hide k, n in post(post(post(post(post(post(init_reg))))))) endhide;
print  hide hCOX2,hINOS,hIDO,hTGFB in acces  endhide;

acces:= hide k, n in post(post(post(init_reg & k=n) & ~k=n)) endhide;
init_reg:=hull(acces) & init_reg;
print  hide hCOX2,hINOS,hIDO,hTGFB in init_reg endhide;
acces:= hide k, n in post(post(post(init_reg & k=n) & ~k=n)) endhide;

init_reg:=hull(acces) & init_reg;
print  hide hCOX2,hINOS,hIDO,hTGFB in init_reg endhide;
```

Analysis Commands For Invarianve Kernel

```
--r_ini:= loc[auto] = loc_0010 & hINOS=0 & hCOX2=0 & hIDO>=0 & hIDO <=
dpIDO0010 & hTGFB>=0 & hTGFB <= dpTGFB0010;
r_ini:= loc[auto] = loc_0010 & hIDO>=0 & hIDO <= dpIDO0010 & hTGFB>=0 &
hTGFB <= dpTGFB0010;

r_new :=hide k,n in hull (post(r_ini & k=n) & ~k=n) endhide;
r_old :=r_ini & ~r_ini;

while not empty(r_new) and empty(r_new & r_ini) do
r_old:=r_new;
r_new:=hide k,n in hull(post(r_new & k=n) & ~k=n) endhide;
endwhile;
-- To verify that the initial zone is accessible from itself
if not empty (r_new & r_ini) then
-- if accessible
r_acc:=hide k,n in hull(post(r_new & k=n) &~k=n) endhide;
r_old:=r_ini & ~r_ini; --empty region initialization
while not empty(r_acc) and not r_new<=r_old do
r_old:=r_new;
while not empty(r_acc) and empty(r_acc & r_ini) do
r_acc:= hide k,n in hull(post(r_acc & k=n) &~k=n) endhide;
endwhile;
r_acc:=hull(r_acc & r_ini);
r_new:=hull(r_acc & r_new);
r_acc:=hide k,n in hull(post(r_new & k=n) & ~k=n) endhide;
endwhile;
if not empty(r_new) then
prints "==========================================================";
prints "Constrained region of the Invariance Kernel in the zone:";
--print hide h in r_new endhide;
prints "==========================================================";
prints "==========================================================";
prints "Delay constraintes:";
print  hide hIDO,hCOX2,hINOS,hTGFB in r_new endhide;
prints "==========================================================";
else
prints "Invariance kernel does not exist from the initial region";
endif;
else
-- if not accessible
prints "The initial region is not accessible from itself hence";
prints "there is no initial condition that leads to an invariance kernel.";
endif;
```