# INVESTIGATING THE INTERACTIONS BETWEEN CYTOKINES INTERLEUKIN-1 AND INTERLEUKIN-6

A Senior Scholars Thesis

by

MITHIL CHOKSHI

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

UNDERGRADUATE RESEARCH SCHOLAR

April 2010

Major: Biomedical Engineering

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Approved by:

Research Advisor: Arul Jayaraman
Associate Dean for Undergraduate Research: Robert C. Webb

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# ABSTRACT

Investigating the Interactions between Cytokines Interleukin-1 and Interleukin-6.
(April 2010)

Mithil Chokshi
Department of Biomedical Engineering
Texas A&M University

Research Advisor: Dr. Arul Jayaraman
Department of Chemical Engineering

The purpose of this research project is to study the signaling pathways of the inflammatory cytokines – Interleukin-1 (IL-1) and Interleukin-6 (IL-6) – and investigate any "cross-talk" between the two. IL-6 plays a central role in the Non-Alcoholic Fatty Liver Disease (NAFLD) and we hypothesize that the progression of NAFLD is caused by the imbalance in downstream transcription factor activity of IL-1 and IL-6. Thus, investigating the possible cross-talk would be beneficial in assessing this imbalance. According to the previous work done by the research group, green fluorescent protein (GFP) reporter plasmids with STAT3 and C/EBPβ transcription factor binding sequences were transfected into human hepatocarcinoma (HepG2) cells. Once they were stimulated with cytokines IL-6 or IL-10, they were analyzed with fluorescence microscopy which showed that the transcription factors STAT3 and C/EBPβ were activated leading to a corresponding expression of GFP. This research project will be focused on the dynamics of IL-6 and the effects of IL-1 (instead of IL-10) on it. IL-1 and IL-6 are both involved in many inflammatory responses of the body and by having a deeper understanding about their pathways to initiation of the inflammatory responses

and any interconnections will help us model a feedback system to control the response. The findings will assist in finding a therapeutic approach to mitigate various inflammatory diseases where IL-1 and IL-6 play a major role in (*e.g.* NAFLD). In addition, these results will also be useful in bioengineering. Inflammatory response of the body towards any bioengineered implants mediated by these cytokines could be controlled on a molecular level. Thus, instead of wasting thousands of dollars on coming up with a new design, bioengineers can manipulate the body's response and use the device that was formerly rejected by the body. Research will be conducted to 1) quantify the transcription factor activity, 2) study the dynamics of the transcription factor activity under varying conditions, and 3) isolate the pathways and monitor the effects of the cytokines on each of pathway. This approach, which is based on Systems Biology, is about putting together rather than taking apart, integration rather than reduction. Thus, once we have researched the above three factors, we can put them together to hypothesize and test the cross-talk.

# DEDICATION

To my parents, who have always supported me in all my endeavors

# ACKNOWLEDGMENTS

The cooperation and the contribution of the graduate students, Colby Moya and Zuyi Huang, the undergraduate student, Cheng Peng, and the guidance of Dr. Arul Jayaraman are greatly appreciated. The teachings on the principles of microscope and the right way to use it received from Tarun Bansal are also appreciated. The distinguished facility at the Jack E. Brown Engineering building, Texas A&M University, and the help of the staff working to preserve it have an equal contribution.

The researcher was awarded the following for this research:

1) 1st place for International Student Diversity Undergraduate Research Award
2) 2nd place for Microbiology Taxonomy award
3) Undergraduate research scholar presenter recognition

at the *13th Annual Student Research Week* at Texas A&M University.

# NOMENCLATURE

| | |
|---|---|
| C/EBPβ | CCAAT Enhancer Binding Protein-Beta |
| ERK | Extracellular-signal Related Kinase |
| GDP/GTP | Guanosine Diphosphate/ Guanosine Triphosphate |
| GFP | Green Fluorescence Protein |
| GP130 | Glycoprotein 130 |
| Hr | Hour |
| HepG2 | Human Hepatocellular Carcinoma (cell line) |
| IL-1 | Interleukin 1 |
| IL-6 | Interleukin 6 |
| JAK | Janus Kinase |
| JNK | c-Jun N-Terminal Kinase |
| MEK | Map-Erk Kinase |
| NAFLD | Non-Alcoholic Fatty Liver Disease |
| NF-IL6 | C/EBPβ |
| OSM | Oncostatin M |
| Raf-1 | Proto-Oncogene Serine/Threonine-Protein Kinase |
| SOS-1 | Son of Sevenless Homolog-1 Protein |
| STAT3 | Signal Transducer and Activator of Transcription-3 |
| TF | Transcription Factor |

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# CHAPTER I

# INTRODUCTION

The main purpose of this research would be to study the pathways to inflammation regulated by IL-6 and to investigate the effects of the cytokine IL-1 on it (interactions between IL-1 and IL-6) by measuring the transcription factor activity associated GFP expression. IL-1 is a pro-inflammatory cytokine and IL-6 is a pleiotropic cytokine that has both pro- and anti-inflammatory actions. Inflammation is the response of the tissue to injury and is characterized in the acute phase by increased blood flow and vascular permeability along with the accumulation of fluid, leukocytes, and inflammatory mediators such as cytokines **[1]**. One of the purposes of researching IL-6 is that, it is responsible for signaling in the Non-alcoholic Fatty Liver Disease. IL-6-type cytokines IL-6, IL-11, LIF (leukemia inhibitory factor), OSM (oncostatin M), ciliary neurotrophic factor, cardiotrophin-1 and cardiotrophin-like cytokines are an important family of mediators involved in the regulation of the acute-phase response to injury and infection **[2]**. The acute phase response up- or down-regulates the expression of certain plasma proteins that take part in the body's response to the injury-induced inflammation **[3]**. IL-6 is responsible for inflammation of liver on injury, or as in this case, for accumulation of fat.

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This thesis follows the style of BMC Systems Biology.

The importance of this research is that if one is able to figure out a link between IL-6 and IL-1 signaling pathways, then one can indirectly alter the IL-6 pathway, simultaneously altering the IL-1 pathway, and thus control the unwanted inflammatory responses of the body. These findings will help to find a solution to inhibit the triggers to several inflammatory diseases wherein, IL-1 and IL-6 play an important role leading to inflammation. According to the *Nutrition Review*, since this early study, a long list of pro-inflammatory cytokines has been shown capable of stimulating osteoclastic bone resorption including interleukin (IL)-1, tumor necrosis factor alpha (TNF-α), IL-6, IL-11, IL-15, and IL-17 **[1]**. Systemic inflammatory mediators, such as the cytokines interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor (TNF) … play an important role in the induction of the inflammatory acute phase response (APR) in the liver upon trauma or injury **[4]**. Thus, IL-1 and IL-6 mediate important inflammatory responses at various places in the body and isolating the pathways to inflammation would be a valuable progress towards understanding and further manipulating these responses.

Detailed description of the IL-6 pathways is out of the scope of this paper, but a brief description of it is as follows (See Figure 1). IL-6-type cytokines exert their action via the signal transducers gp (glycoprotein) 130, LIF receptor and OSM receptor leading to the activation of the JAK/STAT (Janus kinase/signal transducer and activator of transcription) and MAPK (mitogen-activated protein kinase) cascades **[5]**. Then it leads to the transcription of STAT3 and C/EBPβ.

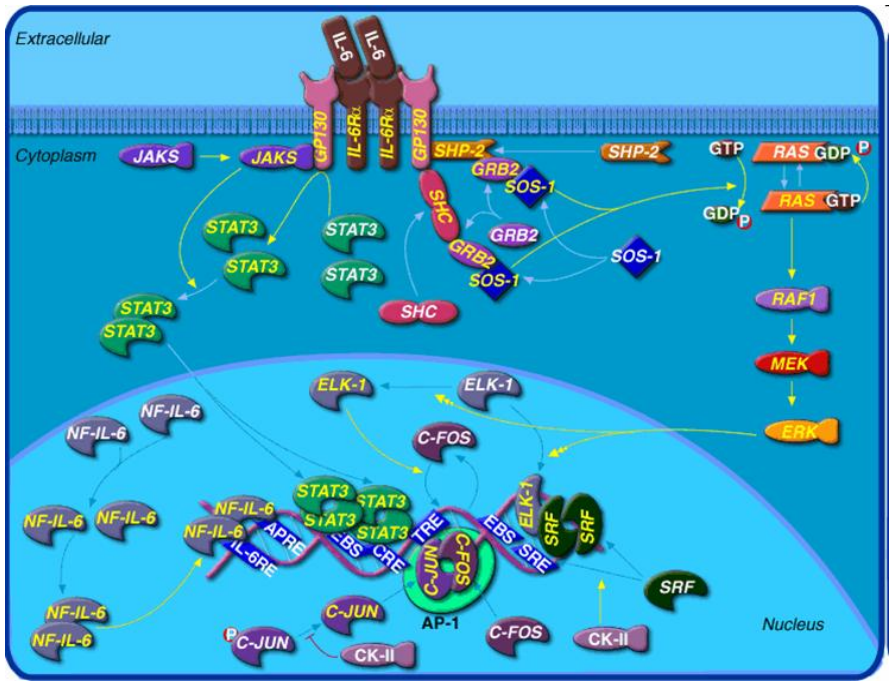



**Figure 1:** IL-6 signaling pathway**[6]**.

Interleukin-1 (IL-1) is a pro-inflammatory cytokine that has several effects in the inflammation process. When it binds to its cell-surface receptor, IL-1 initiates a signaling cascade that leads to activation of the transcription factor NF-kB and is relayed through the protein TRAF6 and a succession of kinase enzymes, including NF-kB-inducing kinase (NIK) and IkB kinases (IKKs) **[7]**.

**Project description**

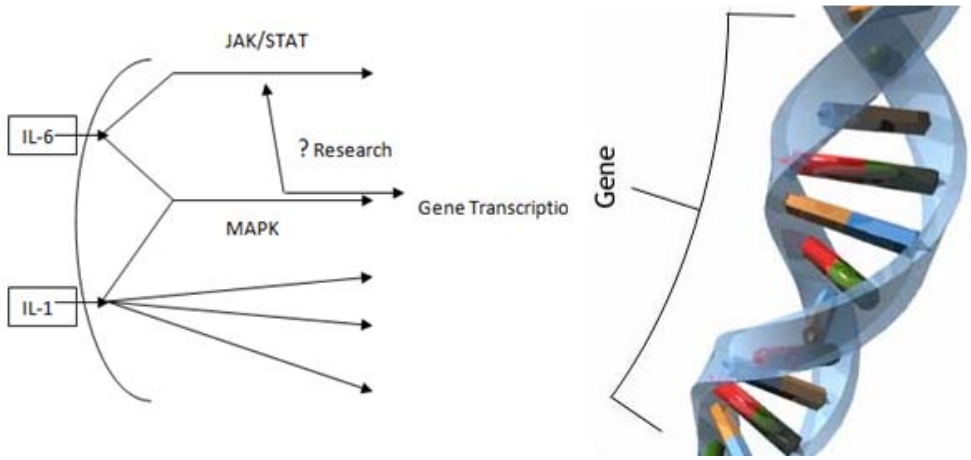



**Figure 2:** Simplified version of what this research is about. Gene product picture, courtesy of Google **[8]**.

Based on the details of the pathways, we have hypothesized that since both the signaling cascades have the mitogen activating protein kinase (MAPK) as an intermediate in one of their pathways, there is a possibility that we can find a way to control MAPK pathway in IL-6 with the help of IL-1. This will be done by stimulating the HepG2 cell line with IL-1 and IL-6 and then analyzing the intensity of the fluorescence to calculate the amount of gene expression under different conditions (concentration, time duration etc.). These conditions would be changed time-to-time and is expected to show some significant changes in gene expression. These changes would be further analyzed with the help of mathematical models used by the research group in order to find the dominant conditions which lead to the most expression. This is just one of the many possibilities that this research will explore. (See Figure 2). “Cross-talk” between IL-1 and IL-6 is very possible because these two cytokines are coexpressed at many sites of

inflammation **[9,10,11]**. The evidence presented herein demonstrates that … IL-1 and TNFα modulate activation of the Jak-STAT signaling pathway by the pleiotropic cytokines related to IL-6 that use the gp130 signaling receptor subunit **[12]**. According to few other studies conducted on various interactions between many pro- and anti-inflammatory cytokines in the body, pro-inflammatory cytokines are able to suppress the anti-inflammatory cytokines at the level of signal transduction. One such model based on the research published in *journal of immunology* is depicted in Figure 3 below, and it shows the inhibition of activation of Stat3 genes by one of the pathways of IL-1 signal transduction cascade. However, many of these inhibitory effects on signal transduction are cell-type specific…**[12],** so this model is not necessarily true for this research.

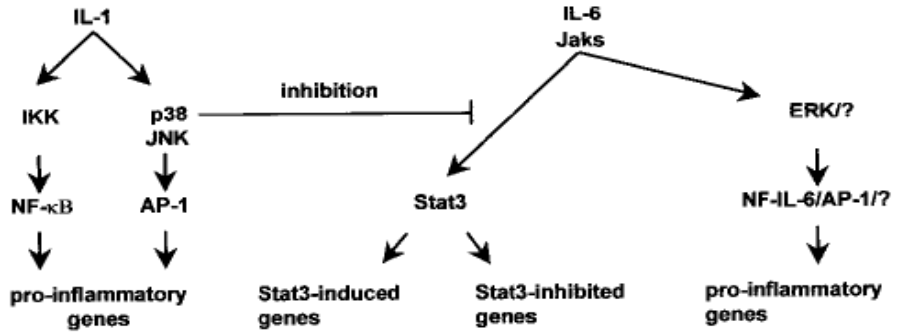



**Figure 3:** Model for the complex interactions of IL-1 and IL-6. IL-1 blocks Stat3 activation and thus inhibits expression of genes that are strongly dependent on Stat3 for expression. In contrast, genes that are repressed by Stat3 would be expressed to a higher level in the presence of IL-1. In addition, IL-6 signaling pathways other than the STAT pathway may not be suppressed by p38 and may work together with IL-1 signaling pathways to coactivate certain genes **[12]**.

# CHAPTER II

# METHODS

This chapter of the research is dedicated to the development of the reporter system and the fluorescence microscopy method to measure fluorescence. This fluorescence was then analyzed by the MATLAB programs in order to quantify it and determine the dynamics of transcription factor activity over time.

**Principle of GFP reporter system**

As shown in Figure 4 below, a low half-life (2hr) GFP was cloned into a plasmid under the control of a minimal promoter ($CMV_{min}$). When a TF bound to its upstream recognition sequence, the promoter was activated and GFP was expressed.

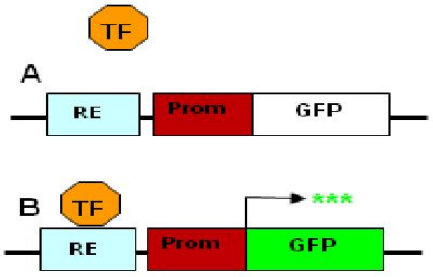



**Figure 4**: Principle of GFP reporter systems.

**Reporter cell line generation**

Reporter plasmids for monitoring activation of STAT3 and C/EBPβ were introduced into HepG2 cells by electroporation. Approximately 5 x $10^6$ cells were mixed with 10 μg of linearized plasmid (pSTAT3-d2egfp or pC/EBPβ-d2egfp) in a pre-chilled sterile 4-mm electroporation cuvette and electroporated at 240V and 950 μF. Growth media (600 μL) was immediately added to the cells after electroporation and allowed to sit for 10 min at room temperature. The mixture was transferred to a 10 cm tissue culture dish and incubated for 2 days prior to addition of 800 μg/mL of G418 as selection pressure. Surviving colonies (i.e., cells with the STAT3 or C/EBPβ reporter plasmid integrated into their genome) were selected and screened for induction of STAT3 or C/EBPβ activity after 3 weeks.

**Fluorescence microscopy**

HepG2 reporter cell lines for STAT3 and C/EBPβ were seeded in 24-well plates and exposed to different concentrations of the cytokines. The cell culture plate was placed on the incubated stage of a Zeiss Axiovert 200M fluorescence microscope where constant temperature and $CO_2$ levels of 37 □ C and 5% respectively, were maintained for HepG2 cells. Phase and fluorescence images were taken every 45 minutes for 24 hrs. Fluorescence images were analyzed by MATLAB to obtain the dynamic profiles of STAT3 and C/EBPβ activation and then compared with a model derived from the TF

calculation. The schematic on page 8 explains the signal transduction model that is followed for the experiments performed in this research.

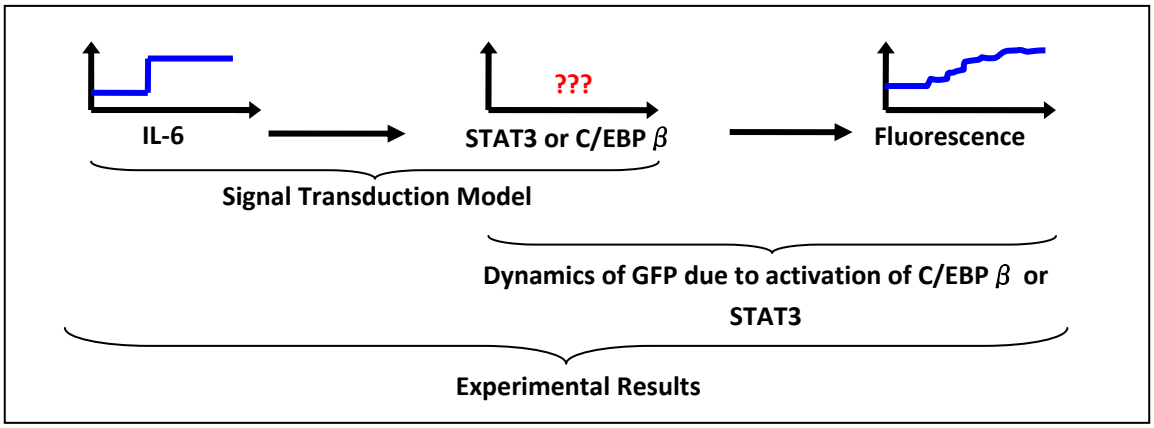



**Figure 5:** Scheme to calculate TF data from GFP data: Relationship between input, output, and concentration of transcription factors with GFP-reporter systems.

The above mentioned model in Figure 5 has been used for all the experiments thus far, with the different cytokines used – IL1, IL6, and OSM. Within five minutes after the stimulation of the cells in the plate, the plate is staged in the incubated chamber of the microscope. A PH2 condenser is used with the 20x lens used to image the cells. The images are focused and saved and then refocused and saved again after 20 minutes and the experiment is setup to run for 24hr period. The microscope exposes the cells to the normal transmitted light and also to UV light (blue) at the saved positions and it does this to each saved position for thirty-three times over the twenty-four hour period. If the signaling pathway is stimulated, the GFP is expressed and a picture of this expression is saved with every exposure. This pictorial data is then fed into the MATLAB program

which analyzes, quantifies, and draws a profile of expression like depicted in graphs on page 15.

**Gel electrophoresis and immunoblotting**

In this part of the project, the actual *cross-talk* proposed thus far was tested. Literature for cytokine research has shown that, the C/EBPβ pathway is inhibited with pharmacological inhibitors like U0126 **[13]**. U0126 was added at different time points to the cells and then the cells were lysed with a lysate and the protein inside it was fractionated and then a gel electrophoresis was ran on it in order to determine the presence of C/EBPβ in the cells. According to the hypothesis, there should be no C/EBPβ present in the cells but it was not entirely true in this case. The band for C/EBPβ was still present in the final image, but the expression was lower than usually seen without U0126. This led to further three hypotheses: 1) There is a possibility that the U0126 is not as effective and potent for HepG2 cells; 2) The C/EBPβ activity is inhibited but there can be some other intermolecular event leading to a change in conformation that is making the U0126 less effective; 3) There is some unknown factor, in the serum that is activating the pathway. In order to test these considerations, it was decided to use a different inhibitor; to speculate other possible intermolecular events that would explain this behavior; and to image the cells in a serum free media in order to know any effects that the serum might have on U0126 during which, it is effective. The runs for gel electrophoresis are shown in figure on page 20.

# CHAPTER III

## PRELIMINARY RESULTS AND DISCUSSION

In order to correctly understand the working of the microscope that the Jayaraman group possesses, the group was given a special lecture on the proper usage of the microscope and the proper ways image culture cells. The different techniques that are necessary to acquire optimum images of the cell culture, including Kohler Illumination, was taught and practiced. The designed and planned experiments were then carried out every time using the established standard protocol for the microscope use.

In order to first test the methodology of using the fluorescence microscopy and the experiment design, a test experiment of stimulation was performed on the Hela cells stimulated with Doxycyclin. They were used since HepG2 tend to grow in clumps while one can see individual cells with Hela cells; also, cytokines are mighty expensive when compared to Doxycyclin. The cells were stimulated with Doxycyclin, commonly used for treating various resistant infections. The resultant expression is shown in Figure 7. Three different concentrations of Doxycyclin were used – 1ng/mL, 10ng/mL, 100ng/mL – per well for each well in a 24 well plate (only 12 wells were used in this case). (See Figure 6).

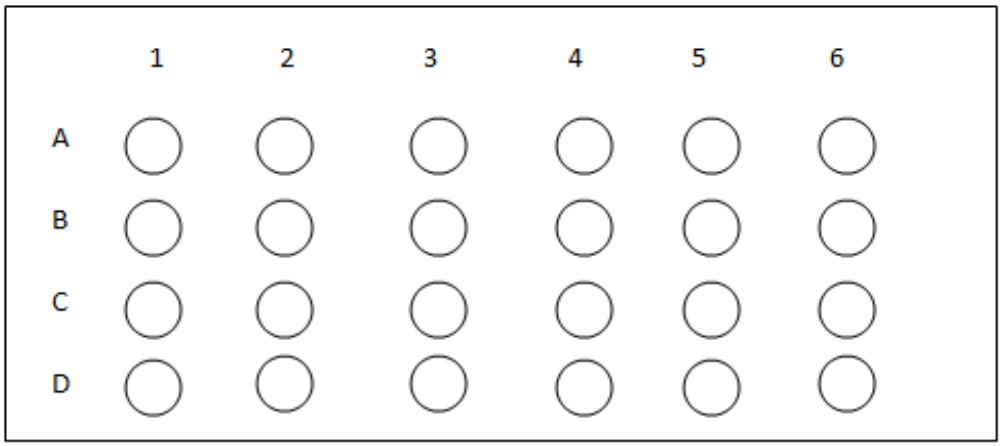
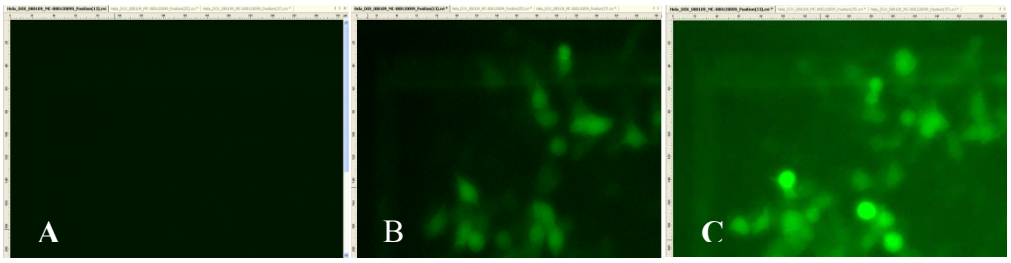



**Figure 6**: This figure shows the 24 well plate used for the experiments throughout this research project. Sometimes only 12 wells are used instead all the 24 wells. An example experimental setup will be 12 wells – A1 to A3, B1 to B3, C1 to C3, and D1 to D3 – of the plate containing Hela cells, and Doxicyclin added to B1 to B3 at 1ng/mL, C1 to C3 at 10ng/mL, and to D1 to D3 at 100ng/mL, while A1 to A3 are control.

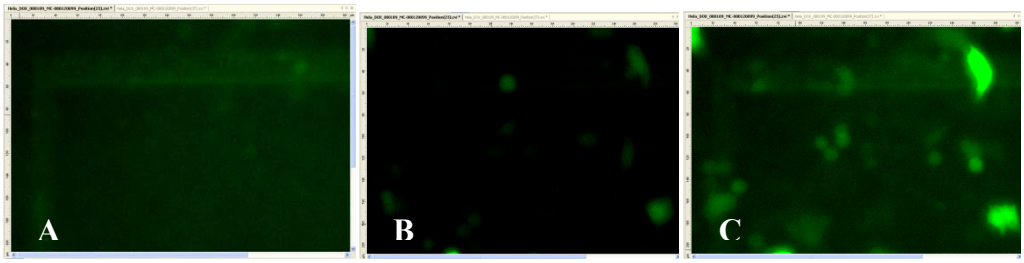
The cells are fed with the Hela media every 48 hours and after an approximate confluence of 90%, the plate is ready for the experiment. 24 hours prior to an experiment, the cells are fed again. Approximately 5-8 minutes before the experiment, the cells in the plate are stimulated with the Doxycyclin and then used for the experiment. The experiment is run for 20 to 24 hours during which the microscope acquires phase contrast and fluorescence images of the cells. The fluorescence images are then analyzed by the MATLAB program written by the graduate students. This MATLAB program gives us the kinetics of the transcription factor activity related expression of GFP over the duration of the experiment.

Fluorescence images observed over a period of 24hrs following the stimulation:

1ng/mL




10ng/mL




100ng/mL

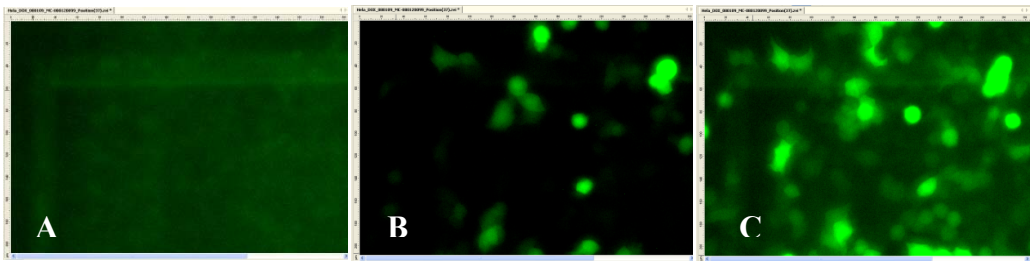



**Figure 7**: The expression resulting from the stimulation of the Hela cells with Doxicyclin. A represents the image at 0 hr, B after 11 hrs, and C after 24 hrs.

Since the kinetics, the graphs of fluorescence versus time, of the Hela with Doxycyclin are of no significant importance to this research, they are not reported.The exact same procedure was followed to investigate the kinetics of the HepG2 cells when stimulated with cytokines OSM, and IL1. The experiment with the HepG2-C/EBPβ with OSM

stimulation was performed twice; the experiment with the HepG2-C/EBPβ with IL1 stimulation was performed thrice; the experiment on HepG2-STAT3 with IL1 stimulation was performed once.

The experimental setup is shown in Figure 8 below. The microscope also has an incubated chamber where the temperature and carbon-dioxide concentration is maintained throughout the experiment.

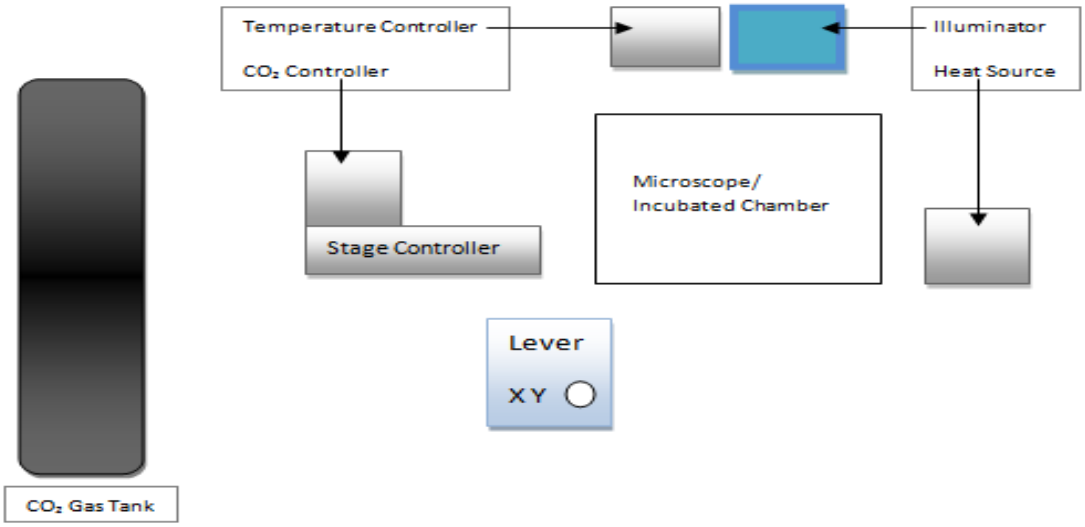



**Figure 8**: The schematic of the experiment facility. The plate is staged inside the incubated chamber.

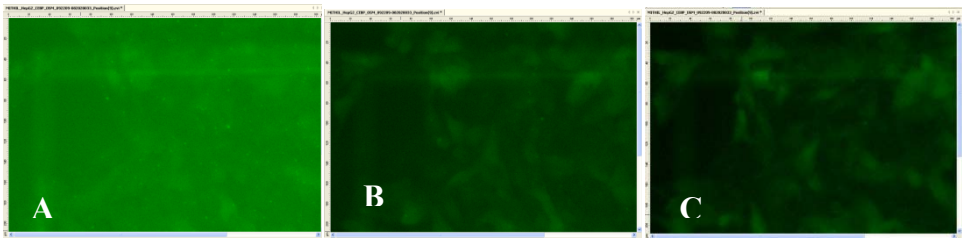
**HepG2 – STAT3 and C/EBPβ – cytokine stimulation**

The HepG2-C/EBPβ were stimulated with OSM at 1ng/mL, 10ng/mL, and 100ng/mL dilutions in a 24 well plate followed by an experiment. The HepG2-C/EBPβ were also stimulated with IL1 at 1ng/mL, 10ng/mL, and 100ng/mL dilutions in a 24 well plate followed by an experiment. In order to observe any change in kinetics, three other

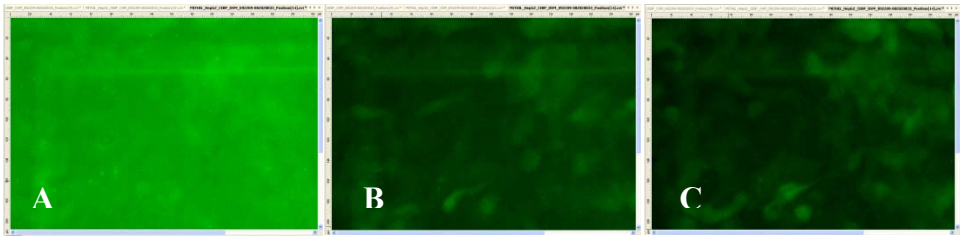
concentrations – 0.5ng/mL, 0.05ng/mL, and 0.01ng/mL – of the cytokines were also used but the resultant intensity does not vary by an appreciable amount. Finally, HepG2-STAT3 were stimulated with three different concentrations – 1ng/mL, 10ng/mL, and 100ng/mL – of IL1. Figure 9 below shows the fluorescence images and the profile of the kinetics followed with different stimulations.

Fluorescence images observed over a period of 24hrs following the stimulation:

1ng/mL




10ng/mL




100ng/mL

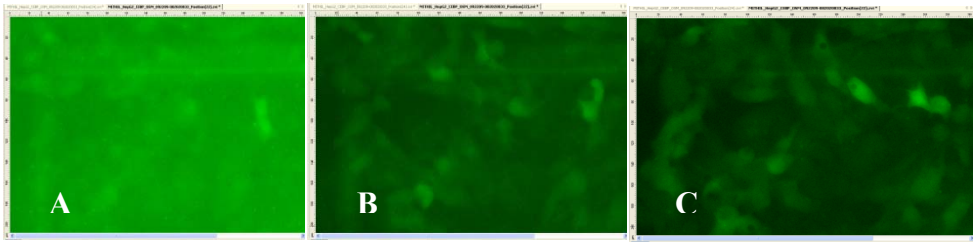



**Figure 9**: The expression resulting from the stimulation of the HepG2 cells with IL1. A represents the image at 0 hr, B after approximately 12 hrs, and C after 24 hrs.

Dynamic profile of the transcription factor activity:

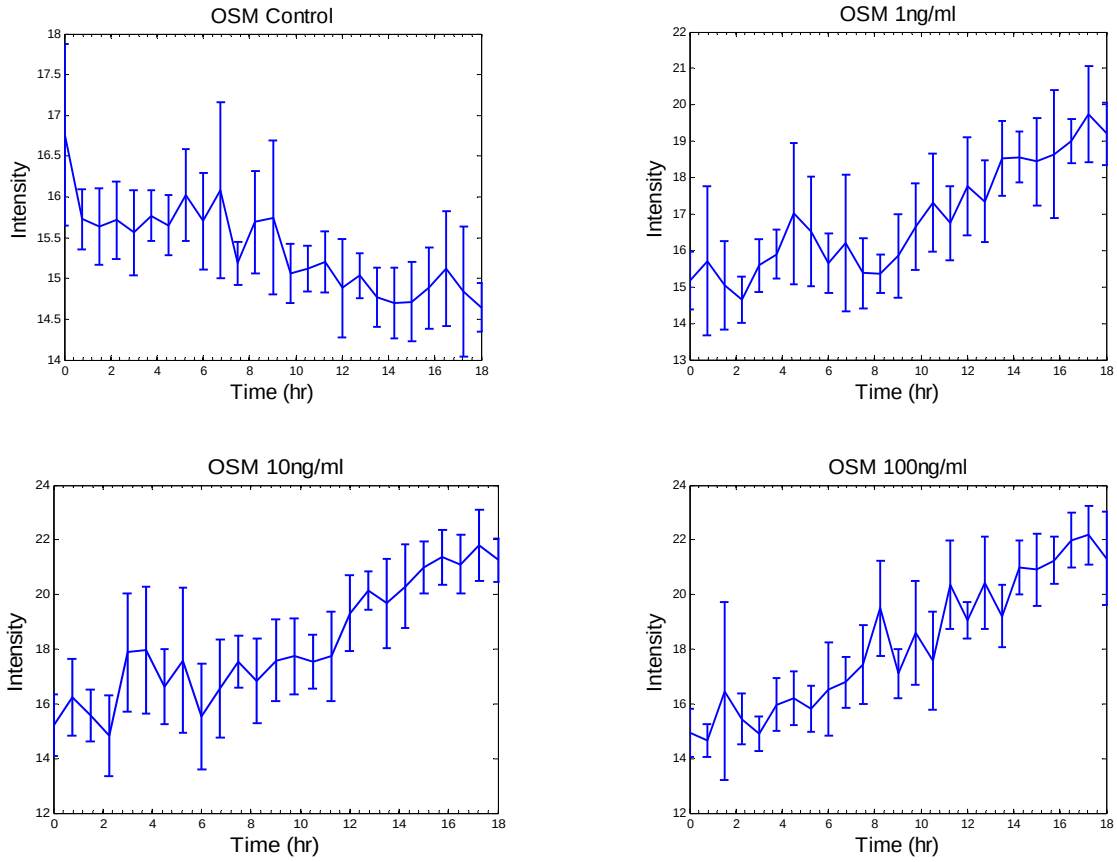



**Figure 10**: The fluorescence images and the profiles shown above display the kinetics of the experiment on HepG2-C/EBPβ with OSM.

When comparing the above shown dynamics (see Figure 10) to the model described before in Figure 5, one may notice that the theoretical model and the experimental results are in excellent agreement with each other. Thus, we have developed HepG2 reporter cell line with which we can isolate each of the pathways initiated by the cytokines IL-1 and OSM, and we can quantify the transcription factor activity with it.

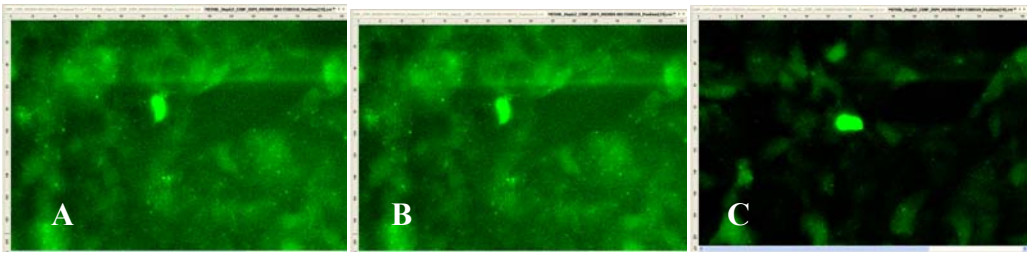
This gives a powerful tool to develop a feedback system that will help model the activity of any transcription factor related to any cytokine.

The kinetics of HepG2 C/EBPβ with IL-1 were also plotted after the fluorescence microscopy experiment conducted on it. Figure 11 shows the profile of GFP expression over the duration of the experiment for HepG2 C/EBPβ when stimulated with IL-1. The transcription factor activity increased steadily with time and after about eleven hours, it stabilized. The graph also shows error bars on it and one can notice that the maximum error for this experiment was less than 5 units of intensity at any given time point. In order to see if there is any change in the transcription factor activity kinetics, two additional 24hr experiments were conducted to monitor the expression when stimulated with 0.01, 0.05ng/mL, and 0.5ng/mL of cytokine IL1. However, as one can see from the profiles on page 18, there was no significant change in the profile, and thus the activity of transcription factor. (See Figure 12).

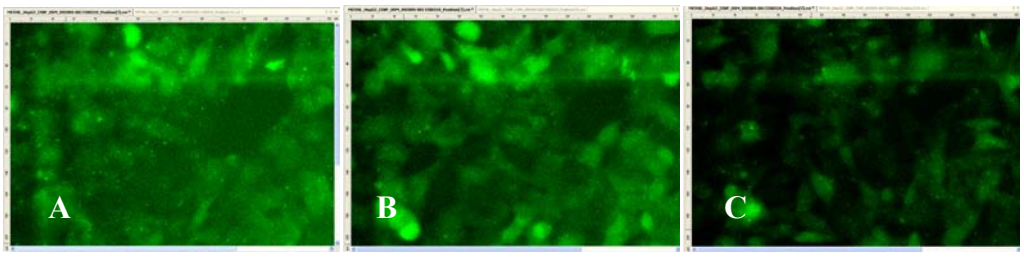
The expression kinetics correspond to the transcription factor activity explained in the Chapter II.

Fluorescence images observed over a period of 24hrs following the stimulation:

1ng/mL




10ng/mL




100ng/mL

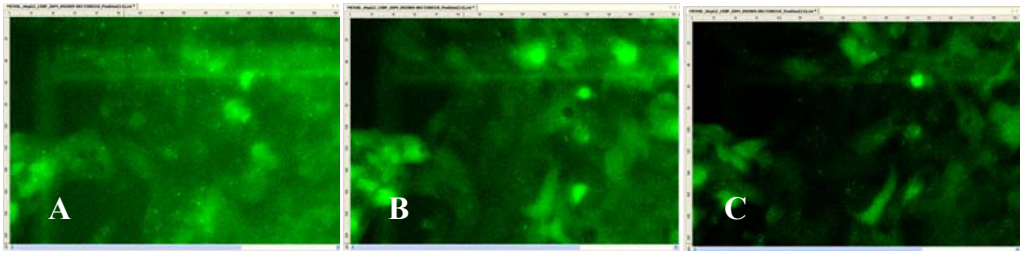



**Figure 11**: The expression resulting from the stimulation of the HepG2-C/EBPβ cells with IL1. A represents the image at 0 hr, B after approximately 12 hrs, and C after 24 hrs.

Dynamic profile of the transcription factor activity:

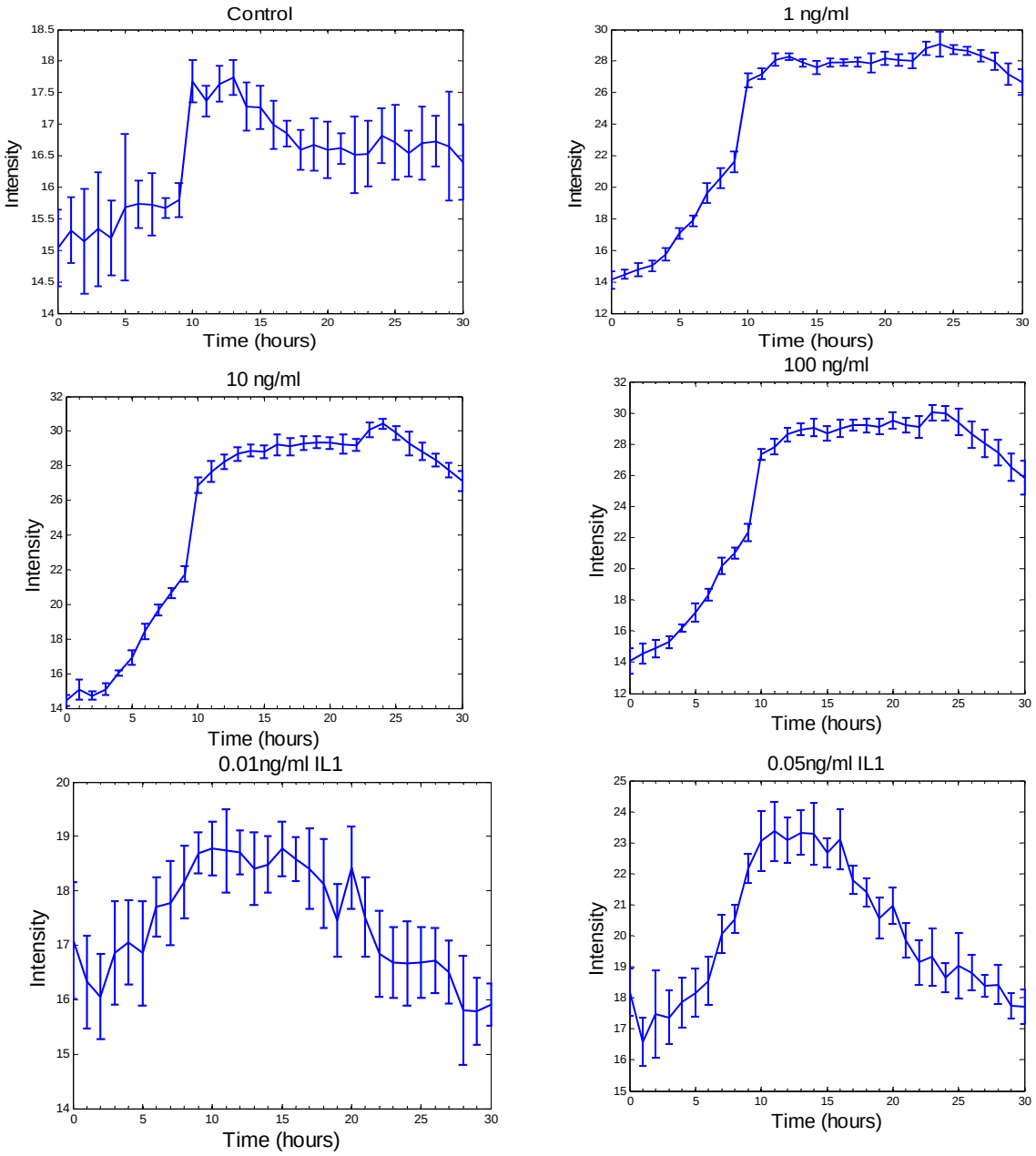



**Figure 12**: The kinetics followed by the experiment with HepG2-C/EBPβ and IL1 at different concentrations. This data set shows less than one unit of error in intensity and represents the best data our experiments have produced.

**U0126 stimulation**

Plate 1:

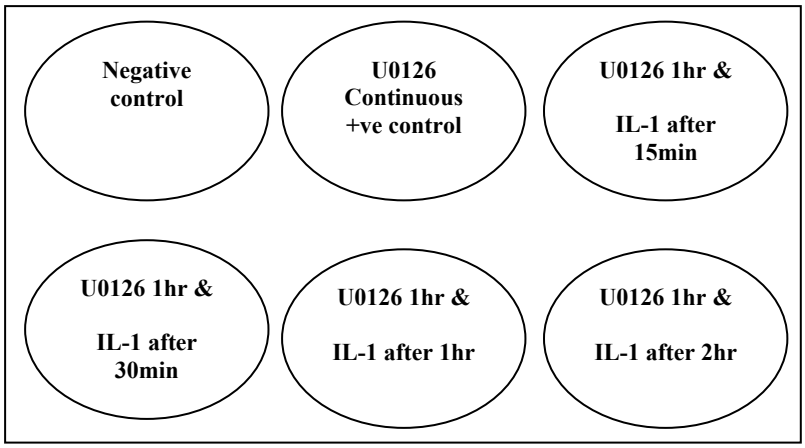



Plate 2:

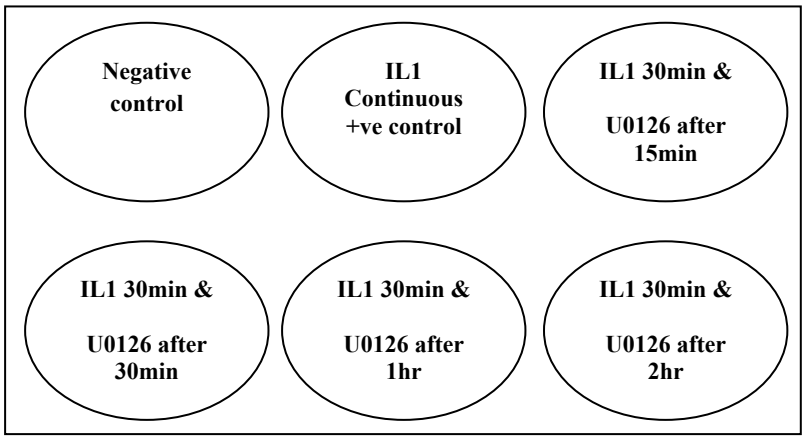



**Figure 13**: Conditions for gel electrophoresis runs.

Gel Electrophoresis Images:

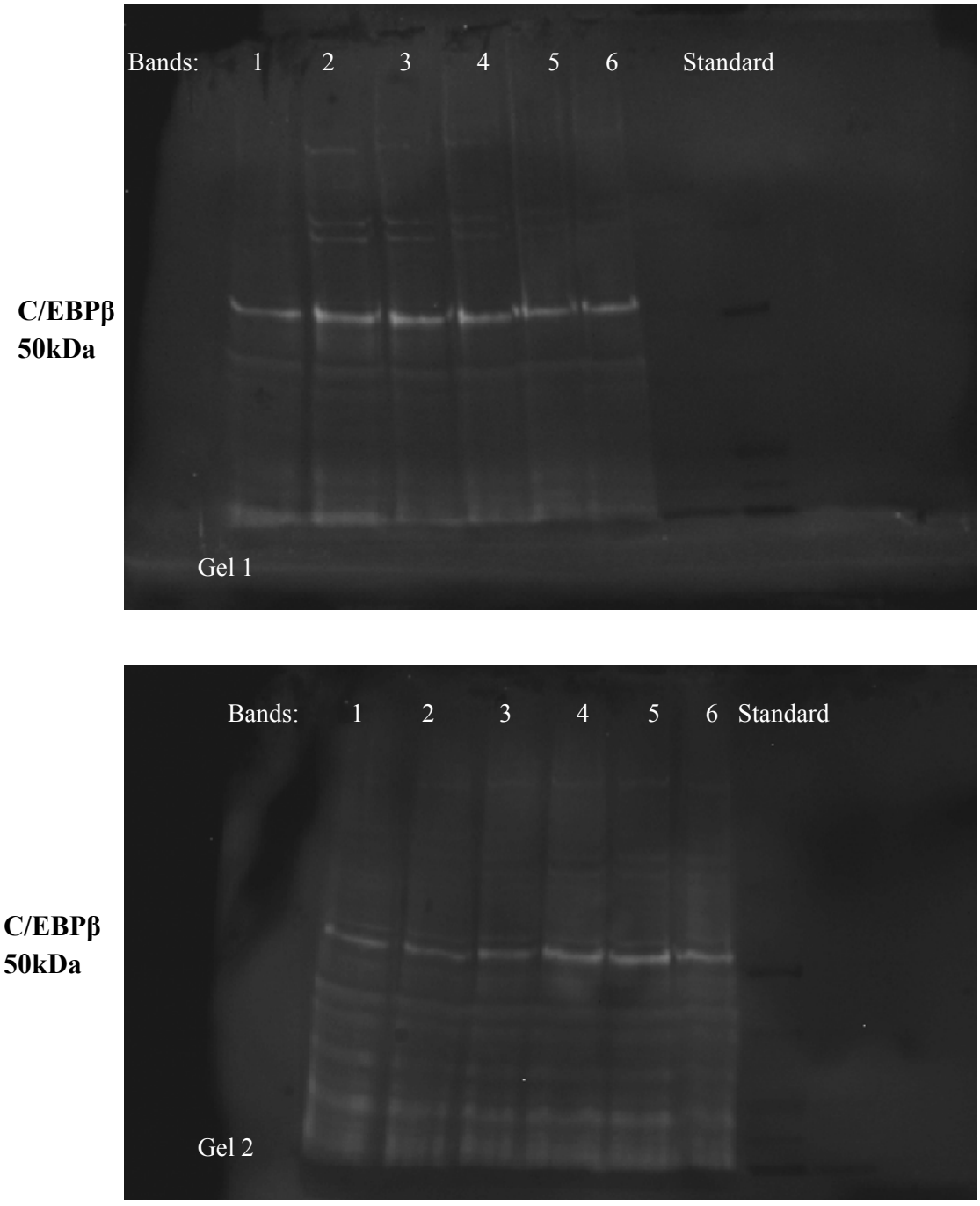



**Figure 14**: The gel electrophoresis runs are shown in this figure.

There were two gels that were run for testing the hypothesis of knocking down the expression of the C/EBPβ pathway with the help of U0126. However, because of the before speculated reasons, U0126 did not completely knock down the expression, which can be seen from gel electrophoresis images. (See Figures 13 and 14). The expression should have increased as usual for about 10hrs and then completely knocked down to zero. Instead we see that the expression was lowered to a certain extent.

Fluorescence images observed over a period of 24hrs following the stimulation:

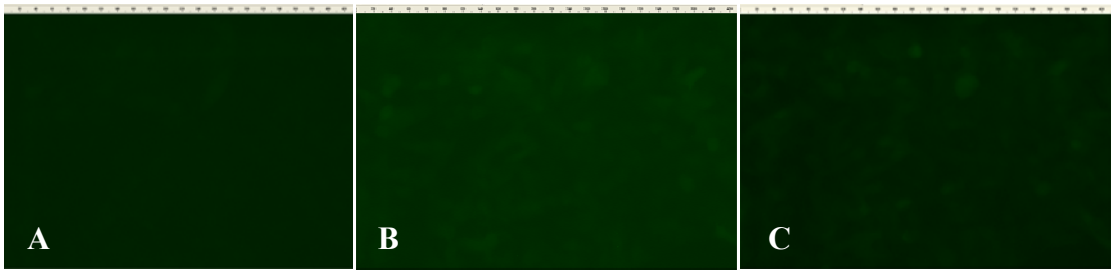



**Figure 15**: This figure shows fluorescence of C/EBPβ with IL-1 and U0126 over 24hrs.

In order to verify the Gel runs with U0126, a fluorescence microscopy experiment was conducted with C/EBPβ stimulated with U0126 present simultaneously with IL-1. (See Figure 15). The expected output would be zero fluorescence over the duration of the experiment but as seen from the Figure 15 above, there was some fluorescence after 10 and 24 hour period. This is also what is seen from the Figure 14, which shows the C/EBPβ activity still present even though the cells were stimulated with U0126 but to a lesser extent. This expression can be compared with the expression in Figure 11, which was the same stimulation, but without U0126.

**Raf-1 involvement**

Recent studies conducted with HepG2 cells for the C/EBPβ pathway has revealed other ways by which the pathway gets activated. Of 71 apoptosis-associated genes, *c-raf*-1 and *S6* were up-regulated in 42.9% and 32.1% of 28 cirrhosis tissues, respectively, and both genes were well correlated in a five-cluster *K*-means analysis. For *c-raf*-1 and downstream genes in the MAPK pathway, *c-raf*-1, *MEK*, and *MAPK* were up-regulated in 40%, 80%, and 86.7% of 45 cirrhosis specimens, respectively, and in 50%, 63.6%, and 59.1% of 22 hepatocellular carcinoma specimens, respectively **[14]**. This means that everytime the Raf-1 was up-regulated, it activated the C/EBPβ pathway again that leads to accumulation of fat, leading to more inflammation and ultimately to cirrhosis.

The other explanation that we hypothesis that there is an unknown factor in the serum, that is activating the MEK, leading to the activation of C/EBPβ. The next part of the research is to investigate these effects.

# CHAPTER IV

# CONCLUSIONS

The results from the fluorescence microscopy of the individual pathways has shown that with the reporter cell line generated for the purpose of this project, the transcription factor activity can be quantified. IL-6 and OSM have been shown to activate the C/EBPβ and STAT3 pathways and IL-1 has been shown to activate the C/EBPβ pathway. The expression of GFP (and hence, the activation of transcription factors) correlated with the specificity of the cytokine used. The dynamic profiles of the STAT3 and C/EBPβ activations obtained from the fluorescence images matched with the model derived from the TF calculation.

Thus, the proposed model and the experimental results have been shown to be in excellent agreement with the experimental data set and having less than 1unit of random error in the fluorescence intensity. Thus, with this model of transcription factor activity, one can easily take into consideration any transcription factor and do a similar analysis of quantifying the transcription factor activity.

The future work will be conducted on – 1) Test the hypothesis of Raf-1 activating the pathway by monitoring the activation of Raf-1 in HepG2, 2) Test the hypothesis of the unknown factor in serum activating MEK, ultimately leading to fat accumulation, 3)

Using a more potent ERK pathway inhibitor, PD184352, to investigate the imbalance in the transcription factor activity.

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