KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY

UNDERSTANDING THE ROLE OF AFLATOXIN B_1 IN MODULATING THE TYPE I INTERFERON RESPONSE

by

PATRICK WILLIAMS NARKWA

A thesis submitted to the Department of Clinical Microbiology, Kwame Nkrumah University of Science and Technology in partial fulfillment for the degree of

DOCTOR OF PHILOSOPHY (PhD)

School of Medical Sciences
College of Health Sciences
February, 2016

Declaration

I therefore declare that this submission is my own work towards the PhD and that to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any degree of any University, except where due acknowledgment has been made in the text.

PATRICK WILLIAMS NARKWA …… ……..

(Exams Number PG7956512) Signature Date

Certified by:

Dr. MOHAMED MUTOCHELUH …… ……..

(SUPERVISOR) Signature Date

Dr. THEOPHILUS .B. KWOFIE ……. ……..

(HEAD OF DEPARTMENT) Signature Date

Acknowledgements

First of all I would like to give thanks to God the Father who has seen me through from the beginning to the end of this work. My sincerest thanks go to my supervisors Dr. Mohamed Mutocheluh and Prof. David J. Blackbourn (University of Surrey, UK) for their unflinching support, guidance and uncountable contributions and directions throughout the work and its write up. I wish to acknowledge many professionals associated with this work; Dr.

Augustina Sylverken Annan and the entire staff at Kumasi Centre for Collaborative Research (KCCR) for their assistance during the RT-qPCR work. Thanks also go to Mr. Charles Osei for his continual advice and encouragement and the entire staff of the

Department of Clinical Microbiology for their support. I would like to thank Royal Society, UK for providing funds for this work.

To everyone that contributed to this work in one way or the other, I say thank you very much and may God richly bless you all.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M_1
AFM_2	Aflatoxin M_2
BER	Base Excision Repair
cAMP	cyclic Adenosine Monophosphate
CBF	Core-Binding Factor,
CBP	CREB-Binding Protein
CD14	Cluster of Differentiation 14
c-jun/ATF2	c-jun, Activating Transcription Factor 2
CpG motifs	Cytosine-phosphate-Guanine
CREB	cAMP-Responsive Element Binding
CYP450	Cytochrome P450
DBD	DNA Binding Domain
DMEM	Dulbecco's Modified Eagle Medium
dsRBD	double stranded RNA Binding Domain
ECACC	European Collection of Authenticated Cells Cultures
eIF2	Eukaryotic Initiation Factor 2
EIF2AK2	Eukaryotic Initiation Factor 2 Alpha Kinase 2
Epo-R	Erythropoietin Receptor
ETR	Electron Transfer Reagent
FAO	Food and Agriculture Organization
G-CSF	Granulocyte-Colony Stimulating Factor
GH-R	Growth Hormone Receptor
GM-CSFR	Granulocyte Macrophage Colony-Stimulating Factor Receptor
GST	Glutathione-S-transferase
HCC	Hepatocellular carcinoma
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IAD	IRF Association Domain
IARC	International Agency for Research on Cancer
ICS	Interferon Consensus Sequence

ICSBP	Interferon Consensus Sequence-Binding Protein
IFNAR	Interferon Alpha Receptor
IFN-α	Interferon-alpha
IFN-β	Interferon-beta
IL	Interleukin
IRFs	Interferon Regulatory Factor
ISGF3	Interferon Stimulated Gene Factor 3
ISGs	Interferon Stimulated Genes
ISRE	Interferon Stimulated Response Element
Iκ B	Inhibitor of NF-κB
JAK	Janus Kinase
JECFA	Joint Expert Committee on Food Additives
LPS	Lipopolysaccharide
MAL	Myelin And Lymphocyte Protein
MAPK	Mitogen-Activated Protein Kinase
MD2	Myeloid Differentiation protein 2
MDA5	Melanoma Differentiation-Associated gene 5
mEH	microsomal Epoxide Hydrolase
MEM	Minimum Essential Medium
MHC	Major Histocompatibility Complex
Myb	Myeloblastosis
MyD88	Myeloid Differentiation Primary Response gene 88
NADH	Reduced Nicotinamide Adenine Dinucleotide
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
NCTC	National Collection of Type Cultures
NER	Nucleotide Excision Repair
NF-κB	Nuclear Factor kappa B
NK-cells	Natural Killer-cells
NLS	Nuclear Localization Signal
OAS	Oligo Adenylate Synthetase
PAMPS	Pathogen Associated Molecular Patterns
PES	Phenazine Ethosulfate
PHH	Primary Human Hepatocytes
PKR	Protein Kinase R
Prl-R	Prolactin Receptor
PRR	Pattern Recognition Receptors
RIG1	Retinoic acid-Inducible Gene 1
SH2	Src (sarcoma) Homology 2
STAT	Signal Transducer and Activator of Transcription
TICAM1	Toll-interleukin 1 receptor domain (TIR)-Containing Adaptor Molecule 1
TICAM2	Toll-interleukin 1 receptor domain (TIR)-Containing Adaptor Molecule 2

TIR	Toll-IL 1 Receptor
TIRAP	Toll-Interleukin 1 Receptor (TIR) Domain Containing Adaptor Protein
TLR	Toll-like Receptor
TNF-α	Tumour Necrosis Factor-alpha
TP53	Tumour Suppressor 53 gene
TRAM	Toll-like Receptor 4 Adaptor Protein
Trif	TIR-domain-containing adapter-inducing interferon-β
Tyk2	Tyrosine kinase 2
WHO	World Health Organization

Abstract

Prolonged dietary exposure to Aflatoxin B_1 (AFB_1) is one of the major risk factors for the development of primary liver cancer or hepatocellular carcinoma (HCC). AFB_1 contamination of some Ghanaian staples has been reported to be very high. AFB_1 causes mutations in the tumour suppressor gene *TP53.* These mutations are known to be involved in the pathogenesis of HCC. However, how AFB_1 affects other anti-cancer pathways such as the type I interferon (IFN) pathway is largely unknown. The aim of the study was to test the hypothesis that AFB_1 inhibits the type I IFN response in human hepatoma cell lines (HepG2 cells) by directly interfering with key signaling proteins and thus increase the risk of HCC. In this study, the effect of AFB_1 on cell viability was investigated by MTS-based assay. The type I IFN response in HepG2 cells was induced using recombinant (r) IFN-α and measured by IFN stimulated response element (ISRE) luciferase reporter gene assay. In addition, the effects of AFB_1 on the mRNA levels of *JAK1, STAT1* and *OAS3* in HepG2 cells stimulated with rIFN-α were determined by RT-qPCR and confirmed by western blotting assay. Exposure of HepG2 cells to AFB_1 up to 3200 µM for 24 hours followed by maintenance of cells in AFB_1 free media for 24, 48 and 72 hours decreased cell viability in a dose dependent manner. AFB_1 also suppressed/inhibited the IFN-α driven activation of a reporter promoter that contained the ISRE. When HepG2 cells which had been stimulated with rIFN-α were treated with AFB_1 the mRNA synthesis of *JAK1, STAT1* and *OAS3* was suppressed/inhibited. AFB_1 also inhibited the protein accumulation of *STAT1*. The biological significance of the inhibition/suppression of the type I IFN pathway by AFB_1 was investigated by employing the replication of chandipura virus in HepG2 cells stimulated with or without rIFN-α and treated with or without AFB_1. Chandipura virus was titred by plaque assay on L929 cells. Paradoxically when chandipura virus was exposed to AFB_1 for 24 hours, the replication of the virus was reduced as measured by a decrease in the virus titer. Results from this study suggest that AFB_1 may also induce HCC by inhibiting the type I IFN response pathway which is known to have anticancer property. Since AFB_1 rather kills the IFN sensitive chandipura virus rather than rescuing them, it is speculated that AFB_1 and viruses might not co-operate directly to amplify HCC development and that further studies are required to determine how viruses co-exist with AFB_1 to catalyse carcinogenesis caused by AFB_1 in human liver.

CHAPTER ONE INTRODUCTION

1.1 Background information

Aflatoxins are naturally occurring mycotoxins that were discovered in the 1960s after the devastating loss of poultry in England in which Turkey X disease caused the deaths of over 120,000 turkeys and other poultry which were fed with meals that contained peanut imported from South America (Blount, 1961). Aflatoxins are produced by fungal species that belong to the genus *Aspergillus*. The commonest species that produce these lethal mycotoxins are *Aspergillus flavus* and *Aspergillus parasiticus*. These fungi can infect grains and produce aflatoxins before the grains are harvested or when the grains are being stored. The grains are usually more prone to *Aspergillus* colonization when they are exposed to high temperatures and high humid environment for longer period of time or when they are damaged by environmental stresses like drought making the cereals more susceptible to *Aspergillus* infection (Wu and Khlangwiset, 2010) Maize and groundnuts are two food crops which are more susceptible to colonization by *Aspergillus* and they form part of meals of many Africans. Due to high levels of poverty in most areas of Africa, most people are unable to afford many food variety and hence rely heavily on maize and groundnut or cereals as a whole thus increasing aflatoxin exposure. Aflatoxin contamination in food is a worldwide health problem especially in the underdeveloped nations (Liu and Wu, 2010). It has been reported that more than 4.5 billion individuals globally are exposed to aflatoxin more especially in the developing nations (Strosnider *et al*., 2006). Food meant for human and animal consumption have been reported to contain aflatoxins in some countries such as Ghana, Togo, Nigeria and Benin in the West African sub region (Oyelami *et al*., 1996, Kpodo *et al*., 2000, Awuah and Kpodo, 1996) probably

because of suboptimal farming practices and poor storage conditions in these countries. For example, a study conducted by Kumi and colleagues in 2014 (Kumi *et al*., 2014) to determine aflatoxin contamination of locally made food (weanimix) from maize and groundnuts reported a high levels of aflatoxin and fumonisin in weanimix in the EjuraSekyedumase district in the Ashanti Region of Ghana. They reported that 83.3% of weanimix samples had aflatoxin levels higher than the national permissible level of 15ppb with mean level of 145.2ppb.

Currently about eighteen (18) different kinds of aflatoxins have been identified. The representatives of the 18 that are of greater importance include aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2). Some minor members of 18 different kinds of aflatoxins include aflatoxin M_1 (AFM_1) and aflatoxin M_2 (AFM_2). AFB_1 is a common contaminant of food stuffs meant for human consumption and is the most lethal carcinogen in both humans and experimental animals (Wild and Gong, 2010, Sudakin, 2003). Evidence from experimental and epidemiological studies has led to the classification of AFB_1 as group 1 human carcinogen by the International Agency for Research on Cancer (IARC) (IARC, 2002). AFB_1 contamination of diet coupled with its subsequent prolonged heavy exposure is a major risk factor that contribute to the development of primary liver cancer or hepatocellular carcinoma (HCC) (Wogan, 1992 , Wild and Hall, 2000). In addition to AFB_1 exposure, other environmental risk factors that predispose individuals to HCC include chronic hepatitis B and C virus infections, heavy alcohol consumption and iron overload Chen *et al*. (1997). A study conducted by Liu and Wu (2010) to assess the extent of aflatoxin-induced HCC in the world reported that of the 550,000 to 600,000 new HCC cases recorded in the world each year, about 25,200 to 155,000 may be attributable to aflatoxin exposure. They reported that majority of the cases

occur in the West African sub-region, Southeastern part of Asia and China where both exposure of people to high levels of aflatoxins and hepatitis B virus infections are common. Liu and Wu (2010) estimated that aflatoxin exposure may be a contributing factor in 4.6-28.2% of HCC worldwide.

HCC rapidly reduces quality of life and typically causes death six month to one year from diagnosis (Bosch *et al*., 2005). According to the GLOBOCAN 2012 reports released by IARC in December 2013 (IARC, 2013), HCC is ranked as the sixth commonest cancer in the world with about 782,000 new cases diagnosed in 2012. According to the report HCC is the second cause of cancer deaths resulting in about 0.8 million (9.1%) of all deaths attributable to cancers globally in 2012. It has been reported that the number of new HCC cases is high in West Africa with an annual death rate of approximately 200,000 (Ladep *et al*., 2014). In fact West Africa is ranked second aside Eastern Asia as the region affected most with HCC (Ladep *et al*., 2014). For example in West African countries like Senegal, Gambia and Guinea Conakry, the incidence of HCC has been reported to be very high ranging from 30-50 cases per 100000 in men and 12-20 cases per 100000 in women (Nordenstedt *et al*., 2010). In West Africa the death rate of HCC is almost equal to its incidence with most HCC sufferers dying within weeks of their diagnosis indicating how aggressive and dangerous HCC is (Ladep *et al*., 2014, Jemal *et al*., 2011).

The development of HCC is also affected by sex and age of the individuals. HCC is the fifth and the seventh most frequently diagnosed cancers in men and women respectively worldwide (Jemal *et al*., 2011). A study conducted by Laryea *et al*. (2014) to review data from Kumasi Cancer Registry at the Komfo Anokye Teaching Hospital (KATH) in Kumasi for the year 2012 reported that HCC is the commonest cancer among males accounting for 21.1% of all cancers. In another study conducted at the Korle Bu Teaching Hospital

(KBTH) in Accra the capital of Ghana to review the cancer mortality patterns in Ghana over a 10- year period (from 1991 to 2000) in 2006, Wiredu and Armah (2006) reported that HCC is the leading cause of cancer deaths in males accounting for 21.5% of all cancer deaths in Ghana. Wiredu and Armah also reported that HCC is the third leading cause of cancer deaths in females accounting for 10.97% of all cancer deaths in Ghana.

Information available suggests that the risk of HCC development may be amplified through the synergistic effects of aflatoxin ingestion and HBV infection. The risk of HCC developing in people with chronic HBV infection and also exposed to aflatoxin is up to 30 times greater than the risk in individuals exposed to either of the two factors (Wu and Khlangwiset, 2010, Liu and Wu, 2010, Groopman *et al*., 2008). These two risk factors

(aflatoxin and HBV) are common in underdeveloped countries in the world including Ghana (Awuah and Kpodo, 1996, Allain *et al*., 2003). Within these resource-limited countries; there is often significant rural-urban difference in HBV prevalence and aflatoxin exposure with both risk factors usually affecting rural populations strongly (Plymoth *et al*., 2009). Information available on mycotoxin-induced changes of immunity and impairment of resistance to disease has been largely obtained from studies with aflatoxins (Hahon *et al*., 1979). Aflatoxins have been reported to reduce greatly serum complement activity

(Thurston *et al*., 1974, Michael *et al*., 1973), decrease or impair phagocytosis (Richard and Thurston, 1975), increase susceptibility to and mortality of certain infectious agents (Hamilton and Harris, 1971 , Edds *et al*., 1973), interfere with acquired resistance (Pier and Heddleston, 1970), or inhibit cellular immune processes (Savel *et al*., 1970 , Pier *et al*., 1972).

In spite of these negative effects of AFB_1 in the human system, the innate immune system is constantly working to protect the individual against the harmful effects of aflatoxins and other disease causing agents. One component of the innate immune system that plays a key role in the first line of defense in eradicating pathogens (such as viruses) and tumour cells is the interferon (IFN) system. Some studies have been carried out to determine the effects of IFNs on human tumours or malignancies. For example in 1970, Gresser examined the capacity of IFN to reverse the phenotype of tumourigenic cells to normal phenotype and reported that IFN ensured a partial reversion in human osteosarcoma cells (Gresser, 1970). Indeed in 1986, IFN-α2a and IFN-α2b were licensed and approved for the treatment of Hairy Cell Leukemia, AIDS-related Kaposi's sarcoma and other malignancies (Bekisz *et al*., 2010). Studies have shown that IFN-treatment induces the tumour suppressor gene p53 which plays a role in the apoptosis of some tumour cells (Bekisz *et al*., 2010). Indeed Aziz *et al*. (2005) showed in their study that IFN-α has a significant protective effect against hepatic fibrogenesis and carcinogenesis. They reported that rat liver cells expressing ectopic IFN-α showed minimized fibrotic and cirrhotic processes when treated with the carcinogens AFB_1 and carbon tetrachloride. Although the study of Aziz *et al*. is yet to be performed in human liver cells to assess the capacity of viruses in inducing IFN production in the context of AFB_1 exposure, it showed IFN-α as a significant protective agent against liver cancer.

The signaling pathways through which IFNs exert their anti-tumour actions can vary. Studies have shown that one of the mechanisms by which IFNs exert their antitumour activity is through the induction of signal transducer and activator of transcription 1 (STAT1) protein (Stephanou and Latchman, 2003). When STAT 1 is activated, many pro-apoptotic and anti-proliferative genes that are required to prevent or reverse the

tumourigenic processes are induced (Elahi *et al*., 2008, Egwuagu *et al*., 2006). The activity of STAT 1 is pivotal for tumour immune surveillance as it drives the induction of major histocompatibility complex molecules Class I (MHC I) required for presentation of antigens to cytotoxic T cells and natural killer cells and also elicits anti-tumour immune response (Rodriguez *et al*., 2007). Another protein that is targeted by type 1 interferon is

2'-5' oligoadenylate synthetase (OAS). The expression of OAS results in the activation of another protein RNase L (a ribonuclease). The OAS/RNase L is an RNA decay pathway known to play significant role in the established endogenous anti-viral pathway (reviewed in Randall and Goodbourn, 2008). This pathway can break down ssRNA which may include mRNA of the host cell and viral RNA and thus inhibit protein synthesis of both the host cell and the viruses (Silverman, 2007). The importance of IFN-α/β in mediating antiviral response is established by the fact that mice that lack IFN-α/β receptors (Tay *et al*., 1999, Nunez, 1999, Muller *et al*., 1994) are unable to mount efficient responses to a large number of viruses.

The fact that AFB_1 causes HCC by inducing mutation in the tumour suppressor gene p53 has been well established. However, information on how AFB_1 could inhibit the type 1 IFN (IFN-α/β) response as a way of causing HCC development is scanty. This study will explore the mechanisms by which AFB_1 could inhibit the type 1IFN response using human hepatoma cell line as a model system. Results from this study could influence future therapeutic strategy for AFB_1- induced HCC and also broaden our knowledge of the role of AFB_1 in HCC biology.

1.2 Problem Statement and Justification

Cereals form a major staple of diet of majority of Ghanaians and other developing nations yet sub-optimal farming practices and poor storage conditions in these developing nations make these food crops highly susceptible to aflatoxin contamination. Prolonged dietary exposures to aflatoxins particularly AFB_1 have been identified to increase the risk of development of HCC. Studies have shown that IFN-α, a member of type I interferon family shows a protective effect against hepatic fibrogenesis and carcinogenesis. However, information on how AFB_1 modulates the anti-viral and anti-tumour properties of type I interferon and possibly increase the risk of HCC is scanty.

Even though quite a number of studies have examined the effects of AFB_1 on the immune system of experimental animals (*in vivo*) and on animals and human immune cells *in vitro*, less is known about the exact role of AFB_1 in modulating the anti-viral and antitumour activities of IFN-α. Understanding the molecular mechanisms by which AFB_1 modulates the type 1 IFN response in human liver cells may provide information that may have practical application in the design and manufacture of new anti-cancer drugs and thereby provide avenues for future therapeutic interventions.

1.3 Aim/Objectives

The aim of the study was to determine whether AFB_1 inhibits the type I IFN response and the mechanism involved.

The specific objectives were:

1. To determine the cytotoxic effects of AFB_1 on human hepatoma cell line (HepG2).

2. To determine the inhibitory effects of AFB_1 on the type 1IFN pathway with luciferase gene reporter assay.

3. To use RT-qPCR to determine the effects of AFB_1 on mRNA levels of key signaling proteins of the JAK-STAT-ISRE pathway

4. To use immunoblot assay to determine the effects of AFB_1 on key signaling proteins of the JAK-STAT-ISRE pathway.

5. To determine the biological effects of AFB_1 inhibition/suppression of the type I IFN response using chandipura virus as a model.

CHAPTER TWO LITERATURE REVIEW

2.1 Distribution of fungi that produce aflatoxins

The fungi that produce aflatoxins mainly belong to the genus *Aspergillus*. The two most important species of *Aspergillus* responsible for the production of most aflatoxins in food crops worldwide are *Aspergillus flavus* and *Aspergillus parasiticus*. Although the two species have similar geographical ranges, *Aspergillus parasiticus* is less ubiquitous and is rare in Southeastern part of Asia. On the other hand *Aspergillus flavus* is ubiquitous and is commonly found in certain cereals that cultivated under environmental stresses such as drought. *Aspergillus flavus* and *Aspergillus parasiticus* colonize agricultural produce such as maize, spices, oilseeds, groundnuts, peanuts, walnuts, almonds, cottonseed, corn, millet etc and produce aflatoxins during growth periods, harvesting, threshing, drying, storage and transportation and usually produce AFB_1 and AFB_2 (Strosnider *et al*., 2006). AFG_1 and AFG_2 are mostly produced by *Aspergillus parasiticus* (Strosnider *et al*., 2006). Other fungal species belonging to the genus *Aspergillus* which also produce aflatoxins include *Aspergillus nomius*, *Aspergillus niger* and *Aspergillus australis*. The *Aspergillus* species are found mostly in soil, decaying vegetation, hay and grains undergoing microbiological deterioration. *Aspergillus* growth and aflatoxin production is dependent upon the temperature, humidity, host plant type, and the strain of fungus; high humidity usually required for growth (Williams *et al*., 2004).

2.2 Chemistry and types of aflatoxins

Aflatoxins are a family of structurally related secondary metabolites that are produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus.* Secondary

metabolites are chemical substances produced that are produced by living organisms that are not essential for the growth of the producing organism. Chemically, the aflatoxins are highly substituted coumarins that contain a fused dihydrofurofuran moiety. There are six main groups of aflatoxins; four main naturally produced aflatoxins known as AFB_1 **(Fig 2.1)**, AFB_2, AFG_1 and AFG_2 and two additional metabolic products known as AFM_1 and AFM_2. AFM_1 is an intermediate products of AFB_1 metabolism in humans and animals while AFM_2 is an intermediate products of AFB_2 metabolism in the milk of cattle fed on contaminated meals. Whereas the B designation of AFB_1 and AFB_2 resulted from the production of blue fluorescence colours under ultraviolet (UV) light on thin layer chromatographic plates, the G designation refers to the green fluorescent colours produced under ultraviolet (UV) light on thin layer chromatographic plates. The AFM_1 and AFM_2 were first isolated from milk of lactating animals fed on aflatoxin preparations hence the M designation. The subscript numbers 1 and 2 indicate the major and minor compounds respectively. AFB_1 and AFG_1 have an unsaturated bond at the 8, 9 position on the terminal furan ring. Studies have shown that epoxidation at this position is crucial for the carcinogenic potency (Groopman and Kensler, 2005).

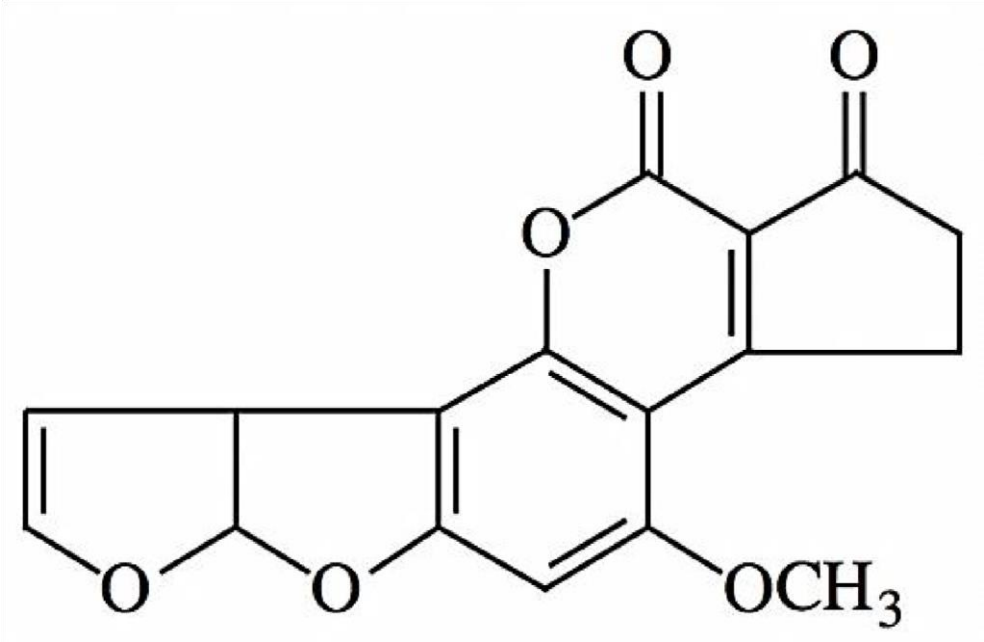

Fig. 2.1: Chemical structure of AFB_1: AFB_1 like all other aflatoxins is made up of dihydrofuran or tetrahydrofuran residues that are fused to a substituted coumarins ring. The difference between the AFB and the AFG molecules is that the AFG molecules have a lactone ring instead of the cyclopentenone ring. The AFB_1 and AFG_1 have double bonds at the positions 8 and 9 on the terminal furan and this confers the distinct carcinogenic properties to AFB_1 and AFG_1. The structure of AFB_1 was adopted from Smela *et al*. (2002).

2.3 Physical and chemical properties of aflatoxins

Aflatoxins exist as colourless to pale-yellow crystalline substances at room temperature (IARC, 1993). They dissolve sparingly in water and hydrocarbons. However, they can be completely dissolved in methanol, acetone and chloroform but are insoluble in non-polar solvents. Aflatoxins can easily be degraded in light and air especially when exposed to oxidizing agents, ultraviolet light or strongly acidic or basic solutions. Aflatoxins breakdown at temperatures between 237 °C and 299 °C. Aflatoxins are not degraded under normal cooking temperatures but can be completely destroyed by autoclaving in the presence of ammonia or by treatment with bleach containing sodium hypochlorite.

2.4 Human exposure to aflatoxins

People usually become exposed to aflatoxins by consuming contaminated food. The fungal species that produce aflatoxins basically grow on corn and other grains, peanuts, tree nuts and cottonseed meal. Consumption of meat, eggs, milk and other edible products from animals that consume aflatoxin contaminated feed are also sources of potential exposure. Maize and nuts especially groundnuts are the major sources by which humans become exposed to aflatoxins because; (i) they are very prone to *Aspergillus* infection and subsequent contamination with aflatoxins, (ii) they are heavily consumed globally (Wu and Khlangwiset, 2010).

Generally food may contain AFB_1 and AFB_2 in concentration ratios of 1.0:0.1. In situations where AFB_1, AFB_2, AFG_1 and AFG_2 occur may be present in concentration ratios of 1.0: 0.1: 0.3:0.03 (Probst *et al*., 2007). Even though moulds contamination is usually seen

as universal problem, the quantity of aflatoxins in cereal products can differ from less than 1µg/kg (1ppb) to more than 12,000 µg/kg (12000ppb) (Probst *et al*., 2007). In a study to investigate the cause of death of some people in Kenya who were suspected to have consumed aflatoxin contaminated food, it was estimated that people were exposed to 50mg of AFB_1 on daily basis (Probst *et al*., 2007). In a study to determine the immune status in relation to aflatoxin levels of Ghanaian adults chronically exposed to aflatoxin in their diets, Jiang *et al*. (2005) reported that on the average Ghanaians who were recruited into the study consumed at least 10µg (32µM) of AFB_1 per day in their diets. It has been reported that infants may become exposed to aflatoxins through the consumption of breast milk (Zarba *et al*., 1992). A study conducted by Somogyi and Beck (1993), to determine the levels of aflatoxins in the breast milk of nursing mothers from Africa and Germany revealed that about 34% of breast milk samples from Africa contained aflatoxins but aflatoxins were not found in all the samples from Germany. They also reported that the commonest aflatoxin found in breast milk was AFM_1 which occurred at concentrations that ranged from 0.02 to about 1.8µg/L. However, the most abundant aflatoxin that was found AFB_1 which was detected at concentrations of 8.2µg/L.

Aflatoxin exposure may also be facilitated by the type of work an individual is engaged in. For example, humans also become exposed to aflatoxins by inhaling aflatoxin containing dust generated during the handling and processing of contaminated crops and feeds. Individuals who are at greater risk of occupational exposure to aflatoxins are farmers and agricultural workers. In a study conducted by Autrup *et al*. (1993) in Denmark in which blood samples of 45 animal-feed factory workers were taken and analyzed, it was reported that aflatoxin was detected in their blood after working for four weeks in the factory or unloading raw materials from the ships. Similarly, a study conducted by Ghosh *et al*. (1997

) to determine the levels of aflatoxins in respiratory samples of workers at rice and corn processing factory in India revealed that aflatoxins were present at concentrations of 0.00002 to 0.0008µg/m^3.

Information gathered from epidemiological studies has revealed that there is a significant statistical correlation between aflatoxin intake and the incidence of HCC globally. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) using epidemiological data from China and data from animal toxicity studies conducted a quantitative risk assessment of aflatoxin in 1998 in order to estimate the impact of different regulatory standards on aflatoxin-induced HCC in populations with or without chronic

HBV infection. Based on previous cohort study that estimated cancer potency in both HBsAg positive and HBsAg negative individuals (Yeh *et al*., 1989), the JECFA aflatoxin risk assessment chose two different cancer potency factors for aflatoxin: 0.01 cases per 100,000 per year per ng/kg bw/day aflatoxin exposure for individuals without chronic HBV infection and 0.30 corresponding cases for individuals with chronic HBV infection. Based on the assumption that all foods containing higher than standard levels of aflatoxins were discarded and that there were enough maize and nuts to preserve consumption patterns, JECFA determined that HCC incidence would decrease by about 300 cases per billion people per year if stricter aflatoxin standard was followed in nations with HBV prevalence of 25% (Joint FAO/WHO Expert Committee on Food Additives (JECFA), 1998). However, in nations where HBV prevalence was > 25%, the stricter aflatoxin standard would only save 2 HCC cases per billion people per year (Henry *et al*., 1999). In 2008, another study conducted by Shephard (2008) who combined the estimates of JECFA with exposure assessments based on data collected from various studies in Africa on staple food

consumption and estimated the population risk for aflatoxin-induced HCC in selected subSaharan African nations reported that there was a very high population risk for HCC based on exposure to aflatoxin.

2.5 Metabolism of AFB_1

Aflatoxins are metabolized into a variety of products such as aflatoxicol, aflatoxin Q_1(AFQ_1), aflatoxin P_1 (AFP_1), and AFM_1 in the liver by cytochrome P450 (CYP450) group of enzymes when ingested (Fig. 2.2). In addition, when aflatoxins especially AFB_1 is transported to the liver, it converted to the reactive oxygen species; AFB_1- 8, 9 epoxide intermediate by CYP450 enzymes especially CYP1A2, CYP3A4 (Wild and Gong, 2010., Sudakin, 2003). The AFB_1- 8, 9 epoxide metabolites formed can be detoxified through conjugation with glutathione mediated by the enzyme glutathione- S- transferase (GST) to form a stable, non-toxic, polar product that is excreted in the bile (Kew, 2008). The aflatoxin-glutathione product also goes through systematic metabolic processes in the liver and kidneys leading to it excretion as mercapturic acid in the urine. However, if the amount of AFB_1 ingested in the diet exceeds the capacity of the GST enzymes to detoxify the epoxides formed or if, for any reason the activity of GST is decreased (for example by polymorphisms of the GST gene), the highly reactive AFB_1-8, 9 epoxide may bind to liver proteins and lead to their failure resulting in acute aflatoxicosis. Alternatively, the 8, 9 epoxides may bind to and modify DNA of liver cells leading to the formation of promutagenic lesions that result in the activation of proto-oncogenes and the inactivation of the tumour suppressor genes. The AFB_1- 8, 9 epoxide reacts with a N^7 atom of guanine to form a promutagenic DNA adduct (aflatoxin-N^7-guanine). This aflatoxin-DNA adduct is

unstable and undergoes depurination leading to its excretion in the urine. The activity of GST is much higher in animal species that are resistant to the carcinogenicity of aflatoxin such as mice than in susceptible animal species such as rats. GST activity in humans is lower than either mice or rats, suggesting that humans are less capable of detoxifying aflatoxin-B_1- 8, 9 epoxide (IARC, 1987).

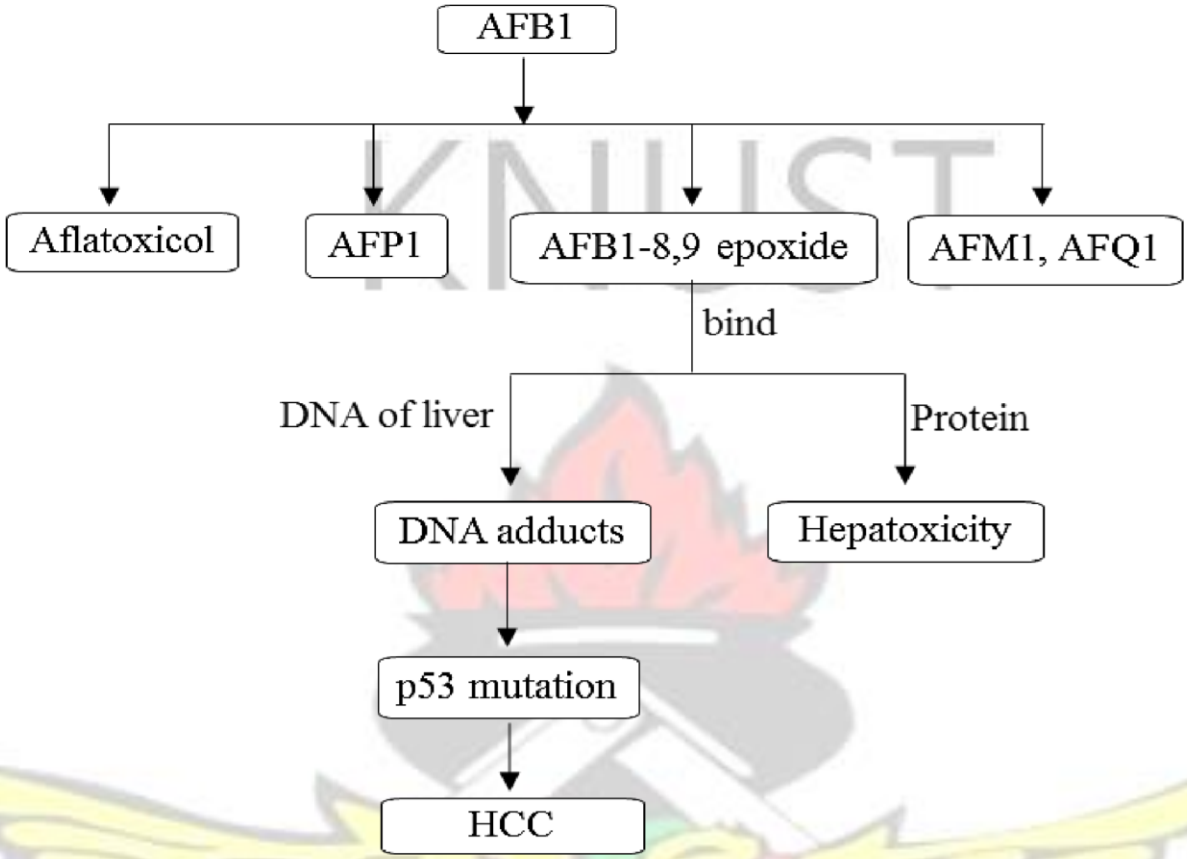

Fig. 2.2: Biotransformation of AFB_1: AFB_1 is converted to AFB_1- 8, 9 epoxide and other products by the CYP450 enzymes in the liver. The highly reactive 8, 9 epoxide formed binds to DNA of liver cells, causes mutation in the DNA which may lead to HCC. AFB_1- 8, 9 epoxide formed may also cause aflatoxicosis when it binds to proteins. The aflatoxicol formed by oxidation-reduction reactions, the AFP_1 formed by demethylation and the AFM_1 and AFQ_1 formed through hydroxylation are less harmful.

2.6 Mechanisms of carcinogenesis of AFB_1

Studies have shown that aflatoxins cause genetic damage in cultured cells from humans and experimental animals and in humans and experimental animals exposed to aflatoxins *in vivo*. Types of genetic damage that are observed in cultured cells and animals include the formation of DNA adduct, albumin adduct, gene mutations, micronucleus formation, sister chromatid exchange and mitotic recombination (IARC, 1987).

In humans and susceptible animal species, AFB_1 is metabolically converted to unstable reactive AFB_1-8, 9-exo-epoxide in the liver by several CYP450 enzymes mainly CYP4501A2 and CYP4503A4 (Mace *et al*., 1997). The 8,9-exo-epoxide metabolites bind covalently to DNA preferentially on the N7- positions of guanines to form primary DNA adducts (8-9, dihydro-8-(N7-guanyl)-9-hydroxy aflatoxin; AFB_1-N7-Gua (Gallagher *et al*., 1994). The primary adduct is rapidly converted to two secondary lesions; an apurinic (AP) site and a stable ring-opened AFB_1-Formamidopyrimidine (AFB_1-FAPY) adduct (Smela *et al*., 2002). The AP site as well as AFB_1-FAPY adduct which is considered as the most mutagenic lesion (Smela *et al*., 2002) are repaired by nucleotide excision repair (NER) or base excision repair (BER) (Waters *et al*., 1992, Sarasin *et al*., 1977). However, improper repair leads to AGG to AGT transversion mutations. In the HCC cells of patients exposed to aflatoxins, the mutations occur at codon 249 in the tumour suppressor gene *TP53* which result in the substitution of the amino acid arginine into serine (R249S) in the p53 protein (Hsu *et al*., 1991, Aguilar *et al*., 1993). Mutated R249S p53 expression may lead to inhibition of apoptosis, inhibition of p53 mediated transcription and stimulation of liver cell growth (Martin and Dufour, 2008). This mutation is detected in over 50% of HCC in high incidence areas of Africa and China (Hsu *et al*., 1991, Bressac *et al*., 1991). However, this

mutation is uncommon in regions where aflatoxin is not present at significant levels in the diet and in cancers other than HCC (Ozturk, 1991).

The tumour suppressor gene *TP53* encodes for tumour suppressor protein p53. p53 is usually maintained at low levels under normal conditions when it binds to ubiquitinligases such as Mdm2 (also referred to as Hdm2 in humans) and then degraded by proteasome enzymes (Lereau *et al*., 2012). However, when certain stress factors are present in the cell, p53 undergoes post- translational processes which may include the phosphorylation on serine 15 (Pser15-p53). The phosphorylated p53 then binds specific DNA response elements which lead to trans-activation of genes involved in cell cycle arrest, apoptosis, DNA repair or senescence (Hollstein and Hainaut, 2010). These responses may result in the repair of damaged DNA which aid in maintaining the genetic integrity of the cells. The response may also stimulate apoptosis of damaged cells leading to their elimination from the system.

2.7 AFB_1-induced HCC in humans and experimental animals

As stated already the aflatoxins are common contaminants of many foods such as cereal grains, groundnuts, oilseeds that are mostly consumed by humans throughout the resource limited nations of the world (Eaton and Groopman, 1994). The greater portions of agricultural land in tropical and sub-tropical regions of the world like Africa and Asia lie in climatic regions favourable for *Aspergillus flavus* and *Aspergillus parasiticus* proliferation. These two species are noted for their high and efficient production of aflatoxins (Liu and Wu, 2010). Aflatoxin is a controllable risk factor in food yet the parts of the world in which the risk of production is particularly high have limited resources to implement most

aflatoxin control strategies (Liu and Wu, 2010). The early evidence that aflatoxins are carcinogens in humans came from epidemiological studies that correlated geographic variation in aflatoxin content of foods with geographic variation in the incidence of HCC. Studies have shown that prolonged exposure to aflatoxin is associated with an increase in the incidence of HCC in humans and sensitive animal species (Groopman *et al*., 2008). Studies in Uganda, Swaziland, Thailand, Kenya, Mozambique and China demonstrated strong, significant positive correlations between estimated aflatoxin intake or aflatoxin levels in food samples and the incidence of HCC (IARC, 1993). In the United States, a 10% excess of HCC was observed in the southeast, where the estimated average daily intake of aflatoxin was high, compared with the North and West areas with low aflatoxin intake (IARC, 1993 , IARC, 1987).

In an experiment to determine the toxicological hazard level of aflatoxins, Wogan *et al.*, 1974) fed rats with aflatoxins at levels of 1, 5, 15, 50 and 100 ppb and reported that these levels induced tumours at incidence of 4.5%, 9%, 19%, 80% and 100% respectively (Wogan *et al*., 1974) thus suggesting that for cancer risk assessment, it is traditionally assumed that there is no threshold of exposure to a carcinogen below which there is no observable adverse effects (National Research Council (NRC), 2008). The adverse health consequences of aflatoxins in populations are quite varied, sometimes causing acute effects such as rapid death (Azziz-Baumgartner *et al*., 2005) and chronic outcomes such as HCC (Ross *et al*., 1992, Qian *et al*., 1994). Aflatoxin-induced HCC risk varies significantly from one geographic region to another and even among different populations within the same country (Liu and Wu, 2010). In their study to assess the global burden of aflatoxin-induced

HCC, Liu and Wu (2010) found that sub-Saharan Africa is the most important region for HCC cases attributable to aflatoxin. In addition, Liu and Wu reported that Southeast Asia and China are also very key areas where aflatoxin related HCC is common. It is worth noting that in Mexico where HBV prevalence is low but aflatoxin contamination in food is relatively high, aflatoxin appears to be a significant risk factor in the development of HCC among those without HBV with an estimate of 152-924 HCC cases per year per 100,000 people (Liu and Wu, 2010). Wild and Hall (2000) reported that rural populations usually tend to have high levels of aflatoxin exposure than do urban dwellers probably because urban dwellers consume more diversified diets than the rural populace and may have food that is controlled for aflatoxin contaminants. It has also been reported that there is a strong seasonal variation in aflatoxin exposure that correlates with food availability (Tajkarimi *et al*., 2007 , Gnonlonfin *et al*., 2008). To elucidate the importance of aflatoxin in the etiology of HCC, a total of 6,487 residents aged 30 to 35 years were recruited in a community based cohort study in the Penghu Islets in Taiwan. It was reported in that study that among the 33 HCC cases recorded, 31 (94%) were chronic hepatitis B surface antigen (HBsAg) carriers (Chen et al., 1996). Chen *et al*. (1996) also reported that among the 20 HCC patients and 86 matched healthy controls whose serum samples were tested for AFB_1, 13 (65%) HCC patients and 32 (37%) matched controls were seropositive for aflatoxins. In many populations in which HCC occurs at a high incidence, HBV infection and high levels of aflatoxin ingestion exist concurrently. Epidemiological studies support the existence of a synergy between chronic HBV infection and exposure to AFB_1. It has been reported that the R249S mutation is almost exclusively present in HCC of patients exposed to both chronic HBV and AFB_1, thus suggesting that DNA damage by HBV and AFB_1 may influence each other (Kew, 2003). Although the biological mechanism underlying this synergy is not fully understood it is believed that HBV and aflatoxin may influence each other through one of the following: firstly, when an individual is exposed to aflatoxin during HBV infection, HBV may decrease the repair of DNA and make the cells more permissive to the formation of mutations (Jia *et al*., 1999). Secondly, during the development of chronic HBV infection, inflammatory stress may enhance AFB_1 metabolism and DNA damage, increasing mutagenesis (Chemin *et al*., 1999). Thirdly, in pre-cancer or cancer HCC cells, the mutant R429S p53 protein may contribute to the survival of cells expressing viral antigens (Gouas *et al*., 2009).

2.8 Toxicological effects of aflatoxins in humans

Aflatoxins are remarkably potent often causing disease even when ingested in minute quantities (NRC, 1983). Aflatoxins cause disease throughout the body, but are most commonly known for causing acute or chronic liver disease and liver cancer. Aflatoxin exerts it toxicity in several ways when it is consumed. In the intestine, it may alter intestinal integrity (Gong *et al*., 2008) or modulate the expression of cytokine proteins that signal to

each other and to immune system components. These effects may result in stunted growth in children and or immune suppression (Wu, 2010). The effects of aflatoxins on humans as with other lower animals depend upon dosage and duration of exposure. Humans usually become exposed to aflatoxins either by ingesting a high amount of aflatoxins in a very short time (acute exposure) or by ingesting a small amount of aflatoxins at a time, but over a long period (chronic exposure).

Acute exposure to high levels of aflatoxins can cause liver disease, mental impairment, abdominal pain, vomiting, and disruption of food digestion, coma and aflatoxicosis with a case fatality rate of approximately 25% (Cullen and Newberne, 1994). Certain conditions of the environment increase the probability of acute aflatoxicosis in humans. These conditions include the unavailability of food, environmental conditions that favour fungal growth and development in crops and commodities and the lack of regulatory systems for aflatoxin monitoring and control. Outbreaks of acute aflatoxicosis are a recurring public health problem throughout the world (CDC, 2004, Lye *et al*., 1995) . Several cases of acute aflatoxicosis have been reported in Africa and they were associated with the consumption of home-grown maize contaminated with aflatoxins. Two of such acute aflatoxicosis outbreaks occurred in Kenya in 1982 during which 12 people died and in 2004 in which

317 cases were recorded with 125 people dying in the central provinces (Strosnider *et al*., 2006, Probst *et al*., 2007). Experimental animal evidence suggests that chronic exposure to aflatoxins may lead to impaired immunity, reduced uptake of nutrients from the diets and growth retardation (Miller and Wilson, 1994 , Hall and Wild, 1994). Several studies of children in Benin and Togo have reported an association between aflatoxin-albumin adduct levels and impaired growth (Gong *et al*., 2003 , Gong *et al*., 2004). A study conducted in

Ghana by Jiang *et al*. (2005) showed that a higher level of AFB_1-albumin adducts in plasma was associated with lower percentages of certain leukocyte immunophenotypes. Another study conducted among Gambian children found an association between serum aflatoxin-albumin levels and reduced salivatory secretory IgA levels (Turner *et al*., 2003). HCC as a result of chronic aflatoxin exposure has been well documented generally in association with HBV or other risk factors (Qian *et al*., 1994,

Groopman *et al*., 2008).

2.9 Mechanism of immunomodulatory effects of AFB_1 in humans

The molecular mechanism (s) by which aflatoxins and other mycotoxins modulate the immune system is poorly understood. Evidence available on the other hand suggests that aflatoxins and other mycotoxins may exert their immunosuppressive effects by one of the following ways; they may inhibit replication of DNA, transcription and protein synthesis of genes needed to activate the innate and adaptive immune response through the use of many different mechanisms (Jolly *et al*., 2008). For example, it has been reported that AFB_1-8, 9-exo-epoxide formed as a result of AFB_1 metabolism binds preferentially to mitochondrial DNA in comparison with nuclear DNA thus hindering ATP production (Verma, 2004 , Bhat *et al*., 1982). The damage that is caused to mitochondrial DNA is through adduction and mutations to the mitochondrial membranes which leads to increased cell size and disruption of energy production (Verma, 2004 , Bbosa *et al*., 2013b). AFB_1 and its metabolites have been reported to affect protein synthesis by binding to biological molecules such as essential enzymes, block ribosomal translocase thus inhibiting protein synthesis (Eaton and Gallagher, 1994). In addition, aflatoxins have been reported to interfere with certain

enzymes and substrates that are required for initiation, transcription and translation processes which are involved in the synthesis of protein (Bbosa *et al*., 2013a).

In addition to the above, quite a number of studies have attempted to look at the immunosuppressive effect of AFB_1 in humans. For example Jiang *et al*. (2015) investigated the effects of AFB_1 on the mRNA expression levels of certain cytokines namely IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ and TNF-α in the small intestines (duodenum, jejunum and ileum) of broilers and reported a general reduction in the mRNA levels of IL-2, IL-4, IL6, IL-10, IL-17, IFN-γ and TNF-α in AFB_1 treated group compared with the untreated or the control group. In addition Jiang et al. reported that AFB_1- treated broilers showed a decreased proportion of intestinal T-cells subset compared to the control group. Other researchers have also demonstrated that AFB_1 suppressed/inhibited the mRNA and protein expression of IL-4, IL-6 and IL-10 from the peritoneal macrophages, splenic lymphocytes and macrophage cell lines (Bruneau *et al*., 2012, Dugyala and Sharma, 1996 , Marin *et al*., 2002). However Li *et al*. (2014) reported that AFB_1 increased the levels of IL-6, IFN-γ and TNF-α mRNA and protein expression in the serum and spleen of broilers fed with diet containing AFB_1 (0.074 mg/kg). A study conducted by Qian *et al*. (2014) to investigate the effects of AFB_1 on splenic lymphocyte phenotypes and the inflammatory cytokine production in male F344 rats reported that exposure of animals to AFB_1 resulted in a general dose-dependent decrease in IL-4 production by $CD4^+$ T cells. In addition AFB_1 suppressed/inhibited IFN-γ and TNF-α production by $CD4^+$T cells and $CD3^-CD8a$ NK cells respectively. It has also been reported that exposure of murine macrophages to AFB_1 *in vitro* resulted in a decreased secretion of

anti-inflammatory IL-10 and increased production of pro-inflammatory IL-6 (Bruneau *et al*., 2012).

In their study to evaluate the effects of AFB_1 on STAT5A gene expression, Forouharmehr *et al*. (2013) treated bovine mammary epithelial cells with AFB_1 and quantified the mRNA levels of STAT5A by RT-qPCR. They reported that AFB_1 significantly suppressed/inhibited STAT5A gene expression at the transcript levels in a dose-dependent fashion and that the suppression/inhibition resulted in the reduction of proliferation and differentiation of mammary epithelial cells which affected milk protein quantity and quality. A study conducted by Rossano *et al*. (1999) to investigate the effects of AFB_1 at low concentrations (0.01-1.0pg/ml) on the release and expression of IL-1α, IL-6α, TNF-α by human monocytes activated with bacterial lipopolysaccharide showed that at concentration of 0.05pg/ml, AFB_1 decreased each of the 3 cytokines and completely blocked the transcription of their mRNAs. The study also showed that the mRNA levels of β-actin was not affected by AFB_1 and this observation lead the authors to conclude that AFB_1 exerts it effects on the production of cytokines probably by specifically inhibiting the synthesis of certain mRNAs without affecting protein synthesis as a whole. They speculated that AFB_1 may inactivate some kinases that are involved in the activation of genes that code for cytokines. However, a study undertaken by Dugyala and Sharma (1996) showed that AFB_1 significantly increased mRNA levels of major cytokines produced by macrophages but suppressed their corresponding protein levels. Indeed, AFB_1 inhibition of viral interferon induction has been reported in experiments performed by infecting monkey kidney cells (LLC-MK) with influenza virus (Hahon *et al*., 1979).

2.10 Effects of AFB_1 in animal and human cell cultures

In vitro studies have been done to study the effects of AFB_1 in animal and human immune system cells. The cells of the immune system undergo continual proliferation and differentiation even though they are susceptible to the toxic as well as the immunosuppressive effect of AFB_1 and other mycotoxins. A study conducted by Lereau *et al*. (2012) to determine the cytotoxic effects of AFB_1 on HepaRG cells reported that AFB_1 had no measurable cytotoxic effect on HepaRG cells upon exposure to concentrations up to 10µM (3.125µg/ml) after treating cells with AFB_1 for 4 hours followed by 48 hours of culture in medium without AFB_1 (Lereau *et al*., 2012).

In another study, Cheng *et al*. (2002) investigated the cytotoxic effects of AFB_1 on duck peritoneal macrophages by incubating the cells with increasing concentrations of AFB_1 (0, 5, 10, 20, 50 and 100µg/ml) for 12 hours and reported that the viability of cells decreased significantly with increasing concentrations of AFB_1 (Cheng *et al*., 2002). Furthermore, Al-Hammadi *et al*. (2014) conducted a study to investigate the toxic effects of AFB_1 on human lymphocytes *in vitro* and reported that exposure of human lymphocytes to AFB_1 resulted in the impairment in cellular oxygen consumption, caspase activation and necrosis underscoring the immunosuppressive activity in humans exposed to this lethal mycotoxin (Al-Hammadi *et al*., 2014). In a related study, Hahon *et al*. (1979) investigated the cytotoxic effects of four basic related aflatoxins namely AFB_1, AFB_2, AFG_1 and AFG_2 on LLC-MK_2 cells in confluent monolayers. They reported that treating the confluent monolayers of LLC-MK_2 with AFB_2, AFG_1 and AFG_2 concentrations up to 500 µg did not affect the viability of the cells. However, when the cells were treated with AFB_1 concentration up to 500 µg, there was a dose-dependent decrease in the viability of the cells

(Hahon *et al*., 1979).

2.11 Effects of AFB_1 on viral replication

Epidemiological evidence available suggests that there is synergy between prolonged AFB_1 exposure and chronic HBV infection in hepatocarcinogensis. Studies have shown that long term exposure to AFB_1 coupled with chronic HBV infection increases the risk of HCC development. Lereau *et al*. (2012) conducted a study to determine the shortterm effects of AFB_1 exposure on HBV replication and reported that exposure up to 5µM AFB_1 concentrations well below the half maximum inhibitory concentration (IC 50) of >

50 µM obtained in HepaRG cells decreased HBV replication after 48 hours as measured by decrease in viral antigens (HBsAg, HbeAg and large envelope proteins) in culture media. They also reported that intracellular HBV DNA, HBV transcript levels as well as HBsAg were also decreased. In addition they reported that HBV infection did not modify significantly the AFB_1-DNA adducts formation or repair and that induction of p53 in response to AFB_1 was similar in infected and non-infected HepaRG cells. These data made the authors conclude that in HepaRG cell line AFB_1 and HBV do not cooperate to increase DNA damage caused by AFB_1 (Lereau *et al*., 2012).

The ability of AFB_1 to suppress/inhibit influenza virus-induced IFN production in cell cultures was demonstrated by Hahon *et al*. (1979). In their study they investigated the effects of AFB_1 (1-500 µg) on IFN induction by influenza virus in confluent LLC-MK_2 cell monolayer and reported that 100 µg of AFB_1 was the most effective concentration in suppression/inhibiting IFN production. Further studies by Hahon *et al*. revealed that the degree of inhibition of IFN production by AFB_1 was dependent on the sequence in which AFB_1 and the viral inducers were administered onto the cell monolayers. They demonstrated that AFB_1 suppressed/inhibited IFN production by 66% when the cells were treated with AFB_1 (50 µg) for at least 20 hours before the addition of the viral inducer. When AFB_1 and the viral inducers were added in tandem, IFN production was suppressed/inhibited by 40%. However, Hahon *et al*. demonstrated that adding AFB_1 2 hours after the addition of the viral

inducers had a negligible influence on IFN production suggesting that AFB_1 does not impair the sequence of viral IFN induction once the action is initiated. In addition, Hahon *et al*. investigated the rate of growth of influenza virus in AFB_1 treated and untreated cells and reported that the levels of virus concentration attained in AFB_1 treated cells were two to four-fold higher compared with the untreated control.

The enhanced influenza viral growth rate observed in AFB_1 treated cells in the study of Hahon et al. was attributed to the ability of AFB_1 to inhibit IFN production (Hahon *et al*., 1979).

Moreover, a study conducted by Barraud *et al*. (1999) to investigate the effects of AFB_1 exposure on the replication of duck hepatitis B virus in Pekin duck model during the initial stage of virus-AFB_1 interaction showed a significant increase in the titer of duck hepatitis B virus in the serum and liver of AFB_1 treated-ducks compared with dimethyl sulfoxide (DMSO) treated controls. The increase in viral replication observed in the Pekin duck model was confirmed in vitro in primary duck hepatocytes (Barraud *et al*., 1999).

2.12 The Interferon system

Interferons (IFNs) are a family of signaling proteins that are manufactured and secreted by host cells in response to the presence of disease-causing agents such as viruses, bacteria and parasites. They are also synthesized and released by host cells in response to the presence of tumour cells. IFNs form one of the several components of the innate immune system that work to eradicate pathogens and tumour cells from the host. Currently three classes of IFNs have been discovered based on the receptor through which they signal and

on the amino acid sequence. These classes include type I, type II and type III IFNs. The type I, type II and type III are differentiated from each other through their amino acid sequence as reviewed in (Killip *et al*., 2015).

The type I IFNs which comprises interferon-alpha (IFN-α), interferon-beta (IFN-β), interferon-omega (IFN-ω) and several other related molecules (Platanias, 2005) have similar amino acids sequence and were the first IFNs that were discovered. In mammals thirteen (13) different IFN-α gene types have been discovered. Also one to three IFN-β genes (one in humans) and other genes such as IFN- omega (IFN-ω), IFN-kappa (IFN-κ),

IFN-delta (IFN-δ) and INF-epsilon (IFN-ε) have been identified as reviewed in (Hagberg and Ronnblom, 2015). The type I IFNs are known for the capacity to confer antiviral resistance on cells. Two members of the type I IFNs which play crucial roles in the innate immune response to pathogens are the IFN-α and IFN-β genes. The other members of the type I IFN class play a relatively less defining role (reviewed in Randall and Goodbourn,

2008). The IFN-α is also referred to as leukocyte IFN. It is usually produced by leukocytes that have been infected with viruses. On the other hand, IFN-β which is also known as fibroblast IFN is produced by epithelial cells or fibroblasts that have become infected with viruses as reviewed in (Killip *et al*., 2015). The type I IFNs signal through a common receptor called type I IFN-α receptor (IFNAR) found on the surfaces of cells. The IFNAR

comprises two chains namely IFNAR1 and IFNAR2 (de Weerd *et al*., 2007). When engaged by the appropriate ligands, the receptor (IFNAR) works in activating a cascade of signal-transduction events in the host cells. This cascading process culminates in the transcription of different genes called IFN-inducible genes or IFN-stimulated genes (ISGs)

in the nucleus of the host cells. The transcription of ISGs ultimately leads to the establishment of antiviral state in the target cells. IFN-α/β the two major representatives of the type I IFN also regulate the immune system by activating effector-cell function. They also promote the development of the acquired immune response (reviewed in Randall and Goodbourn, 2008).

The type II IFNs are also known as the immune IFNs. The only representative of type II IFNs in humans is interferon-gamma (IFN-γ). Unlike type I IFNs which are produced in direct response to viral infections, the type II IFNs is produced after receptor engagement of T cells and natural killer (NK) cells during immune responses (reviewed in Hagberg and Ronnblom, 2015). Type II IFNs signal through a receptor known as interferon-gamma receptor (IFNGR) which has two subunits (Cohen and Parkin, 2001). The interaction between type II IFN and its receptor results in the phosphorylation and activation of STAT1 homodimer which ultimately induces the expression of genes containing IFN-γ activated sequence (GAS) (reviewed in Hagberg and Ronnblom, 2015).

The type III IFNs were recently described and this group of cytokines consists of IFN- lambda1 (IFN-λ1), IFN-lambda2 (IFN-λ2) and IFN-lambda3 (IFN-λ3) and IFNlambda4 (IFN-λ4) as reviewed in (Hagberg and Ronnblom, 2015). The IFN-λ1, IFN-λ2 and IFN-λ3 are also referred to as interleukin-29 (IL-29), interleukin- 28A (IL-28A) and interleukin-28B (IL-28B) respectively (Baccala *et al*., 2005, Donnelly and Kotenko, 2010, Prokunina-Olsson *et al*., 2013). Type III IFNs bind to IFN-λ (IFNL) receptor which is mainly expressed by epithelial cells and also by plasmacytoid dendritic cells (pDCs), B cells

and monocytes (Sommereyns *et al*., 2008, Yin *et al*., 2012) and elicit an antiviral response similar to that of IFN-α & β (reviewed in Hagberg and Ronnblom, 2015).

In addition to the antiviral properties of IFNs which was established by Isaacs and Lindenmann (1957), IFNs have been proven to exhibit a myriad of biological effects which may include immunomodulatory and anti-tumour activities (Theofilopoulos *et al*., 2005).

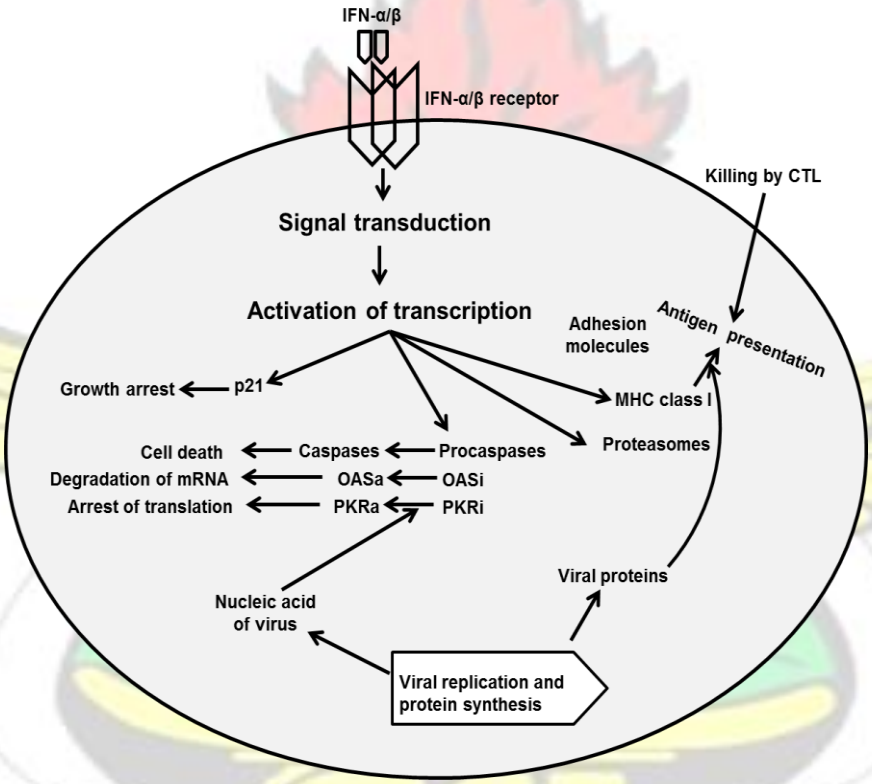

Fig. 2.3: Biological effects of IFN-α and IFN-β. When IFN-α/β binds to its receptor, a sequence of signaling processes is initiated (see Fig. 2.4). The signaling process ultimately leads to the activation of IFN responsive proteins such as PKR, OAS, Mx etc. These proteins are normally enzymatic in nature and also lie latently in the host cells. The activation of these enzymes is dependent on the presence of viral co-factors such as dsRNA. When the viral co-factors are made available, these enzymes bring about a host of changes in the functions of the cells. Some of the changes may include translational arrest, apoptosis etc. All these changes cumulatively confer

antiviral state on the cell. There are other IFN-inducible factors that initiate cell cycle arrest and facilitate the processing and presentation (MHC class I) of viral particles to CTL for degradation.

Moreover, IFN-α/β has immunomodulatory functions which include: they promote the maturation of DCs, they up-regulate the activities of NK cells and $CD8^+$ T cells. They also induce the synthesis of IL-15 which promotes the division of memory $CD8^+$ T cells. Fig. 2.3 was redrawn from Randall and Goodbourn (2008).

2.13 Interferon regulatory factors

Interferon regulatory factors (IRFs) are a family of proteins or transcription factors that are involved in host immune response, haemotopoietic differentiation and immunomodulation (Paun and Pitha, 2007, Huang *et al*., 2010). Nine members of the IRF family of transcription factors have been identified. These include IRF1, IRF2, IRF3, IRF4 (also known as PIP or ICSAT), IRF5, IRF6, IRF7, IRF8 (also known as ICSBP), and IRF9 (also known as ISGF3 γ) (Taniguchi *et al*., 2001, Mamane *et al*., 1999). IRFs contain a conserved amino (N)- terminal DNA binding domain (DBD) with five tryptophan repeats, that bear resemblance to the DBD of Myeloblastosis (myb) transcription factors (Veals *et al*., 1992 , Taniguchi *et al*., 2001). The conserved N-terminal region is made up of about 120 amino acids, which folds into a structure that binds specifically to the IFN consensus sequence (ICS) which is located upstream of the IFN gene (Weisz *et al*., 1992). All the IRFs except IRF1 and IRF 2 have carboxy (C)-terminal regions that have an IRF association domain (IAD) that is responsible for homo- and heteromeric interactions with other family members or transcription factors such as signal transducer and activator of transcription (STAT) (Taniguchi *et al*., 2001, Mamane *et al*., 1999).

Of the IRFs that have been identified, four of them namely IRF1, IRF3, IRF5 and IRF7 have been found to be positive regulators of type 1 IFN gene transcription (Honda *et al*., 2006a). It has been shown that IRF1 is the first of the IRFs that was discovered to activate type 1 IFN gene promoters (Miyamoto *et al*., 1988). The over-expression of IRF1 results in the induction of endogenous type 1 IFN genes. Matsuyama *et al*. (1993) reported in a study that although IRF1 induces type 1 IFN gene in some facets of TLR signaling, the induction of type 1 IFN was normally observed in virus-infected Irf1-/-fibroblast. Genetargeting study has shown that IRF5 regulates the expression of inflammatory cytokine genes such as interleukin (IL)-12 and TNF-α but IRF5 is not needed for type 1IFN gene induction by viruses or TLR agonists (Takaoka *et al*., 2005). This indicates that the exact role of IRF1 and IRF5 in type 1 IFN induction is still not clear.

IRF3 and IRF7 are highly homologous and are now considered as key regulators of type 1 IFN gene expression induced by viruses. IRF3 is constitutively expressed and resides in latent form in the cytosol. Upon viral infection, IRF3 undergoes phosphorylation, dimerization and nuclear translocation (Yoneyama *et al*., 1998, Lin *et al*., 1998). The Cterminal region of human IRF3 has potential virus-mediated phosphorylation sites. These sites include serine 385, 386 (2 S site) and serine 396, 398, 402, 405, and threonine 404 (5 ST site). The phosphorylation of serine 396 was first observed in a study with a phosphospecific antibody (Servant *et al*., 2003). Another report demonstrated that the phosphorylation of serine 386 is critical determinant for the activation of IRF3 (Mori *et al*., 2004). Studies have shown that there is no direct evidence for the phosphorylation of the remaining 5 serine or threonine site (Honda *et al*., 2006b). Unlike IRF3, IRF7 is expressed at a low concentration in most cells and is strongly induced by type 1 IFN- mediated signaling (Sato *et al*., 1998b, Marie *et al*., 1998). When IFNs bind to type 1IFN receptor,

the hetero-trimeric transcriptional activator known as IFN stimulated gene factor 3 (ISGF3) is activated. ISGF 3 comprises IRF9, STAT1 and STAT2 and is responsible for the induction of the IRF7 gene. Like IRF3, IRF7 is found in the cytosol and upon viral infection, IRF7 undergoes serine phosphorylation in its C-terminal region which causes its dimerization and translocation into the nucleus. IRF7 forms a homodimer or heterodimer with IRF3 and each of these different dimmers differentially acts on the type 1 IFN gene family members. Studies have shown that whereas the IRF3 is more potent in activating the IFN-β gene than IFN-α genes, IRF7 activates both IFN-α and IFN-β genes efficiently (Sato *et al*., 1998b, Marie *et al*., 1998).

The events in the cell that control the activity of IRF3 and IRF7 have been elucidated in recent years. These events constitute crucial antiviral pathways that are triggered by the detection of molecular patterns derived from viruses. There are at least two PRR systems in cells that detect the presence of viruses. These include Toll-like receptors (TLRs) found in the cell membrane and cytosolic PRRs such as retinoic acidinducible gene 1 (RIG-1) and melanoma differentiation-associated gene 5 (MDA5).

2.14 Virus induction of IFNs

Viral infections induce the expression of type I IFNs genes. When pathogens invade the system, certain protein molecules such as glycoproteins, nucleic acids, bacterial endotoxin (lipopolysaccharides), bacterial flagella, CpG motifs also referred to as pathogen associated molecular patterns (PAMPs) bind to specific receptors called pattern recognition receptor (PRRs) such as Toll-like receptors (located in the cell membranes) or RIG-1 and

MDA 5 (cystolic) (Honda *et al*., 2006b). The binding of PAMPs to PRRs activate a cascade of events that finally lead to the release of IFNs.

Studies have shown that the release of IFN-β is largely dependent on the activation of nuclear factor kappa B (NF-κB) and IFN regulatory factor-3 (IRF-3) (Paun and Pitha, 2007, Honda and Taniguchi, 2006). Before the cell is induced to release IFN, both NF-κB and IRF-3 lie latently in the cytoplasm. When the cell receives the appropriate signal, the

C-terminus of the IRF3 becomes phosphorylated resulting in conformational changes of IRF 3 protein. These changes cause IRF 3 to form dimmers which leads to unveiling of a nuclear-localization signal (NLS) (Panne *et al*., 2007, Lin *et al*., 1998, Dragan *et al*., 2007). The phosphorylated IRF-3 moves to the nucleus of the cell where it is retained until it is dephosphorylated (Kumar *et al*., 2000). Conversely, the NF-κB is usually bound to an inhibitor molecule called inhibitor of NF-κB (IκB) and lies inactive in the cytoplasm.

During viral infection, the signal that is generated causes phosphorylation of IκB. The phosphorylated IκB then combines with ubiquitin and becomes degraded by proteasomes.

When the NF-κB is freed from its inhibitors, the NLS of the p65 subunits of the NF-κB then becomes accessible and the NF-κB is translocated to the nucleus (Wullaert *et al*., 2006, Hayden and Ghosh, 2004). For the optimal induction of IFN-β gene, a c-jun/ATF2 heterodimer bind to the promoter of the IFN-β gene. The IRF-3, NF-κB and c-jun/ATF2 complexes assemble in a co-operative manner to form what is called enhanceasome. The enhanceasome promote the assembly of basal transcriptional machinery which ultimately results in the production of IFN-β.

The induction of IFN-α gene is not well understood (Civas *et al*., 2002). Whereas the promoters of the IFN-β genes have NF-κB sites, the promoters of the IFN-α gene lack the NF-κB sites. However, the promoters of the IFN-α gene contain several binding sites for members of the IRF family (reviewed in Randall & Goodbourn, 2008). It is not certain

as to which member of the IRF family stimulates the transcription of IFN-α gene but available evidence suggests that IRF-7 has a more relaxed DNA-binding specificity for IRF sites than other members of the IRF family (Morin et al., 2002) and that IRF-7 stimulates IFN-α gene preferentially (Lin *et al*., 2000, Au *et al*., 1998) probably in association with IRF-3 (Morin *et al*., 2002).

2.15 Mechanisms of type I IFN signaling and gene activation

Type I IFNs are one of the major subsets of cytokines that signal through Janus tyrosine kinases (JAKs) and STATs. There are four JAKs namely JAK1, 2, 3 and Tyk2 (O'Shea *et al*., 2013). The STAT family of proteins consists of seven (7) different members: STAT1, 2, 3, 4, 5A, 5B and STAT 6 as reviewed in (Dutta and Li, 2013). The co-operative activities of JAKs and STATs regulate the expression of certain genes of the immune system.

All members of the type I IFN family use the same receptor called IFN-α receptor to initiate signaling (reviewed in Hagberg and Ronnblom, 2015). The IFN-α receptor has two subunits, IFNAR1 and IFNAR2 (de Weerd *et al*., 2007). The IFNAR1 which has a molecular weight of 110-130kD is constitutively bound to tyrosine kinase 2 (Tyk2) and IFNAR2 which has a molecular weight of 55-95kD is constitutively bound to Janus kinase 1 (JAK1) (Mogensen *et al*., 1999, Domanski and Colamonici, 1996). Prior to induction, STAT2 is also bound to IFNAR2 and it is weakly associated with STAT1 (Tang *et al*., 2007 , Stancato *et al*., 1996, Precious *et al*., 2005). When ligand (IFN-α/β) binds to the receptor, both chains are induced to undergo dimerization and the receptor undergoes a

conformational change. This brings the Tyk2 and JAK 1 closer to each other resulting in the phosphorylation and activation of Tyk2 which in turn trans-phosphorylates JAK1 thereby activating it (van Boxel-Dezaire *et al*., 2006, Novick *et al*., 1994, Colamonici *et al*., 1994). The activated Tyk2 phosphorylates tyrosine 466 on IFNAR1. The phosphorylation of IFNAR1 then creates a strong docking site for STAT 2. The STAT2 is in turn phosphorylated on tyrosine 690 by Tyk2. On the other hand, the activated JAK 1 phosphorylates STAT1 on tyrosine 701 (Stark *et al*., 1998, Muller *et al*., 1994a). Once STAT1 and STAT2 have become phosphorylated, they come together and form a heterodimer complex that is highly stable. When STAT1 is phosphorylated and subsequently forms a dimer with STAT2, a novel nuclear localization signal (NLS) is created (Banninger and Reich, 2004). The creation of NLS and the simultaneous phosphorylation of STAT2 inactivates the nuclear export of STAT2 (Frahm *et al*., 2006) so that dimmers become translocated into the nucleus and retained until they become dephosphorylated (Reich and Liu, 2006). The heterodimer complex of STAT1-STAT2 that is transported to the nucleus combines with a cystolic transcription factor known as IRF-9 to form IRF-9-STAT1-STAT2 heterotrimer complex called the interferon stimulated gene factor-3 (ISGF3). The ISGF3 enters the nucleus and binds to the interferon-stimulated response element (ISRE) (Williams, 1991, Kessler *et al*., 1988) that is present in the promoters of most IFN-responsive genes and brings about transcriptional activation of genes that switch on the anti-viral and anti-tumour effects.

It was thought at first that the assembly of ISGF3 occurred in the nucleus but recent study by Tang *et al*. (2007) seem to suggest that assembly may be coordinated at the receptor. Tang *et al*. (2007) reported that IFNAR2 chain of the interferon receptor recruits the transcriptional co-factor CBF when stimulated by interferon. The CBF that is recruited

to the IFNAR2 catalyses the acetylation of IFNAR2. The IFNAR2 acetylation creates a docking site for IRF-9 that in turn also gets acetylated as do STAT1 and STAT2 that are bound to the receptor. The IRF-9 acetylation is required for DNA binding and the acetylation of the STAT factors may help in the assembling of ISGF3 complex (Tang *et al*., 2007).

In addition to the above signaling pathways, other pathways are activated by interferon and these pathways can influence the outcome of IFN-induced transcription

(Platanias, 2005). Transcription activation by STAT1, whether activated by IFN-α/β or IFN-γ also require phosphorylation on serine 727 for full activity and for mounting a full antiviral response. When serine 727 of STAT1 is phosphorylated, the interaction between STAT1 and basal transcription machinery as well as other adaptor proteins becomes facilitated. Conversely, STAT2 is not serine phosphorylated in response to IFN. However, STAT2 binds CBP/p300 and facilitates interaction with the basal transcriptional machinery

(Bhattacharya *et al*., 1996).

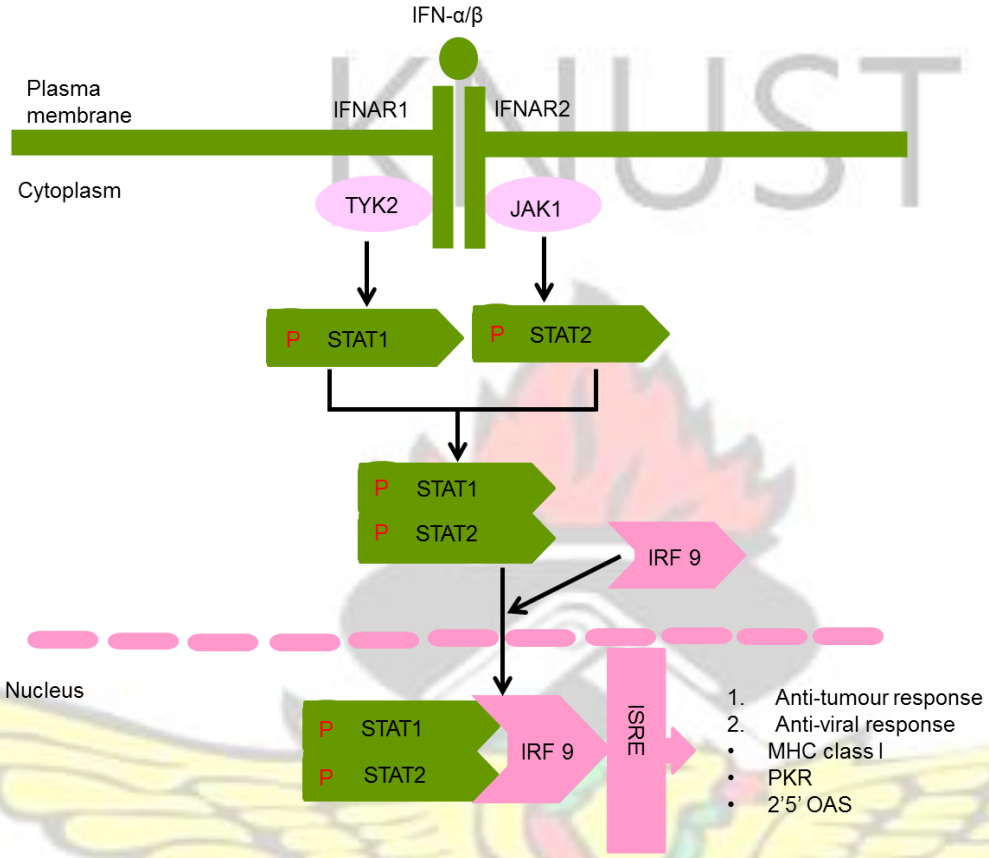

Fig. 2.4: Model diagram of signaling pathway that is activated by IFN-α/β. The biological activities of IFN-α/β are started when a ligand such as IFN-α/β binds a receptor called interferonalpha receptor (IFNAR). The IFNAR consists of the subunits IFNAR1 and IFNAR2 which associate with Tyk2 and Jak1 respectively. When IFN-α/β binds it receptor, the Jaks become phosphorylated and activated. The activated Jaks in turn phosphorylate Stat1on tyrosine 701 and Stat2 on tyrosine 690. The phosphorylated Stat1 and 2 come together and form heterodimer. The dimerized Stat1 and 2 associate with IRF-9 and form a trimeric complex known as ISGF-3. When ISGF-3 enters the nucleus, it binds ISRE and initiates transcription of IFN-inducible genes that switch on the antiviral and anti-tumour effects. It is worth noting that in order for Stat 1 to become fully functional, it is also phosphorylated on tyrosine 727.

2.16 IFN responsive genes or elements

The ability of IFNs to fight against viruses is essential for the survival of the higher vertebrates against viral infection. IFNs are the first line of defense against viral infection; acting rapidly within hours or few days after infection. Studies have shown that when cells are treated with IFN-α/β, the activation and expression of several genes are up-regulated and the expression of all these genes combine to establish an antiviral state (reviewed in Randall and Goodbourn, 2008). For example a study conducted by Clemens (2005) to elucidate the role of IFN in establishing antiviral state reported that mice that were deficient in IFN-α and IFN-β were highly susceptible to viral infections indicating that IFN-α and IFN-β are crucial in fighting against viruses. It is important to note that no single gene is pivotal in the establishment of the antiviral state and that several genes work together to limit the replication of any given virus. Several genes and their resultant proteins are involved in the antiviral response. Information on the role of IFN responsive proteins such as PKR, OAS-3, Mx, ISG-15 and PML in establishing anti-viral state is available in literature. So under this section, I will attempt to discuss the role of the following IFN responsive proteins: PKR, OAS-3, Mx, ISG-15 and PML in: (i) regulating protein synthesis, (ii) regulating cell proliferation, (iii) regulating apoptosis and (iv) prevention of tumours.

The OAS is one of the enzymatic pathways induced by IFNs. When activated the OAS pathway initiates a cascade of events which culminate in the inhibition of protein synthesis which lead to the destruction of both viruses and infected cells and thus establishes the antiviral state (Sen, 2001, Muller *et al*., 1994b).

The protein kinase R (PKR) encoded by the gene eukaryotic translation initiation factor 2- alpha kinase 2 in humans (EIF2AK2) (Feng *et al*., 1992) is produced in inactive form. When activated by dsRNA (Patel *et al*., 2000, Ito *et al*., 1999), PKR phosphorylates eukaryotic translation initiation factor known as eIF-2. The eIF-2 then forms a complex with another protein eIF-2B and becomes inactivated. The inactivation of eIF-2 leads to the rapid inhibition of cellular mRNA translation and thereby prevents viral protein synthesis within the cell. Active PKR is also able to induce cellular apoptosis through complex mechanisms to further prevent the spread of viruses. PKR has also been involved in regulating cell proliferation, playing a role as tumour suppressor (reviewed in Randall and Goodbourn, 2008).

The Promyelocytic leukaemia (PML) gene is known to direct the synthesis of a protein referred to as PML protein. The PML protein in association with other PMLnuclear bodies (PML-NBs) performs functions which may include establishing antiviral state, suppression of growth of tumours; apoptosis etc (reviewed in Randall and Goodbourn, 2008).

2.17 Anti-cancer activity and mechanism of IFNs

When Isaacs and Lindenmann first discovered IFNs in 1957, they described them as proteins capable of inducing resistance to viral infections (Isaacs and Lindenmann, 1957). Some years later, the role of IFNs in preventing the growth of tumours was described. In 1960, Atanasiu and Chany showed that hamsters that were pre-treated with IFN preparations before being inoculated with polyoma virus showed delay in the appearance

of tumours (Atanasiu and Chany, 1960). Following the work of Atanasiu and Chany, Paucker *et al*. (1962) also described the anti-proliferative effects of IFNs by showing that L cells exposed to either UV-irradiated Newcastle Disease virus or to IFN led to a temporary slow-down in the cell growth. A year later Lampson *et al*. (1963) demonstrated that both crude and semi-purified IFN preparations administered into chicks prior to infection with the Rous sarcoma virus inhibited the development of tumours. Thereafter, the capacity of IFNs to inhibit the growth of viral-induced and transplantable tumours have been extensively studied. It has been shown that interferon treatment of mice after infection with leukemia virus was detected to reduce the features related to leukemia with an associated increase in mouse survival (Sarma *et al*., 1969).

A host of studies have identified endogenous type I IFNs as playing key role in the recognition of tumours by the innate immune system and this role serves as a conduit to instant adaptive T cell response (Fuertes *et al*., 2011, Diamond *et al*., 2011). For example, it has been demonstrated that endogenous IFN-β secreted by $CD11^{+}$c dendritic cells (DCs) in response to the existence of tumour activate $CD8\alpha^{+}$ DC family of cells and this enhances the detection of tumour antigens by $CD8^{+}$T cells *in vivo* (Fuertes *et al*., 2013). The type I IFN-activated tumour recognition by the innate immune response is critically necessary for steering the early adaptive immune response against many tumours found in mice (Gajewski *et al*., 2013, Spranger *et al*., 2013). A study conducted by Indraccolo (2010) demonstrated that type I IFNs have antiangiogenic property which indirectly repress the growth and development of tumours (Indraccolo, 2010). Another evidential proof of the anti-cancer activity of type I IFNs was obtained from a study conducted by Jablonska *et al*.

(2010) who reported that in total absence of IFN-β, neutrophils are greatly attracted to the place where the tumour is developing and that the increased attraction of neutrophils improves the vasculature and thus promotes the progression of the cancer (Jablonska *et al*., 2010). Spaapen *et al*. (2014) conducted a study to determine the effects of intra-tumoural production of type I IFNs on tumour cells in murine melanoma cell line B16-F10. They inserted the IFN-β gene into the B16-F10 cell lines which enabled the cells to produce large amount of IFN-β. They reported that the introduction of the B16-F10 tumour cells into syngeneic C57BL16 mice culminated in total repression of tumours. In order to decipher the involvement of the type I IFN signaling in the therapeutic activity of IFN-β on the target cells, the IFN-β secreting B16-F10 tumour cells were transplanted into type I IFN receptor deficient mice by Spaapen *et al*. (2014). They reported that tumours grew progressively in these mice indicating that the role of IFN-β in eradication of tumours depends on signaling through the host cells. Similarly the same results in the context of tumour repression was obtained by Spaapen *et al*. (2014) when the experiment was done with IFN-α.

The exact mechanism by which IFNs prevent the growth of certain tumours is not clear. However, a sizeable number of studies have attempted to unravel the molecular mechanism by which IFNs inhibit tumour growth. For the purposes of this thesis, the mechanism by which key elements (with emphasis on STAT1) of the JAK-STAT-ISRE arm of the type I IFN pathway prevent growth of tumours will be discussed.

2.17.1 Anti-cancer property and mechanism of STAT1

The STAT1 signaling pathway activated by IFNs has been thought about to be tumour suppressive (Dunn *et al*., 2006) and also activate genes that modulate immune

functions such as the establishment of antiviral state and apoptosis induction (Khodarev *et al*., 2012). The anti-tumour function of STAT1 is considered to be critically crucial at the beginning of the tumour development and its role is to promote the eradication of cells that have become transformed by both the innate and the adaptive immune system (Tymoszuk *et al*., 2014). Studies have shown that activation of STAT1 leads to the up-regulation of genes needed for processing and presentation of antigens in dendritic cells (Tymoszuk *et al*., 2014). Aside its functions on immune cells; STAT1 has been shown in some studies to prevent tumour development in tumour epithelium (Raven *et al*., 2011, Klover *et al*., 2010) probably by promoting apoptosis and inhibiting cell proliferation in response to the presence of stimuli such as oncogenic cells (Kim and Lee, 2007). It has been reported that STAT1 induces apoptosis by promoting the expression of cell surface death receptor family and their ligands. For example, STAT1 has been shown to increase the expression of Caspases, Fas and FasL (Ouchi *et al*., 2000, Lee *et al*., 2000). STAT1 has been shown to suppress Mdm2 an inhibitor of the tumour suppressor protein p53 and as a result functions as co-activator of p53 (Pensa *et al*., 2008, Kominsky *et al*., 2000, Chin *et al*., 1997, Allione *et al*., 1999).

The tumour suppressive function of STAT1 has also been linked to the ability of STAT1 to regulate angiogenesis. It has been reported that STAT1 negatively regulate tumour angiogenesis and thus prevent the growth of tumour and metastasis by inhibiting VEGF biological activity and also suppresses the expression of pro-angiogene FGF-β (Pensa *et al*., 2008). STAT1 has also been implicated in preventing the metastasis of breast cancer. For example, Ilkovitch *et al*. (2008) reported that STAT1 can suppress the

expression of the urokinase-type plasminogen activator (uPA) gene in breast cancer and that this inhibition aid in preventing the spread of breast cancer.

Another evidential proof to support the fact that STAT1 plays a crucial role in inhibiting the growth and development of tumours was obtained from studies done with STAT1 deficient mice which were shown to develop tumours within shorter duration compared to STAT1 expressing controls (Raven *et al*., 2011, Klover *et al*., 2010).

2.17.2 Anti-cancer property and mechanism of STAT2

Another component of the type I IFN dependent JAK-STAT-ISRE pathway that has been thought of to mediate the anti-tumour effects of the type I IFN is STAT2

(Schindler *et al*., 1992, Fu *et al*., 1992, Darnell Jr *et al*., 1994, Romero-Weaver *et al*., 2010, Du *et al*., 2009, Clifford *et al*., 2003). Some *in vivo* studies have been done to support the role of IFN-activated STAT2 in inhibiting the growth of tumour. For example Wang *et al*. (2003) reported in their study using STAT2 deficient transgenic mice which constitutively produced IFN-α (GIFN/Stat2 $^{-/-}$) in the central nervous system (CNS) that the mice died prematurely because there was a spontaneous formation of medulloblastoma (Wang *et al*., 2003). In another study conducted by Yue *et al*. (2015) using wild type (WT) and Stat2 $^{-/-}$ mice, it was reported that when WT and Stat2 $^{-/-}$were injected with murine B16-F1 melanoma tumour cell lines, the WT mice developed relatively smaller tumours compared to the Stat2 $^{-/-}$counterparts (Yue *et al*., 2015).

Even though there are some evidence to support the tumour suppressor function of STAT2, the exact mechanism by which this is achieved is still under investigation. It has

been reported that loss of STAT2 could possibly lead to the down-regulation of some genes such as Ifi204 and Cxcl9 that have immunomodulatory function (Yue *et al*., 2015). Ifi204 for example is a type I IFN target gene that plays a key role in cell growth inhibition and macrophage cell differentiation (Lembo *et al*., 1998, Dauffy *et al*., 2006). Cxcl9 on the other hand is an IFN target gene that is induced by IFN-α/β and IFN-γ and its function is to act as T-cell chemo-attractant (Padovan *et al*., 2002, Guirnalda *et al*., 2013). Studies have shown that knocking down Cxcl9 in mice could result in the formation of larger tumours due to immuno-surveillance escape (Guirnalda *et al*., 2013).

Another mechanism by which STAT2 may inhibit tumour growth could be due to its ability to activate DCs in presenting tumour antigens. Yue *et al*. (2015) reported that STAT2 ⁻/ ⁻DCs were unable to effectively present tumour antigens to target cells *in vivo* (Yue *et al*., 2015). The defective nature of the DCs in STAT2 ⁻/ ⁻mice could make STAT2 ⁻ / ⁻more permissive to form tumours due its inherent IFN signaling defect (Yue *et al*., 2015, Park *et al*., 2000).

2.17.3 Anti-cancer properties and mechanisms of JAK1, Tyk2, OAS-3 and IRF-9,

Under this sub-section, the role of JAK1, Tyk2, OAS-3 and IRF-9 as drivers in preventing the growth and development of cancers will be discussed.

As stated already, JAK 1 is a member of the JAK family of proteins that is considered to be very essential in normal cell signaling processes that have been implicated in tumourigenesis (Aaronson and Horvath, 2002, Schindler *et al*., 2007, Zhou *et al*., 2014). It has been reported that JAK1 plays a role in the formation of tumour through its interaction

with STAT3 (Casey *et al*., 2015). The activation of STAT3 by JAK1 leads to survival of cancer cells, proliferation, metastasis and promotion of angiogenesis (Kortylewski and Yu, 2008). In addition Song *et al*. (2011) reported that JAK1 is responsible for STAT3 activation in lung cancer and that inhibition of JAK1 with small molecules or RNA interference resulted in the loss of STAT3 activation and prevented lung cancer growth (Song *et al*., 2011).

Another member of the JAK family which transduces cytokine and growth factor signaling is Tyk2. Tyk2 is commonly associated with five different receptor chains namely IFNAR1, interleukin (IL) 10 receptor 2 (IL-10R2), IL-12 receptor β1 (IL-12Rb1), IL-13 receptor α1 (IL-13Rα1) and glycoprotein 130 (gp130). Although Tyk2 has been reported to play a role in inflammation that may contribute to tumour development, progression and spread (Elinav *et al*., 2013, Grivennikov *et al*., 2010) due to its association with several receptor chains, its importance in tumour immuno-surveillance has also been reported (Ubel *et al*., 2013). Tyk2 has been reported to play a critical role in the anti-cancer property of cytokines such as type I IFNs and IL-2 (Colombo and Trinchieri, 2002, Zitvogel *et al*., 2015). In addition, the role of Tyk2 in type I IFN induced apoptosis in primary pro-B cells and pancreatic cells has also been reported (Gamero *et al*., 2006, Marroqui *et al*., 2015, Potla *et al*., 2006). Studies have shown that the induction and interaction of Tyk2 with proapoptotic factors is highly essential in IFN-α induced B lymphocyte growth arrest or apoptosis (Shimoda *et al*., 2010, Shimoda *et al*., 2002).

The OAS pathway is another component of the type I IFN induced JAK-STATISRE pathway that has been reported to have tumour suppressive activity. The tumour suppressive activity of OAS3 pathway is executed by RNase L (endoribonuclease) a component of OAS

that requires 2-5A synthetase for its activity. A study conducted by Silverman (2003) reported that mutations in RNase L predispose men to an increased incidence of prostate cancer (Silverman, 2003). The role of RNase L in counteracting prostate cancer is believed to be due to its ability to degrade RNA, thus initiating a cellular stress response that leads to apoptosis (Silverman, 2003).

Initially identified as transcriptional regulatory factors, IRFs have also been reported to regulate cell differentiation, cell growth and apoptosis in different cells (Fragale *et al*., 2013). Studies have shown that mutation is IRFs can increase the susceptibility to and progression of several cancers (Fragale *et al*., 2013). Even though all IRFs have been reported to play one role or the other in the regulation of oncogenesis as they modulate cellular responses involved in anti-tumour immune functions (Savitsky *et al*., 2010, Tamura *et al*., 2008), only the role of IRF-9 will be discussed under this sub-section. The role of IRF-9 in inhibiting tumour development is linked to the anti-tumour property of type I IFNs (Belardelli *et al*., 2002, Tsuno *et al*., 2009). As already stated IRF9 is part of trimeric complex, known as ISGF3, which also include STAT1 and STAT2 (Kessler *et al*., 1990, Taniguchi *et al*., 2001). Studies have shown that type I IFNs activate *TP53* through ISGF3 binding to ISREs that are located in *TP53* promoter (Takaoka *et al*., 2003). As one of the component of ISGF3, IRF9 has been reported to stimulate the p53 pathway upon exposure of cells to endogenously induced or exogenously administered type I IFNs (Yanai *et al*., 2012). In addition, it has been reported that most of the ISGs that have anti-tumour activity require IRF-9 to be activated including some IRFs such as IRF-5 and IRF-7

(Fragale *et al*., 2013).

CHAPTER 3 MATERIALS AND METHODS

3.0 Introduction

Under this chapter, the experimental procedures that were used in this research were clearly described. References were made to background literature where necessary. It must be noted that most of the experiments were performed at the Virus Research Laboratory at the Department of Clinical Microbiology of the School of Medical Sciences at the Kwame Nkrumah University of Science and Technology (KNUST) in Kumasi. However, the RTqPCR experiments were performed at the Kumasi Centre for Collaborative Research (KCCR) in Kumasi. All reagents and chemicals used and their components have been comprehensively listed at the appendices.

3.1 Large-scale expansion and purification of plasmid DNA using the EndoFree Plasmid Maxi Kit

The plasmid DNA (pISRE-luc) was extracted from the *Escherichia coli* (*E. coli)* strain 5DHα (kindly donated by Professor David J. Blackbourn, University of Surrey). The *E.coli* strain 5DHα harboured plasmid DNA vector that expressed pISRE-luc. Frozen *E.coli* strain 5DHα which was revived on Luria Bertani (LB) agar (Sigma Aldrich, USA) containing 100µg/ml ampicillin (Thermo scientific, Germany) and incubated (37^{o}C, 18 hours). A single colony of the bacteria was picked and inoculated into a 5 ml LB broth (Sigma Aldrich, USA) containing 100 µg/ml ampicillin (Thermo scientific, Germany) and incubated (37^{o}C, 8 hours) with vigorous shaking (300 rpm) to make a starter culture. After

8 hours of incubation, 500µl of the starter culture was transferred into 250ml of LB broth (Sigma Aldrich, USA) containing 100µg/ml of ampicillin (Thermo scientific, Germany) at a dilution of approximately 1:500 and incubated (37^{o}C, 16 hours) with vigorous shaking (300 rpm). At a cell density of approximately 3-4 x 10^{9}cells/ml, the bacterial cells were harvested by centrifugation (6000 xg, 4^{o}C, 15 minutes). The supernatants were discarded and the bacterial pellets were used for the plasmid DNA extraction using the EndoFree Maxi Prep Kit (Qiagen, USA) following the manufacturer's instruction. Compositions of the buffers P1, P2, P3, ER, QBT, QC and QN used during the extraction process are shown in the appendix **(Appendix B, Table 1)**.

A 10 ml of buffer P1 was added to each sample tube to the bacterial pellets to completely suspend the bacterial cells and begin the lysing process. Next 10 ml of buffer P2 was added to each tube and vigorously mixed to lyse the bacterial cells. The samples were then incubated at room temperature (25^{o}C, 5 minutes). The proteins, genomic DNA and cell debris after lysis were precipitated by adding 10 ml of chilled buffer P3 to each sample and were immediately and thoroughly mixed with vigorous inverting. The lysates were then transferred into the barrel of the QIAfilter cartridges and then incubated at room temperature (25^{o}C, 10 minutes). After 10 minutes, the lysates were filtered by gravity into fresh 50 ml falcon tubes. After this stage, 2.5 ml of buffer ER was added to each filtered lysate and then incubated on ice for 30 minutes. The ice-incubated filtered lysates were then transferred into the QIAGEN-tips which had been equilibrated with buffer QBT. The lysates were allowed to filter by gravity in the QIAGEN-tips after which the tips were washed two times by adding 30 ml of buffer QC in each wash step. After washing, the DNA of each sample was eluted into fresh tubes by adding 15 ml of buffer QN to each QIAGEN-tip. The eluted DNA of each sample was precipitated by adding 10.5 ml of room temperature isopropanol to each tube and then centrifuged (5000 xg, 4^{o}C, 60 minutes). The supernatants were discarded and the pelleted DNA washed with 5 ml of endotoxin-free room temperature 70% ethanol to remove the precipitated salts followed by centrifugation (5000 xg, 4^{o}C, 60 minutes). The supernatants were discarded and the pelleted DNA was

air-dried for 10 minutes. The dried DNA of each sample was then redissolved in 100µl of endotoxin-free buffer TE. The quantity and the purity of the plasmid DNA was determined using NanoDrop 1000 version 3.8.1 (Thermo Scientific, USA). Agarose gel (Invitrogen, Spain) electrophoresis was later used to indicate the presence of the plasmid DNA (pISREluc) **(Fig. 3.1).**

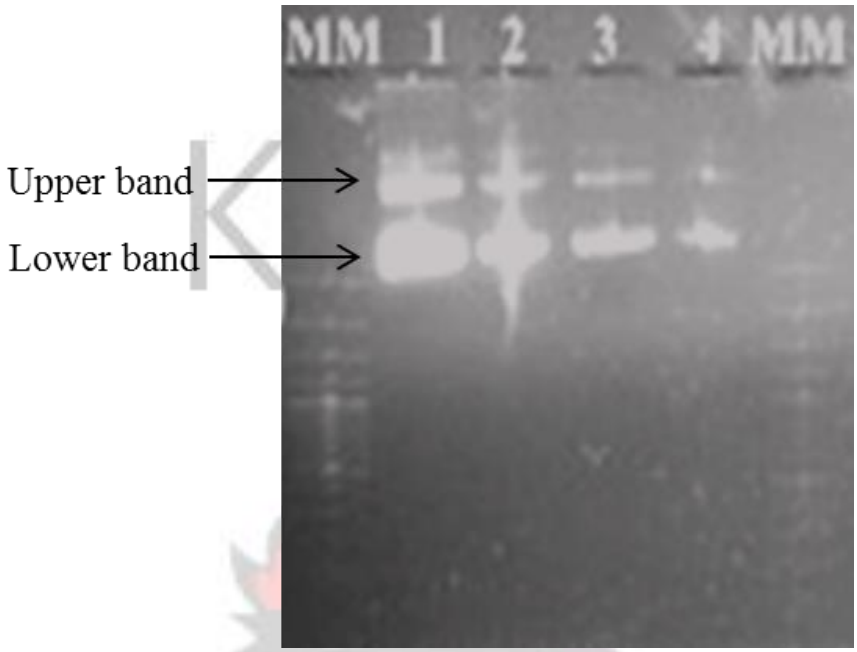

Fig.3.1: Agarose gel analysis of the plasmid DNA purified from *E.coli* 5DH-α using EndoFree Plasmid Maxi Kit from Qiagen. The plasmid DNA eluted was diluted 1:10 (lane 1); 1: 100 (lane 2); 1: 1000 (lane 3) and 1: 10,000 (lane 4). Five microliters (5µl) of each of the dilutions was run on a 1% agarose gel at 60V for 50 minutes using ethidium bromide as a stain. MM: 1kb DNA ladder. The eluted sample contained pure plasmid DNA in supercoiled (lower band) and open circular (upper band) forms.

3.2. Culturing of cells

All the cells were kindly donated by Professor David J. Blackbourn of the University of Surrey, UK. The cell lines used in this research were human hepatoma cell line HepG2 (ECACC 85011430) and mouse fibroblast cell line L929 (NCTC) (ECACC 85103115).

The cells were usually cultured in Dulbecco's Modified Eagle's Medium (DMEM) that contained high glucose, sodium pyruvate, L-glutamine and HEPES (Sigma Aldrich, Germany) supplemented with 10% v/v heat inactivated foetal bovine serum (FBS) (Sigma Aldrich, Germany), 1% v/v non-essential amino acids (Sigma Aldrich, Germany) and 1% v/v penicillin-streptomycin (Gibco by life technologies, UK) (this medium shall be referred to as growth medium in the succeeding sections) in T-75 and T-25 tissue culture flasks and incubated (37^{o}C, 5% CO_2, under humidified condition). At about 80-100% confluence, the cells were washed with phosphate buffered saline (PBS) and trypsinized with 0.25% trypsin-EDTA (Gibco life technologies, UK). Depending on the nature of the experiment, the cells were sub-cultured in 6 or 96-well plates. Before seeding in 6 or the 96-well plates, the cells were counted using a Neubauer haemacytometer and trypan blue dye exclusion method under x10 objective of the Nikon microscope.

The percentage viability was determined using the equation:

$$\% \text{ Viability} = \frac{\text{number of unstained cells (live cells)}}{\text{total number of cells (both dead and live cells)}} \times 100$$

3.3 Determining the cytotoxic effects of AFB_1 on HepG2 cells

The AFB_1 (Sigma Aldrich, Germany) which was used in the study was dissolved in dimethyl sulfoxide (DMSO) (Sigma Aldrich, Germany) to a stock concentration of 3200

µM. The stock solution was stored in working aliquots wrapped in aluminium foil at -20 ^{o}C until ready to use. HepG2 cells were cultured in growth medium in duplicate wells of 96-well plates at density of 5 x 10^4 cells per well. At 60% confluence, the cells were treated with increasing concentrations of AFB_1 (Sigma Aldrich, Germany) (0, 3.2, 32, 320 & 3200 µM) and incubated (37^{o}C, 5% CO_2, under humidified condition, 24 hours). Cells which were treated with 0.2% DMSO were used as controls because the AFB_1 was dissolved in DMSO. At 24 hours post-treatment, the AFB_1 containing medium was removed and the cells were treated with fresh growth medium without AFB_1. The cytotoxic effect of AFB_1 on the HepG2 cells was evaluated after 24, 48 and 72 hours using the CellTiter 96®

Aqueous One Solution (Promega, USA) following the manufacturer's instruction. The CellTiter 96® Aqueous One Solution Cell Proliferation Assay is used to determine the number of cells viable in cytotoxicity or cell proliferation assays. The assay is based on the reduction of tetrazolium salt to water soluble formazan dye by metabolically active cells. The CellTiter 96® Aqueous One Solution contains an active tetrazolium compound called MTS ([3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4sulfophenyl)-2H-tetrazolium, inner salt; MTS]). In addition to the tetrazolium salts, the One Solution also contains an electron transfer reagent (ETR) called phenazine ethosulfate (PES). The PES is reduced by reducing equivalents such as NADH or NADPH inside the cells. The reduced PES then reduces the MTS to intensely-coloured formazan outside the cell. The quantity of formazan produced is directly proportional to the number of living cells present in the culture well. The degree of cell proliferation or death can therefore be quantified by reading the absorbance of the formazan produced at wavelength of 490 nm using 96-well plate reader.

At the time point of treatment, 20µl of the Cell Titer AQueous One Solution (Promega, USA) pre-warmed to room temperature was added to each well of cells and incubated (37^{o}C, 5% CO_2, under humidified condition, 4 hours). At 4 hours of incubation the absorbance of each well of cells was measured at 490 nm wavelength using iMark Micro plate reader (Bio-Rad, USA). Each experiment was conducted three times in duplicates. Each of the three experiments was conducted independently.

$$\% \text{ viability} = \frac{\text{Average absorbance of } AFB_1 \text{ treated cells} - \text{Average blank absorbance}}{\text{Average absorbance of } AFB_1 \text{ untreated cells} - \text{Average blank absorbance}}$$

The percentage viability was calculated as:

3.4 Determining the minimum concentration of rIFN-α that induces the maximum activity of rIFN-α inducible ISRE promoter

After determining the maximum concentration of AFB_1 that was not toxic to HepG2 cells, the next stage was to determine the minimum concentration of rIFN-α that could induce the maximum activity of the rIFN-α responsive promoter (pISRE-luc) using the dual luciferase reporter gene assay technique. The rIFN-α will be referred to as IFN in the subsequent sections of this thesis.

3.4.1 Transient transfection of cultured cells

HepG2 cells were grown to about 80% confluence in growth medium in duplicate wells of 96-well plates. A transfection mixture that contained the appropriate amount of

DNA and Lipofectamine-2000 (Invitrogen, USA) **(Appendix C, Table 2)** in serum and antibiotic free medium was prepared and incubated for 20 minutes at room temperature. Then 10µl of the transfection mixture was added drop-wise to different areas of each well of cells and incubated (37^{o}C, 5% CO_2, under humidified condition, 24 hours). A reporter plasmid that expressed the firefly luciferase was co-transfected with a plasmid that constitutively expressed the *Renilla* luciferase to which the firefly luciferase activity was normalized.

At 24 hours post transfection, the cells were stimulated with or without increasing concentration of IFN (100, 200, 300 and 400 IU/ml) (PBL, Biomedical laboratories, USA). At 24 hours post-stimulation, the cells were harvested and luciferase reporter gene assay performed with a luminometer.

3.4.2 Dual luciferase reporter gene assay

The dual luciferase reporter gene assay which allows the simultaneous expression and measurement of two individual reporter enzymes produced by two reporter genes within a single system was used. The firefly luciferase from the firefly beetle called *Photinus pyralis* was used as the experimental reporter while the *Renilla* luciferase from *Renilla reniformis* also called sea pansy was used an internal control to normalize the data obtained from the experimental reporter. The firefly luciferase catalyses the oxidation of luciferin to oxyluciferin and emits light at about 560 nm. The *Renilla* luciferase oxidizes coelenterazine into coelenteramide and emits light at about 480 nm. During measurement, 100 µl of the firefly luciferase reagent (LAR II) is injected into each well that contains a sample cell lysate. The light output over 10 seconds is measured. Immediately after the first

measurement, 100 µl of the reagent for the second luciferase (Stop & Glo® Reagent) is injected into each well and again the light output is measured. The function of the second reagent is to quench the first reaction and delivers the substrate for *Renilla* luciferase reaction.

The luciferase activity was therefore measured using the dual luciferase assay (DLA) (Promega, USA) following the manufacturer's instruction. The required volume of 1X passive lysis buffer (PLB), Luciferase Assay Reagent II (LARII) and Stop & Glo buffer were prepared according to the manufacturer's instruction. For example, the appropriate volume of the 1X PLB was prepared by adding 1 volume of 5X PLB to 4 volumes of distilled water. The appropriate volume of 1X Stop & Glo solution was prepared at a ratio of 1:50; for example by adding 20 µl of the 50X Stop & Glo substrate to 980 µl of Stop & Glo buffer in an eppendorf tube wrapped in aluminium foil to protect the solution from the direct effect of light. The LAR II solution was prepared by dissolving the lyophilized luciferase assay substrate in 10 ml of luciferase assay buffer II and then distributed into working aliquots and stored frozen at -80^{o}C until ready to use.

Prior to performing the DLR assay, all the needed reagents were pre-warmed to room temperature. Also each of the two auto injectors (one for LAR II reagents and the other for Stop & Glo reagent) of the luminometer was primed with 400 µl of distilled water. After priming with distilled water, the injector lines were finally prepared by priming injector 1 with 400 µl of LAR II and injector 2 with 400 µl of Stop & Glo reagents. The injectors 1 and 2 of the luminometer were automatically programmed to dispense 100 µl of each reagents. The luminometer was also programmed to make a 10-second measurement for the firefly with a delay of 10 seconds followed by a 10 second measurement for the

Renilla luciferase. The data which was automatically saved to Excel spread sheet was retrieved and the pISRE-luc activity normalized to *Renilla* activity performed.

3.5 Determining the effects of AFB_1 on the type I IFN pathway

HepG2 cells were grown in duplicate wells of 96-well plates at density of 5 x 10^4 cells per well. At about 60% confluence, the cells were transiently co-transfected as described previously **(section 3.5.1).** At 24-hours post-transfection, the cells were stimulated with or without IFN (400 IU/ml) (PBL, Biomedical laboratories, USA) and treated with or without increasing concentration of AFB_1 (0.8, 1.6, 3.2, 6.4, 10 and 32 µM) (Sigma Aldrich, Germany). At 24-hours post-stimulation and treatment, the cells were harvested, lysate prepared and dual luciferase reporter gene assay performed as previously described **(section 3.4.2).**

3.6 Quantifying the mRNA levels of some of the key genes of the JAK-STAT-ISRE pathway

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was employed to quantify the mRNA levels of representative genes such as JAK1, STAT1 and OAS-3 of the JAK-STAT-ISRE arm of the type I IFN response pathway. The various stages of RT-qPCR experiments are described in the subsequent sub-sections.

3.6.1 Isolating and purifying total RNA

The cellular total RNA was isolated and purified using GeneJET RNA purification kit (Thermo scientific, Germany) **(Appendix B, Table 1)** following the manufacturer's instruction. Briefly HepG2 cells which were grown in 6-well plates and stimulated with or without IFN (PBL, Biomedical laboratories, USA) and treated with or without AFB_1 (Sigma Aldrich, Germany) were harvested in 1.5 ml of cold PBS (Oxoid limited, UK).

Cells were transferred into RNase/DNase-free eppendorf tube and after centrifugation at (250 xg, 4^{o}C, 5 minutes) the supernatant was discarded. Six hundred microliters (600 µl) of lysis buffer supplemented with 14.3M β-mercaptoethanol (Bio-Rad, USA) was added to each tube to re-suspend and lyse the pelleted cells. The lysate was passed through a blunt 20-gauge needle fixed onto RNase-free syringe 7 times to ensure complete homogenization of the lysate. Three hundred and sixty microliters (360 µl) of 99% analytical grade ethanol (Fisher scientific, UK) was added to the cell lysate. After gentle mixing, the homogenized lysate was transferred into the GeneJET RNA purification column inserted into a collection tube and then centrifuged (12000 xg, 4^{o}C, 1 minute). The ethanol added (together with guanidine thiocyanate a chaotropic salt contained in the lysis buffer) caused the RNA to precipitate and bind to the silica membrane while the lysate is spun through the column thus separating the RNA from the rest of the solution. This step was repeated until all the lysate had been centrifuged. The GeneJET RNA purification column was washed 2 times to remove impurities such as residual salts from the RNA bound to the silica membrane of the column. Seven hundred microliters (700 µl) of wash buffer 1 was added to the GeneJET

RNA purification column and then centrifuged (12000 xg, 4^{o}C, 1 minute) to wash the membrane of the column. Six hundred microliters (600 µl) of wash buffer 2 was added to the GeneJET RNA purification column and then centrifuged (12000 xg, 4^{o}C, 1 minute) to

wash for the second time. Two hundred and fifty microliters (250 µl) of the wash buffer 2 was added and centrifuged (12000 xg, 4ºC, 2 minutes). The flow-through in the first, second and the third wash steps were discarded and the GeneJET RNA purification column was re-spun (20000 xg, 4ºC, 1 minute) to remove any residual fluid in the column. The collection tubes containing the flow-through solutions were discarded and each GeneJET RNA purification column was transferred into 1.5 ml sterile RNase-free micro-centrifuge tube. Hundred microliters (100 µl) of nuclease free water was added to each GeneJET RNA purification column membrane and then centrifuged (12000 xg, 4ºC, 1 minute) to elute the RNA. The quantity and the purity of the total RNA was determined using NanoDrop 1000, version 3.8.1 (Thermo scientific, USA) and was later confirmed by 1% agarose (Invitrogen, Spain) gel electrophoresis **(Fig. 3.2).** The eluted total RNA was stored at -80ºC until ready to use.

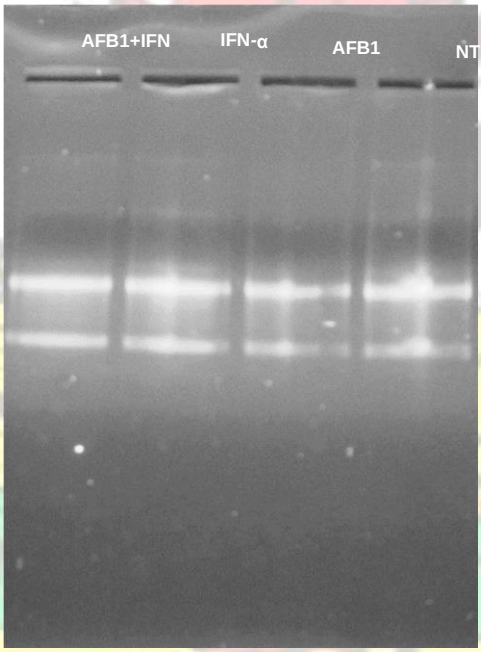

Fig.3.2: Agarose gel analysis of total RNA purification procedure using Thermo Scientific GeneJET RNA purification kit. Five microliters (5µl) of total RNA from each treatment parameter was run on a 1% agarose gel at 60V for 50 minutes using ethidium bromide as a stain. This indicated the presence of RNA. NT: No treatment; AFB_1: treated with AFB_1; IFN: treated with rIFN-α; AFB1+ IFN: treated with AFB_1 and IFN.

3.6.2 Synthesizing complementary DNA (cDNA) from total RNA

RNA was converted to cDNA using the Maxima First Strand cDNA Synthesis kit with dsDNase (Thermo Scientific, Germany) following the instructions of the manufacturer. Any traces of genomic DNA present in the isolated cellular total RNA was removed in a reaction buffer in an RNase free microcentrifuge tube (1µl of 10 X ds DNA buffer, 1 µl of ds DNase, 1µl of RNA and 7 µl of nuclease free water bringing the reaction volume to 10 µl). After gentle mixing and brief centrifugation (250 xg, 4 ^{o}C, 1 minute), the reaction mixture was incubated (37^{o}C, 2 minutes) in water bath. The mixture was quickly placed on ice and after brief centrifugation it was maintained on ice. While on ice 4 µl of 5 X reaction mix, 2 µl of Maxima Enzyme mix and 4 µl of nuclease free water were added to bring the final reaction volume to 20 µl. The mixture was gently mixed and centrifuged (250 xg, 4 ^{o}C, 1 minute) after which it was incubated (10 minutes, 25 ^{o}C). The mixture was incubated for further 15 minutes at 50^{o}C. The entire reaction was stopped by heating the mixture at 85^{o}C for 5 minutes. The cDNA synthesized was stored frozen at -80^{o}C until ready to use in the qPCR reaction. It must be stated that reverse transcriptase minus (RT-) negative control as well as no template control (NTC) were included in the cDNA synthesis processes to monitor genomic DNA as well as reagent contamination respectively.

3.6.3 Designing primers and probes for qPCR

The primers and probes that were used in analyzing *STAT 1, JAK 1* and *OAS3* in the qPCR experiments with GAPDH which was included as endogenous control were purchased from Biomers in Germany **(Table 3.1).**

Table 3.1: Sequences of probes and primers

Name of gene	Sequence of primers and probes	Fluorophores
JAK1	Probe: 5'AGCAGTCAGTGTGGCGTCATTCTCC-3' Forward primer 5'- CAATTGGCATGGAACCAACGAC-3' Reverse primer 5'-CAAATCATACTGTCCCTGAGCAAAC-3'	5' FAM- 3' BHQ-1
STAT1	Probe: 5'-CGCTCTGCTGTCTCCGCTTCCACTCC-3' Forward primer: 5'GTTGCTGAATGTCACTGAACTTACC-3' Reverse primer: 5'- AGCTGATCCAAGCAAGCATTGG-3'	5' FAM- 3' BHQ-1
OAS3	Probe 5'- AGCCTGGTGCCTGCCTTCAATGTCC-3' Forward primer: 5'-TCCGCCTGACATCCGTAGATC-3' Reverse primer: 5'-TCCTCCGCAGCTCTGTGAAG-3'	5' FAM- 3' BHQ-1
GAPDH	Probe: 5'- CCGTTGACTCCGACCTTCACCTTCC-3' Forward primer: 5'- AGCCACATCGCTCAGACACC-3' Reverse primer: 5'- TGACCAGGCGCCCAATACG-3'	5'HEX- 3'TAMRA

All the primers were designed to suit the following thermal cycling condition: (i) an optimal melting temperature (Tm) of 60 ^{o}C; (ii) the difference in Tm of any two pairs of primers was not more than 2 ^{o}C; (iii) the probes were designed to suit Tm of 68-70 ^{o}C about 8-10 ^{o}C higher than the Tm of primers.

3.6.4 Measuring the efficiency of primers and probes

The efficiency of the probes and the primers that were used to amplify and detect the *STAT1, OAS3, JAK1* and *GAPDH* were determined using the C_T slope method. With this method a 10-fold serial dilutions of cDNA samples prepared from the total RNA of the different samples were used to construct standard curves for the targets (*STAT1, OAS3* and *JAK1*) and the endogenous reference (*GAPDH*). After amplification, the C_T values of the target and the endogenous reference genes were plotted against the log concentration of the starting cDNA used in the amplification process. The slope of each curve which is a regression coefficient calculated from the regression line of the standard curve was used to determine the efficiency. Normally the slope of the 10-fold serial dilution of the cDNA template is expected to be -3.32 which indicate an efficiency of 100%. The % of amplification efficiency was preferred to the slope values in this study. The recommended amplification efficiency range is 90-110% (Livak and Schmittgen, 2001). The calculations as well the data analysis were done using the Bio-Rad CFX 96 manager software (Bio-Rad, USA).

3.6.5 Determining the limiting primer concentrations of the GAPDH

One target gene and the endogenous reference gene in this case the GAPDH were co-amplified at a time in the same tube. For example the *STAT1* and the *GAPDH* were initially successfully amplified in the same tube using the primer and probe concentrations **(Appendix D, Table 1)** recommended by Thermo Scientific the manufacturers of the

Maxima Probe/Rox qPCR enzyme master mix (2 X) used in the qPCR experiments. However, when either *OAS3* or the *JAK1* was amplified with *GAPDH* in the same tube, either the *OAS3* or the *JAK1* was not amplified. This was due to the fact that the concentration of the *GAPDH* which was used as the endogenous reference was probably far greater than the concentrations of either the *OAS3* or the *JAK1* in the starting sample. As a result, when the *GAPDH* was amplified with either *OAS3* or *JAK1* in the same tube using the recommended primer concentrations, the *GAPDH* used up the reaction components faster and that impaired the amplification of the more scare target (*OAS3* or *JAK1*). To overcome this challenge so that both the target and the *GAPDH* could be amplified in the same tube, different combinations of the forward and the reverse primer concentrations of *GAPDH* **(Appendix D, Tables 2A, 2B and 2C)** were used to amplify *GAPDH*. The lowest concentrations of the forward and reverse primers of the *GAPDH* that gave the same C_T value as the maximum recommended concentrations **(Appendix D, Table 2D)** were then used to amplify the target and the *GAPDH* in the same tube.

3.6.6 Determining the relative gene expression level using the delta delta C_T ($\Delta\Delta C_T$) method

The qPCR reactions were run using the cDNA produced **(section 3.6.2)** on a CFX 96 real time PCR analyzer (Bio-Rad, USA) using relative quantification. Each reaction was conducted in triplicates and negative controls were always included. The $\Delta\Delta C_T$ method was employed to calculate the relative level of expression of the target genes when the amplification efficiency of the target and the amplification efficiency of the endogenous control were similar (+/–10). The $\Delta\Delta C_T$ analysis of the data was automatically done using

the CFX 96 manager software (Bio-Rad, USA). The data were presented as fold change in gene expression normalized to an endogenous control (*GAPDH*) and relative to the untreated control sample usually referred to as the calibrator sample.

3.7 Determining the effects of AFB_1 on protein synthesis by western blotting

The experimental procedures that were followed in determining the effects of AFB_1 on the protein synthesis of some of the key genes of the JAK-STAT-ISRE arm of the type I IFN pathway shall be discussed under this section of the thesis.

3.7.1 Extracting total proteins from adherent cells

The total proteins were isolated using Radioimmunoprecipitation assay (RIPA) buffer (Thermo scientific, Germany) following the instructions from the manufacturer. Briefly HepG2 cells which were grown in quadruplicates wells of the 6-well plates and stimulated with or without IFN (PBL, Biomedical laboratories, USA) and treated with or without AFB_1 (Sigma Aldrich, Germany) were harvested in appropriate volume of cold PBS and transferred into 2 ml eppendorf tubes. The tubes were centrifuged (1500 RPM; 4^{o}C; 5 minutes) after which the supernatants were discarded. Hundred microliters (100 µl) of cold RIPA buffer (Thermo scientific, Germany) containing protease and phosphatase inhibitor cocktail as well as EDTA (all reagents were purchased from Thermo scientific, USA) were added to the cells in each tube. The tubes were incubated on ice for 30 minutes with intermittent vortexing every 10 minutes and then centrifuged (16,000 x g; 4^{o}C; 10 minutes). After centrifugation the supernatants were gently transferred into newly labelled

1.5 ml eppendorf tubes already kept on ice and the pellet were discarded. The proteins were divided into working aliquots and stored at -20^{o}C until ready to use.

3.7.2 Determining protein concentration using Bradford assay

The concentrations of the protein samples were quantified by Bradford assay. The Bradford reagent (Bio-Rad, USA) contains Coomassie dye which binds to protein to form dye-protein complex. The absorbance of the dye-protein complex is measured at 595 nm using microplate reader or spectrophotometer. Prior to the assay, a standard curve was first constructed from known bovine serum albumen (BSA) protein standards (Bio-Rad, USA). Before starting the assay 1:10, 1:100, 1:1000 and 1:10000 dilutions of the unknown protein samples were prepared. Five microliters (5µl) of each of the pre-diluted BSA standards and the unknown samples were pipetted into designated duplicate wells of the 96-well. Using the multichannel pipette 150 µl of the 1x Bradford reagent pre-warmed to room temperature was dispensed into each well and the reagent was carefully mixed with the proteins. The plate was incubated at room temperature for 5 minutes after which absorbance of the standards and the unknown were measured at 595 nm using iMark Microplate reader (Bio-Rad, USA). A blank containing only the Bradford reagent and distilled water was included in the assay. After reading the absorbance of the standards and the samples, the average of the blank values was subtracted from the averaged values of the standards and the unknown sample. A standard curve was constructed by plotting the absorbance values at 595 nm (y-axis) against the concentration of the standards in µg/ml (x-axis) **(Fig. 3.3).** The concentrations of the unknown samples were determined using the equation of the standard curve. The final concentrations of the unknown samples were adjusted by multiplying the value obtained by the dilution factor used.

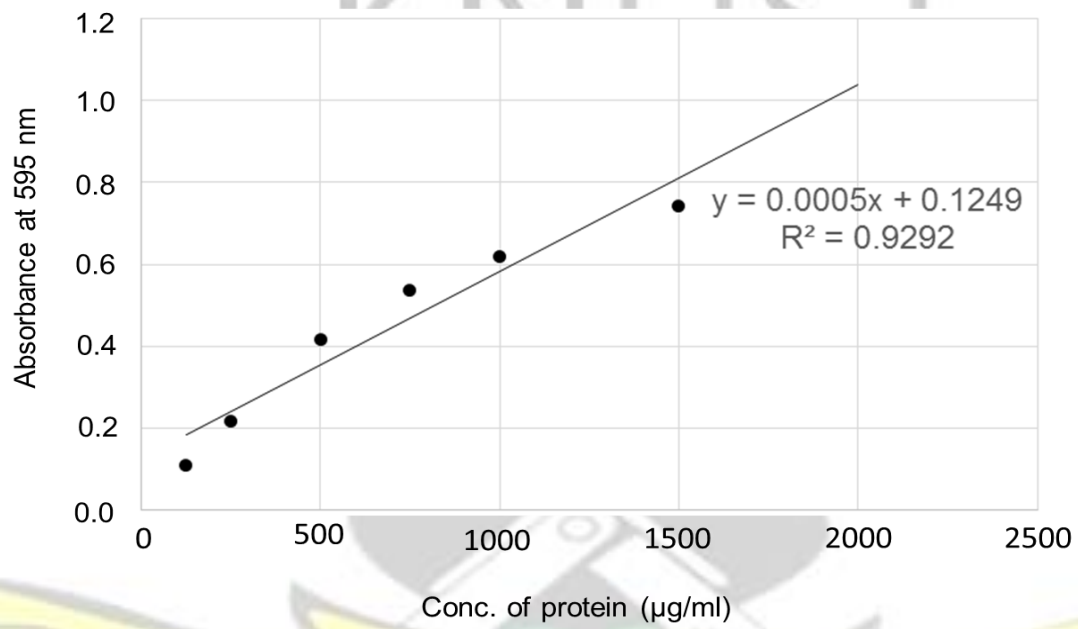

Fig.3.3: Standard curve generated using BSA. A standard curve constructed by plotting the absorbance values at 595 nm (y-axis) against the concentration of the standards in µg/ml (x-axis). The concentrations of the unknown protein samples were determined using the equation of the standard curve.

3.7.3 Separating protein by Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE (Bio-Rad, USA) is a common technique that is used to separate proteins based on their molecular sizes. The glass plate sandwiches were prepared after which 10% resolving gel (Bio-Rad, USA) was made using the recipe in the **Table 3.2**. When ready to pour the ammonium per sulfate (APS) (Sigma Aldrich, Germany) and the tetramethylethylenediamine (TEMED) (Sigma Aldrich, Germany) were added and the mixture well mixed by vortexing. Immediately the glass plate sandwiches were filled with the resolving gel to about 2/3. A layer of isopropanol was added to the top of the resolving gel to provide a uniform surface. The gel was allowed to polymerize for about 30 minutes and the isopropanol layer was poured off. Next the stacking gel was prepared using the recipe in the **Table 3.3**. The stacking gel (Bio-Rad, USA) was added to the resolving gel to the top of the glass plate sandwiches. The combs were inserted and the stacking gel was allowed to polymerize for about 30 minutes. While the stacking gel was polymerizing, the samples were prepared by adding equal volumes of fresh 2X sample buffer (Bio-Rad,

USA) supplemented with 2-β mercaptoethanol (BME) (Bio-Rad, USA) to the samples (ratio of 1:1). The heating block was pre-heated up to 95^{o}C, the samples well mixed and then heated at 95^{o}C for 5 minutes. The samples were quickly placed on ice. To ensure that all the samples had equal volumes the smaller volumes were topped up to the required level using 1X loading dye. When the gel had polymerized, the gel sandwiches were mounted in the electrophoresis apparatus. The comb was removed and 1X electrophoresis buffer was poured into the apparatus. Five microliters (5 µl) of the protein ladder was loaded into the

designated well. To ensure that equal amount (20 µg) of protein was loaded into each well, the entire volume of each sample mixture was loaded into the respective well. The gel was run at 95V to concentrate the proteins in the wells and at 150 V for 85 minutes until all the dye run out of the apparatus.

Table 3.2: Chart for preparing resolving gel (10%)

Stock concentration	**Volume**
30% w/v Acry/bis Acrylamide	5.0 ml
4x Tris-SDS-HCl, pH 8.8	3.75 ml
Distilled water	6.25 ml
10% Ammonium per sulfate (APS)	0.05 ml (50 µl)
TEMED	0.01 ml (10 µl)

Table 3.3: Chart for preparing stacking gel

Stock concentration	**Volume**
30% w/v Acry/bis Acrylamide	0.65 ml (650 µl)
4x Tris-SDS-HCl, pH 6.8	1.25ml
Distilled water	3.20 ml
10% Ammonium per sulfate (APS)	0.025 ml (25 µl)
TEMED	0.0l ml (10 µl)

3.7.4 Staining of polyacrylamide gels with Coomassie Blue

Where necessary, the quality of separation of protein was checked by staining a parallel polyacrylamide gel with Coomassie Blue solution with shaking for 1 hour at room temperature **(Fig. 3.4).** The gel was then destained in a destaining solution.

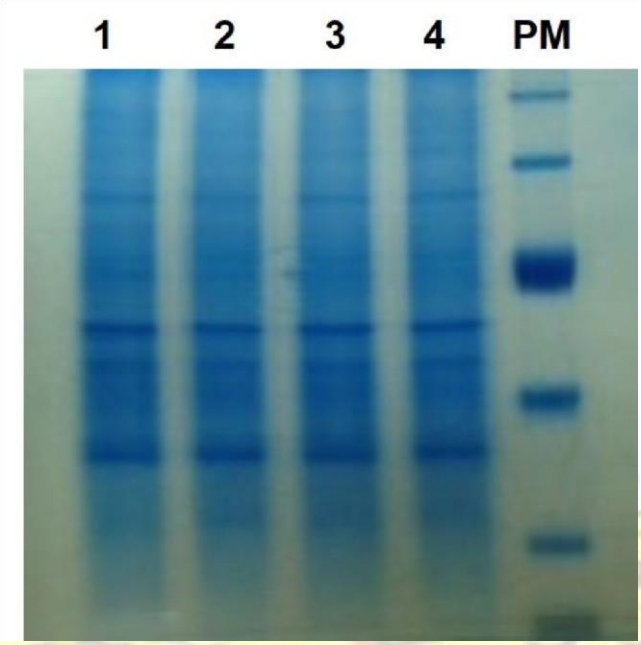

Fig.3.4: Analysis of the quality of separation of protein by Coomassie Blue staining. Coomassie Blue staining of SDS-PAGE showing protein bands. PM: 10 kDa pre-stained protein marker; lane 1: lysate from cells which were not treated; lane 2: lysate from cells treated with AFB_1; lane 3: lysate from cells stimulated with rIFN-α; lane 4: lysate from cells stimulated with rIFN-α and simultaneously treated with AFB_1.

3.7.5 Electrotransfer of proteins onto the polyvinylidene fluoride (PVDF) membrane

The gel was removed from the glass plate sandwich and then incubated in the transfer buffer for 10 minutes to equilibrate it. Quickly the gel sandwich was prepared. The sandwich was mounted in the transfer apparatus. The transfer buffer was added. The cooling block was placed in the unit and the proteins transferred for 1 hour at 200V. After transfer, the blot was removed and the side of the membrane that was facing the gel noted. The membrane was stored in 1X Tris-buffered saline with Tween-20 (TBST) (Bio-Rad, USA).

3.7.6 Antibody probing

Before probing the PVDF membrane with the primary antibody, the nonspecific sites of the blot were blocked with 5% nonfat dried milk (marvel) in 1X TBST at room temperature for 60 minutes with shaking. The blot was probed with the primary antibody (1: 2500 dilution (*GAPDH*) and 1:1000 dilution (*STAT1*) of the primary antibody in 2% blocking solution) and incubated overnight at 2-8^{o}C without shaking. After approximately 15 hours of probing, the blot was washed 6 times with 1X TBST for 5 minutes each. The blot was then probed with the horseradish peroxidase (HRP) conjugated secondary antibodies (1: 5000 dilution of the HRP-conjugated secondary antibody in 2% blocking solution) and incubated for 1 hour at room temperature with constant and gentle shaking. The blot was thoroughly washed 6 times with 1X TBST for 5 minutes each. The blot was stored in 1X TBST until images were taken. The primary and secondary antibodies were purchased from Thermo scientific (Germany).

3.7.7 Chemiluminescent detection and data analysis

The PVDF membrane was incubated with the Pierce ECL Western Blotting chemiluminescent detection substrate buffers 1 and 2 (Thermo scientific, Germany) following the manufacturer's instructions. The detection substrate buffers 1 and 2 were allowed to equilibrate to room temperature. Briefly the ECL chemiluminescent substrate buffers 1 and 2 were mixed in a 1:1 ratio immediately before used. About 3 ml of the chemiluminescent reagent was dispensed onto the glass surface of the C-DIGIT blot scanner (Li-COR Bioscience, USA). The blot was carefully placed with the protein side facing downward on the scanner to avoid trapping air bubbles. A plastic sheet protector was carefully placed on the blot and the blot was incubated with chemiluminescent reagent for 5 minutes at room temperature. An absorbent tissue was used to remove excess liquid.

The chemiluminescent signals were acquired and analyzed using the C- DIGIT blot scanner (Li-COR Bioscience, USA).

3.8 Determining the effects of AFB_1 on the antiviral activity of IFN-α

The experimental work that involved the use of viruses was used to quantify viruses that were cultured in the presence and or absence of IFN (PBL, Biomedical laboratories, USA) and or AFB_1 (Sigma Aldrich, Germany). The Chandipura virus used in the study was kindly donated by Professor David J. Blackbourn (University of Surrey, UK). Chandipura virus is an enveloped single-stranded negative sense RNA virus that belongs to the family Rhabdoviridae. The sensitivity of the virus to interferon makes it very useful in interferon bioassays (Berger and Zimmer, 2011).

HepG2 cells were grown in 6-well plates at densities of 5 x 10^5 cells per well. At about 80% confluence, the cells were stimulated with increasing concentrations IFN (4, 40, and 400 IU/ml) and simultaneously treated with AFB_1 (10 µl). Cells which were neither stimulated with IFN nor treated with AFB_1 were included as control. At 24 hours post stimulation and treatment, the IFN and AFB_1 containing media were removed and the cells were infected with Chandipura virus at multiplicity of infection (MOI) of 0.1 PFU/cell. No virus was added to the control wells. The plates were incubated (37ºC, 5% CO_2, 1hour) to allow the viruses to adsorb onto the surfaces of the cells. At 1 hour post-infection, the virus containing media were removed and the cells were washed twice with PBS. After the wash step, 2 ml of fresh growth media without IFN and AFB_1 was added to each well. The plates were incubated (37ºC, 5% CO_2, 24 hours) after which the cell culture supernatant from each sample was harvested and stored frozen in aliquots at -80ºC until the virus titer was quantified by plaque assay.

3.8.1 Quantifying Chandipura virus using plaque assay

Plaque assay, a technique developed by Renato Dulbecco in 1952 is used to quantify viruses (Dulbecco and Vogt, 1953).

Plaque assay was done in L929 cells. The cells that were used in the plaque assay were the L929 cells. The L929 is a fibroblast-like cell line that was cloned from the parent strain L in 1948 (Sanford and *et al.*, 1948). L929 cell line is a very useful tool that is employed in many experimental processes such as material biocompatibility testing (Bretagnol and *et al.*, 2008, Serrano and *et al.*, 2005), drug cytotoxicity testing (Faria *et al.*,

2009, Nordin *et al.*, 1991) and cell biology studies (Roelofs *et al*., 2006, Taniguchi and *et al*, 2006).

L929 cells were grown in DMEM containing high glucose, sodium pyruvate, Lglutamine, 25mM HEPES (Sigma Aldrich, Germany) supplemented with 10% heat inactivated FBS (Sigma Aldrich, Germany),, 1% NEAA (Sigma Aldrich, Germany), and

1% penicillin-streptomycin (Gibco by life technologies, UK) in 6-well plates and incubated (37ºC, 5% CO_2) for 72 hours until the cells reached 100% confluence. A 10-fold serial dilutions of the virus stocks from the different treatment groups were made up to the 10^{-6} using DMEM supplemented with 1% V/V penicillin-streptomycin. The L929 cell monolayers were then infected with 500 µl per 6-well plate and then incubated (37ºC, 5% CO_2) for 30 minutes. At 30 minutes of incubation the infected cell monolayers were covered with an overlay medium that contained 10% V/V of 1X MEM with Earle's salt, 1% V/V L-glutamine, 2% V/V of FBS, 1% V/V penicillin-streptomycin and 0.6% agarose.

After the agarose containing medium had solidified, the plates were incubated (37ºC, 5% CO_2) for 72 hours. The plaques that were formed were stained by adding 2 ml of crystal violet stain to each 6-well plate. The plates were incubated at room temperature with gentle shaking for 30 minutes. The stain and the agarose overlay were carefully and gently removed by washing the plates under slow running tap water. After air-drying the plates, the numbers of plaques were counted and the virus titer for each sample was calculated as

PFU/ml.

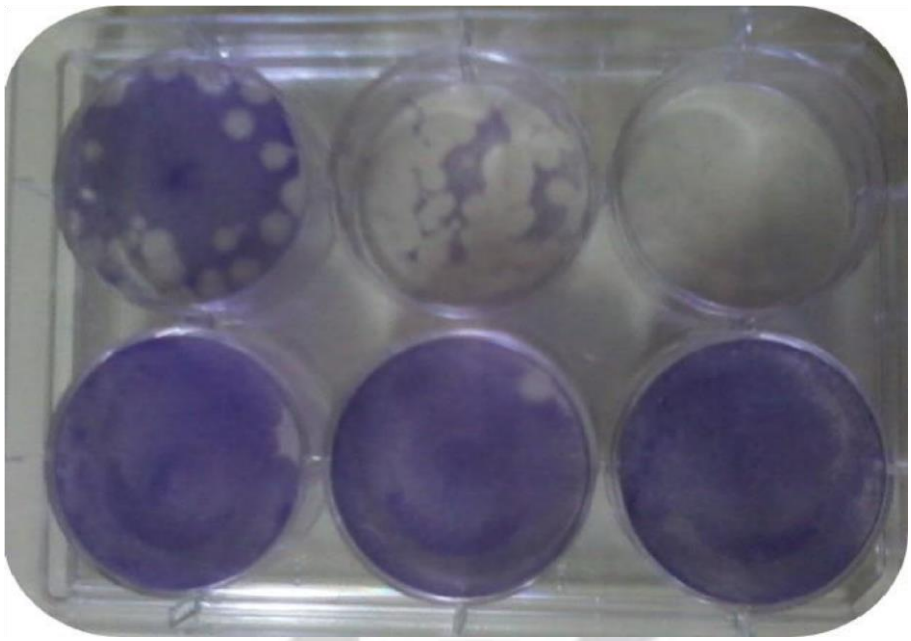

Fig.3.5: Quantification of Chandipura virus titer using plaque assay. A plate showing Chandipura virus plaques identified after 3 days of incubation. Plaques appeared as clear circular zones in crystal violet stained cells. This is a representative plate of the virus work.

3.9 Statistical analysis

All experiments were conducted in triplicates. Data were entered into excel spread sheet and analyzed using excel and Graph pad. Averages of the three independent experimental results as well as the standard deviations were calculated using excel. The pISRE-luc activity of cells stimulated with IFN and cells treated with IFN and AFB_1 were compared. Differences between IFN treated sample and IFN and AFB_1 combined treated sample were analyzed using two-tailed student's t-test. The difference between the different samples was considered to be statistically significant when p-value ≤ 0.05.

.

CHAPTER FOUR THE EFFECTS OF AFB_1 ON THE TYPE I IFN RESPONSE

4.1 Introduction

AFB_1 is the most potent hepatocarcinogen among the different classes of aflatoxins known (Sudakin, 2003, Wild and Gong, 2010). It is produced mainly by the fungi, *Aspergillus flavus* and *Aspergillus parasiticus* under warm and humid conditions mainly in cereals such as maize, millet and groundnut (Wu and Khlangwiset, 2010). Two crops that are most susceptible to infection by *Aspergillus* are maize and groundnut and these constitute staples in many African countries. AFB_1 contamination in food is a serious global health problem particularly in the developing countries (Liu and Wu, 2010) where sub-optimal farming practices and poor storage conditions persist. For example Kumi and co-workers reported that 83.3% of locally made food (weanimix) from maize and groundnuts in the Ejura-Sekyedumase district in the Ashanti Region of Ghana had aflatoxin levels higher than the national permissible of 15 ppb (Kumi *et al*., 2014) suggesting that many infants as well as adults Ghanaians are either directly or indirectly being exposed to high levels of aflatoxins.

As discussed previously **(section 2.12)** the IFN system which comprises the type I, II and III IFNs constitute a major components of the innate immune system that work to eradicate pathogens and tumour cells from the hosts. Studies have shown that prolonged exposure to AFB_1 is associated with increase in the incidence of HCC **(section 2.7)** (Groopman *et al*., 2008, Hamid *et al*., 2013) especially in the developing countries.

Therefore researching into the effects of the AFB_1 on the type I IFN pathway using the human hepatoma cell line (HepG2 cell) as a model system will provide better understanding of the mechanism by which AFB_1 could suppress the type I IFN pathway. The HepG2 cell line derived from the liver tissue was used since the liver plays a major role in the metabolism of many compounds including AFB_1 and also represents an important target organ in systemic toxicity (Davila *et al*., 1998).

4.2 Methods

The methods that were employed in this section of the study were MTS-based cell proliferation assay and the dual luciferase reporter gene assay **(sections 3.3, 3.4.1 and 3.4.2).**

4.2.1 The cytotoxic effects of AFB_1 on HepG2 cells in culture

The AFB_1 (Sigma Aldrich, Germany) is an extremely toxic compounds which could not have been used directly on the cells. Therefore it was paramount to determine the working concentration of AFB_1 (Sigma Aldrich, Germany) that was not toxic to the cells. This concentration was used in the subsequent experiments. To accomplish this aim, the cytotoxic effects of AFB_1 on HepG2 cells was evaluated using the Cell Titer 96 AQueous One Solution (Promega, USA). The experiment was done by treating monolayers of HepG2 cells with increasing concentrations of AFB_1 (0-3200 μM) for 24 hours after which the AFB_1 containing media were replaced with fresh growth media without AFB_1. The viability of the cells was determined after 24, 48 and 72 hours using the MTS-based assay **(section 3.3).**

4.2.2 Activation and measurement of type I IFN response pathway in HepG2 cells

Having established the working concentration of AFB_1, the next task was to determine the concentration of IFN that would induce maximum activity of the IFN inducible promoter activity using the dual luciferase reporter gene assay. The dual reporter system allowed the simultaneous expression and measurement of two different reporter enzymes (one being experimental reporter with the other being control reporter) within a single system. The experimental reporter used in this study was pISRE-luc while pRLSV40 was used as the control reporter to which the activity of pISRE-luc was normalized. To achieve the purpose of this experiment, monolayers of HepG2 cells were transiently cotransfected with pISRE-luc (500 ng) and pRLSV40 (1 ng) using Lipofectamine-2000 as a transfection reagent in 96-well plate **(section 3.4.1).** At 24 hours post-transfection, the cells were stimulated with increasing concentrations of IFN (0 to 400 IU/ml). Twenty hours later, the pathway activity was measured using the dual luciferase reporter gene assay **(section 3.4.2).**

4.2.3 The effects of AFB_1 on the IFN inducible ISRE pathway in HepG2 cells

After establishing the working concentrations for AFB_1 and IFN, the impact of AFB_1 on the type I IFN signaling in HepG2 cells was assessed, by measuring the pISREluc activity in cells stimulated with or without IFN and treated with or without AFB_1.

HepG2 cells were cultured and monolayers of cells were transiently co-transfected with pISRE-luc (500 ng) and pRLSV40 (1 ng) as previously described **(section 3.4.1)** using Lipofectamine 2000 as a transfection reagent in 96-well plate. At 24 hours posttransfection,

the cells were stimulated with or without IFN (400 IU/ml) and simultaneously treated with increasing concentrations of AFB_1 (0.8-32 μM). Twenty hours later, the type I IFN signaling pathway activity was measured using the dual luciferase reporter gene assay **(section 3.4.2).**

4.2.4 Statistical analysis

All data were entered into excel spread sheet and analyzed using excel and Graph Pad Prism (version 5.01). The differences in the viability of cells treated with AFB_1 at 24, 48 and 72 hours were analyzed using one way analysis of variance (ANOVA) with 95% confidence interval (CI). The type I IFN activity of cells stimulated with IFN and cells stimulated with IFN and simultaneously treated with AFB_1 were compared. Two-tailed student's t-test was used to compare the difference between the type I IFN activity of IFN stimulated cells and IFN and AFB_1 treated cells. The differences between the different samples were considered to be significant when the p-value ≤ 0.05.

4.3 Results

The results from the cytotoxicity assay revealed that AFB_1 killed the HepG2 cells in a dose-dependent fashion after exposure to AFB_1 concentrations up to 3200 μM. The concentration of AFB_1 at which $\geq 85\%$ of the cells survived was established to be between 10-20 μM. It was observed that there was no significant difference in the viability upon AFB_1 exposure of HepG2 cells for 24, 48 and 72 (p-value ≤ 0.977) **(Fig. 4.1A).**

The results of the dual luciferase reporter gene assay indicated that increasing concentration of IFN resulted in corresponding increase in the type I IFN activity with

maximum activity being produced with concentrations of 200-400 IU/ml of IFN. It was observed that there was no significant difference in the type I IFN activity of cells stimulated with 200-400 IU/ml of IFN **(Fig. 4.1B).**

When HepG2 cells were stimulated with or without IFN and simultaneously treated with or without AFB_1, the results indicated that the type I IFN pathway activity was highest in cells stimulated with IFN alone and that AFB_1 suppressed the type I IFN pathway activity in HepG2 cells in a dose-dependent fashion by inhibiting the pISRE-luc activity. It was observed that there was a significant difference in the type I IFN pathway activity in cells which were stimulated with IFN alone compared to cells which were stimulated with IFN and simultaneously treated with 10 µM of AFB_1 **(Fig. 4.1C)**

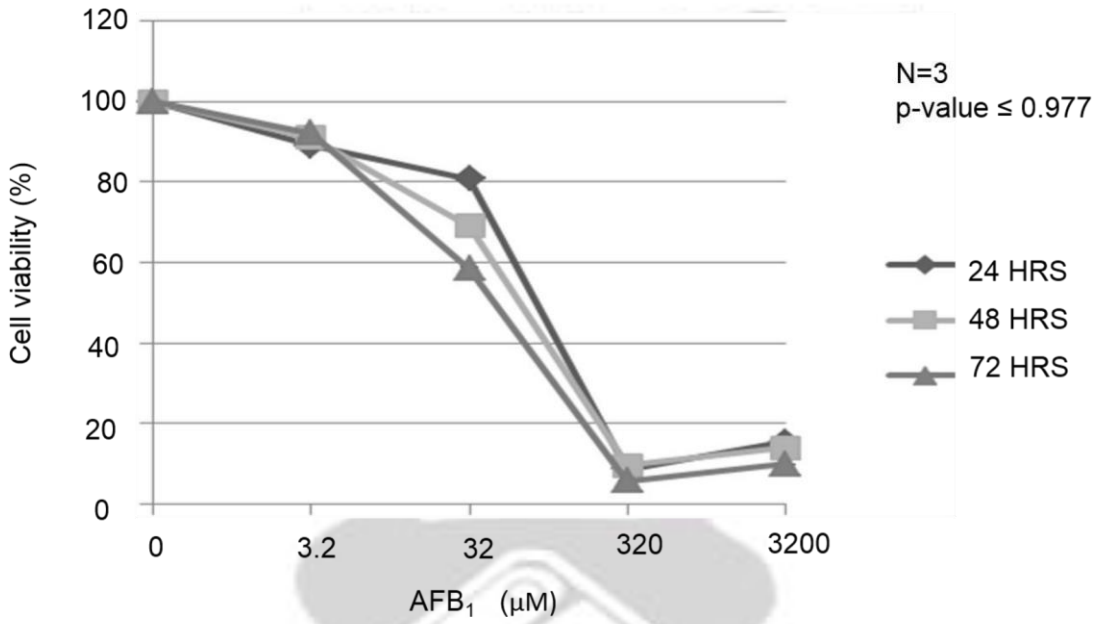

Fig. 4.1A: A graph showing the percentage viability of HepG2 cells as determined by MTS assay. The percentage viability of cells was calculated as ratio between AFB_1 treated cells and untreated cells. Data are presented as mean and standard deviation of three independent experiments each performed in duplicates, p-value ≤ 0.977 as determined by one-way ANOVA.

Error bars = STD (standard deviation) were too small to show.

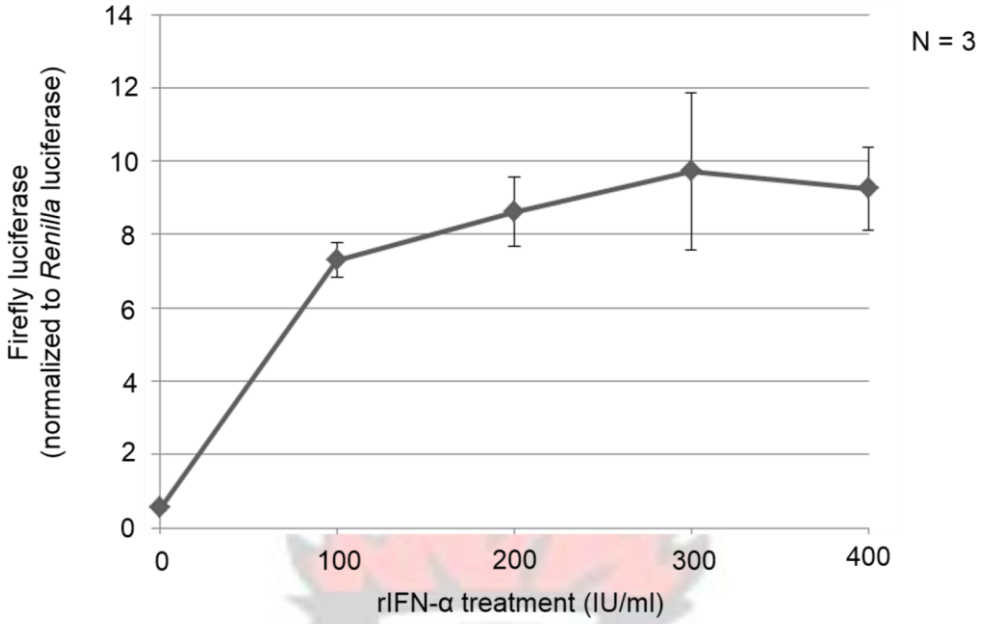

Fig. 4.1B: A graph showing the induction of pISRE driven luciferase reporter gene activity by IFN-α. HepG2 cells were transiently co-transfected with pISRE-luc and pRLSV40. At 24 hours post-transfection, the cells were stimulated with increasing concentration of IFN-α. Luciferase activity was measured 24 hours later. The data are presented as mean and the standard deviation of three independent experiments each conducted in duplicate. There was no significant difference in pISRE-luc activity of cells treated with 300 and 400 IU/ml of IFN-α (pvalue ≤ 0.7527), Error bars = STD.

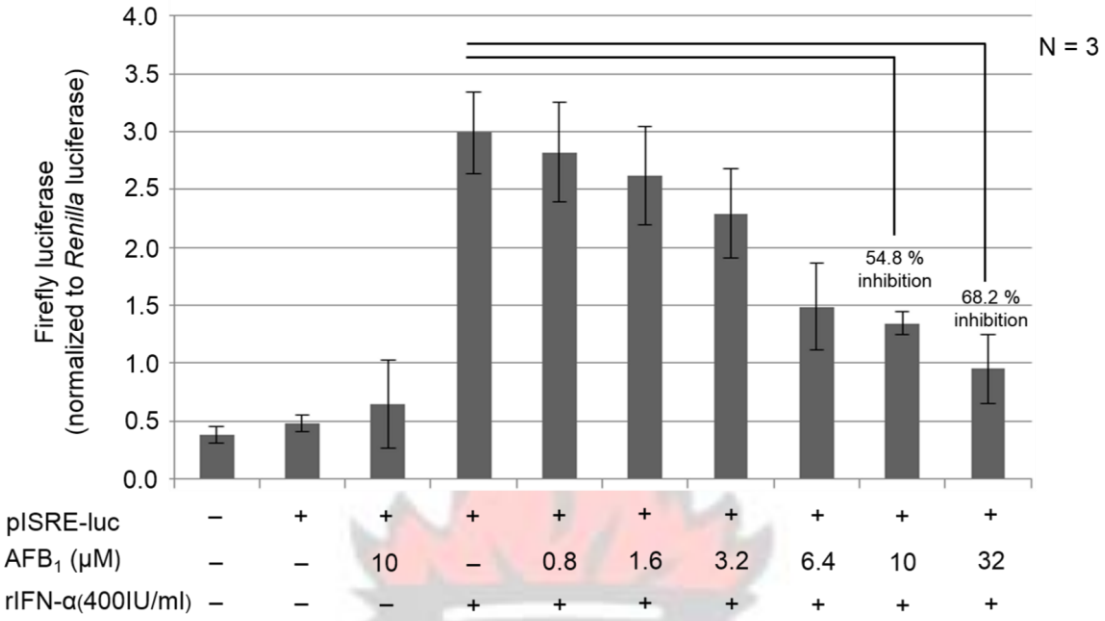

Fig. 4.1C: A graph showing that AFB_1 suppressed the type I IFN response by inhibiting the activity of pISRE-luc. HepG2 cells were transiently co-transfected with pISREluc and pRLSV40. At 24 hours post-transfection, the cells were stimulated with or without IFN-α and simultaneously treated with or without AFB_1. Transfected cells which were stimulated with IFN-α but not treated with AFB_1 were calculated to have 100% pISRE-luc activity. The data are presented as mean and the standard deviation of three independent experiments each conducted in duplicate wells. There was a significant difference in pISRE-luc activity of cells treated with IFNα alone compared to cells treated with IFN-α and 10μM of AFB_1 (p-value ≤ 0.047) and cells treated with IFN-α and 32μM AFB_1 (p-value ≤ 0.024), Error bars = STD.

4.4 Discussion

Information on studies on the effects of AFB_1 on the type I IFN pathway in HepG2 is very limited. However, quite a number of studies have attempted to investigate the effects

of AFB_1 on the viability of cells *in vitro*. For example Cheng *et al*. (2002) demonstrated in their study that the viability of duck peritoneal macrophages declined after exposure to AFB_1 concentration up to 320 μM for 12 hours. Conversely, Lereau *et al*. (2012) reported that AFB_1 had no measurable cytotoxic effects on HepaRG cells after treating the cells with AFB_1 for 4 hours at concentration up to 50 μM followed by 48 hours of maintenance in culture medium without AFB_1. The reported results of Cheng *et al*.

(2002) is consistent with findings from this study. However, the results of (Lereau *et al*., 2012) is at variance with the findings from this study. The difference between findings from this study and that of Lereau *et al*. (2012) could be attributed to differences in concentrations used as well as the length of exposure of cells to AFB_1.

While in this study and that of Cheng *et al*. (2002) HepG2 and macrophages were exposed to AFB_1 concentrations of up to 3200 μM and 320 μM for 24 and 12 hours respectively, Lereau *et al*. (2012) exposed HepaRG cells to AFB_1 concentration up to 5 μM for 4 hours. Therefore the decline in cell viability observed in this study could be attributed to continual disturbance of the physiology of the cells owing to prolonged exposure to AFB_1.

Another factor that could account for the difference observed in the study of Lereau *et al*. (2012) and this study was the types of cell lines that were used in the respective studies. While Lereau *et al*. (2012) used HepaRG cells, in this study HepG2 cells were used. Both HepaRG and HepG2 cells are human hepatoma cells. While HepaRG was derived from a female hepatocarcinoma patient, HepG2 was derived from male

hepatocarcinoma patient. It has been reported that the basal gene expression levels of phase

I and phase II biotransformation enzymes in HepG2 are generally lower compared to HepaRG cells (Guillouzo *et al*., 2007 , Kanebratt and Andersson, 2008, Turpeinen *et al*., 2009) suggesting that HepaRG cells could have a greater capacity of handling chemicals than HepG2 cells hence the difference observed in the two studies.

Aflatoxin contamination of both human and animal food has been found to be common in some West African countries like Ghana, Togo, Nigeria and Benin (Awuah and Kpodo, 1996, Kpodo *et al*., 2000, Oyelami *et al*., 1996) and this suggests that many Ghanaians are either directly or indirectly being exposed to high levels of aflatoxins primarily by eating contaminated food. Because prolonged heavy exposure of humans to AFB_1 is one of the significant environmental risk factors that contribute to the development of HCC (Hamid *et al*., 2013, Wild and Hall, 2000) the concentrations of AFB_1 used in this study as well as the duration of exposure of cells to AFB_1 adopted could be appropriate since it is likely to represent what happens in humans who have been exposed to AFB_1 for decades.

As stated earlier, data or information on the effects of AFB_1 on the type I IFN pathway in literature is very limited. In this study, the luciferase reporter gene pISRE-luc was used to measure the activity of ISRE in response to IFN stimulation and AFB_1 treatment in HepG2 cells. The ISRE is a DNA motif that is found in the promoter regions of genes whose activities are regulated by the type I IFN. These genes are known as IFN stimulated genes (ISGs). The sequence of events which leads to the activation of ISRE begins when type I IFNs interact with their specific receptors. The interaction between the type I IFNs and their receptors set in motion a cascade of events that culminates in the transcription of certain genes **(section 2.15; Fig. 2.4).** Therefore, the suppression of type I IFNs pathway by AFB_1 through the inhibition of ISRE activity could weaken the immune system of

individuals who are heavily exposed to AFB_1 for a longer period and hence make them more prone to infections.

CHAPTER 5 THE EFFECTS OF AFB_1 ON THE mRNA AND PROTEIN LEVELS OF THE TYPE I IFN PATHWAY

5.1 Introduction

The molecular mechanism by which aflatoxins and other mycotoxins modulate the immune system is poorly understood. However, available evidence in literature suggests that aflatoxins and other mycotoxins may exert their immunosuppressive effects by one of the following ways: they may inhibit (i) DNA replication, (ii) transcription and translation through the use of different mechanisms (Jolly *et al*., 2008). It has been reported that many complicated and different post-transcriptional mechanisms could be involved in translating mRNAs into protein (Rasooly *et al*., 2013). This observation suggests that higher mRNA levels will not always translate into higher protein synthesis. In the same manner, lower mRNA levels will not necessarily translate into lower protein synthesis suggesting that there could be a direct correlation between mRNA levels and protein production or mRNA levels may be inversely correlated to protein levels. For example Rasooly *et al*. (2013) reported that an increased mRNA levels does not correspondingly result in increase in the rate of protein synthesis. They reported in their study that even though low levels of AFB_1, ricin and milk enhanced the production of recombinant proteins: luciferase firefly, βgalactosidase and green fluorescent protein (GFP) in Human Embryonic Kidney 293 (HEK 293) and Vero African Green Monkey adult kidney cells, the mRNA levels were more than protein abundance (Rasooly *et al*., 2013). The observation of Rasooly *et al*. (2013) was found to be consistent with the results of Guo *et al*. (2008) who reported that mRNA expression patterns could be accompanied by a 20-fold lower protein expression.

As previously stated, IFNs form a major component of the innate immune system that protect the body against cancers and viruses. The signaling pathways by which IFNs exert their anti-cancer actions can vary. One mechanism through which IFNs exert their anti-cancer activity is through the induction of STAT1 protein (Stephanou and Latchman, 2003). As a result this study focused on the JAK-STAT-ISRE arm of the type I IFN pathway. The JAK-STAT-ISRE pathway include several regulatory elements. However this study focused on *JAK1, STAT1* and *OAS3* which are the key signaling components of the JAK-STAT-ISRE arm of the type I IFN pathway which has been described previously **(section 2.15)**.

As discussed previously **(section 2.17.1)** STAT1 signaling pathway activated by IFNs has tumour suppressive function (Dunn *et al*., 2006) and also induces genes that modulate immune functions such as the establishment of antiviral state and induction of apoptosis (Khodarev *et al*., 2012). Aside its involvement in the fighting against viruses, OAS3 has been reported to play a role in the induction of apoptosis and anti-proliferative responses of IFNs **(section 2.18.3)**.

Because information available indicates that not all transcripts activity translate to protein, western blotting was used together with RT-qPCR to unravel the possible mechanism by which AFB_1 could suppress the type I IFN signaling pathway. Results generated will help in confirming the results of the dual luciferase reporter gene assay **(Fig. 4.1C).**

5.2 Methods

The methods that were employed in this section of the study were reverse transcriptase –quantitative polymerase chain reaction (RT-qPCR) and western blotting or immunoblot assay **(sections 3.6-3.7.7).**

5.2.1 Quantifying the mRNA levels of the key components of the type I IFN pathway

To determine whether AFB_1 had any measurable effects on the type I IFN signaling pathway at the transcription level, RT- qPCR was used to analyze the mRNA expression levels of *JAK1, STAT1* and *OAS 3* in HepG2 cells where the pathway was activated compared to the counterparts cells where the pathway was not activated.

RT-qPCR is normally used to quantify target nucleic acid by using either the absolute quantification or the relative quantification method. In this study the relative quantification method was employed to analyze the data from the RT-qPCR experiment. In relative quantification the ratio between the amount of target PCR products and control is determined. The normalized data from the RT-qPCR allows for comparison of

differential gene expression in different samples. In this study the $\Delta\Delta C_T$ method of relative quantification was used. To use the $\Delta\Delta C_T$ method standard curves were constructed to determine the amplification efficiencies of the target and the endogenous reference gene in duplex RT-qPCR experiment. HepG2 cells were cultured as previously described **(section 3.2)**. Monolayers of HepG2 cells were stimulated with IFN for 24 hours after which total RNA was extracted and then converted to cDNA as described previously **(sections 3.6.1 and 3.6.2)**. A 10-fold serial dilutions ($1:10^{-1}$-$1:10^{-6}$) of the cDNA were made with nuclease

free water. Primers and probes specific for *JAK1, STAT1, OAS3* and *GAPDH* were used to determine the primer/probe binding efficiencies using qPCR. The primers and probes binding efficiency values were measured using the C_T slope method.

Having established that the amplification efficiencies of the target and the endogenous reference gene were comparable **(Fig. 4.2A)**, qPCR was used to quantify mRNA levels of *JAK1, STAT1*and *OAS3*. HepG2 cells were cultured as previously described **(section 3.2)**. Monolayers of HepG2 cells were stimulated with or without IFN and simultaneously treated with or without AFB_1 for 24 hours after which total RNA was extracted and then converted to cDNA as described previously **(sections 3.6.1 and 3.6.2)**. The mRNA levels of *JAK1, STAT1* and *OAS3* were quantified using specific primers and probes using Bio-Rad CFX 96 machine at KCCR.

5.2.2 Does the inhibition of the transcripts of *JAK1, STAT1* and *OAS3* by AFB_1 affect their translation into proteins as well?

As previously stated **(section 5.1)**, some genes can be affected at the transcription level but not at the translation phase. Therefore, after demonstrating through the RT-qPCR experiments that AFB_1 suppressed/inhibited the transcripts expression levels of *JAK1, STAT1* and *OAS3*, it was hypothesized that AFB_1 would ultimately suppress/inhibit the translation of *JAK1, STAT1* and *OAS3* mRNA into proteins as well. Since proteins are the functional end products of genes, if AFB_1 was to suppress the translation of the key components of the type I IFN signaling pathway, it would mean that the immune system of individuals exposed to AFB_1 would be compromised making those individuals more susceptible to infections and cancers. To test the hypothesis, western blotting was used to

analyze the effects of AFB_1 on *STAT1* at the protein synthesis or translation level. HepG2 cells were cultured as previously described **(section 3.2)**. Monolayers of HepG2 cells were stimulated with or without IFN and simultaneously treated with or without AFB_1. Twenty four hours later, the cells were harvested and total proteins extracted following standard protocol **(section 3.7.1).**

The concentrations of the unknown protein samples were determined by Bradford assay **(section 3.7.2).** First a standard curve was constructed from known BSA protein standards. The concentrations of the unknown protein samples were determined from the equation of the standard curve **(Fig. 3.3).**

Having determined the concentrations of the protein samples, equal amounts of the proteins were resolved by SDS-PAGE. After transfer onto PVDF membrane, the membrane containing the proteins was immunoblotted with antibodies to *STAT1* and *GAPDH* epitopes. The *GAPDH* was used to ensure that equal amounts of the proteins were loaded **(sections 3.7.3, 3.7.5 and 3.7.6 for details).**

5.2.3 Statistical analysis

The data from the qPCR experiment was automatically entered into excel spread sheet on the Bio-Rad CFX 96 machine. The data was analyzed using the Bio-Rad CFX 96 manager software to determine the relative gene expression levels of *JAK1*, *STAT1* and *OAS3*. The differences in the gene expression levels of *JAK1*, *STAT1* and *OAS3* in HepG2 cells which were stimulated with IFN alone and in the cells which were stimulated with IFN and simultaneously treated with AFB_1 was analyzed using unpaired student's t-test.

The differences were considered to be significant when the p-value ≤ 0.05.

5.3 Results

The results of qPCR amplification efficiencies of *JAK1, STAT1* and *OAS3* in duplex reactions with *GAPDH* were 91.6%, 98.2% and 93.5% respectively **(Fig.5.1A).** The results of the qPCR indicated that the relative expressions of *JAK1, STAT1* and *OAS3* was highest in cells which were stimulated with IFN alone. The results revealed that AFB_1 suppressed the mRNA transcript levels of *JAK1, STAT1* and *OAS3* by 49.1% **(Fig. 5.1B),** 47% **(Fig. 5.1 C)** and 39% **(Fig. 5.1D)** respectively. It was observed that there was a significant difference in the relative mRNA expressions in cells stimulated with IFN alone and cells stimulated with IFN and simultaneously treated with AFB_1 (JAK 1, p-value ≤ 0.0001; STAT1, p-value ≤ 0.03; OAS3, p-value ≤ 0.05).

Western blotting analysis showed that HepG2 cells that were stimulated with IFN alone showed the highest concentration of *STAT1* protein levels. Conversely, the *STAT1* protein level decreased in cells that were stimulated with IFN and then simultaneously treated with AFB_1. In other words AFB_1 reduced or inhibited *STAT1* protein accumulation in the cells **(Fig. 5.1E).**

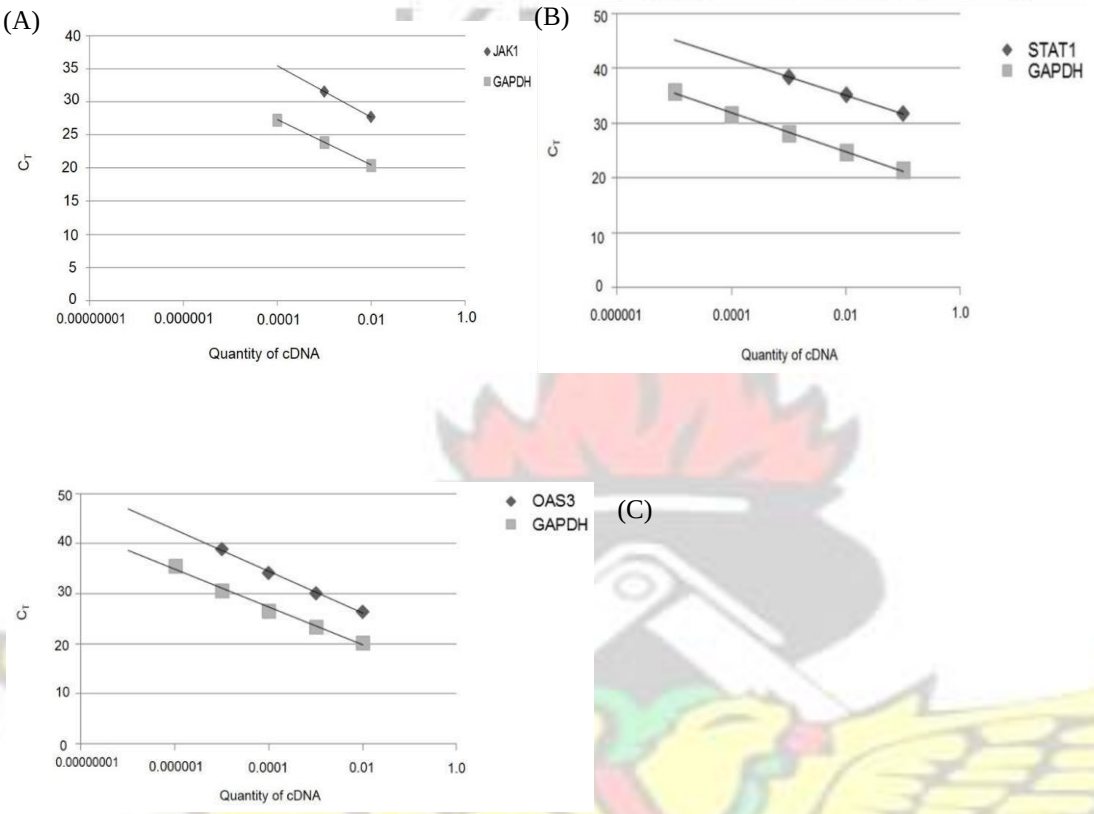

Fig. 5.1 A: Primers and probes amplification efficiency study. The efficiencies of the primers and probes for the targets *JAK1*, *STAT1*, *OAS3* and the endogenous control *GAPDH* in a duplex qPCR were determined by amplifying a sequential 10-fold dilutions of the cDNA. Primers and probes efficiency values were expressed in percentage and were measured using the C_T slope method. The amplification efficiencies were calculated using the slope of the regression line in the standard curve. (A) The amplification efficiency of *JAK1* in a duplex reaction with *GAPDH* was 91.6%. (B) The amplification efficiency of *STAT1* in a duplex reaction with *GAPDH* was 98.2% and (C) The amplification efficiency of *OAS3* in a duplex reaction with *GAPDH* was 93.5%. Data are a representative of three independent experiments each conducted in triplicates. The amplification efficiency reference values were 90-110%.

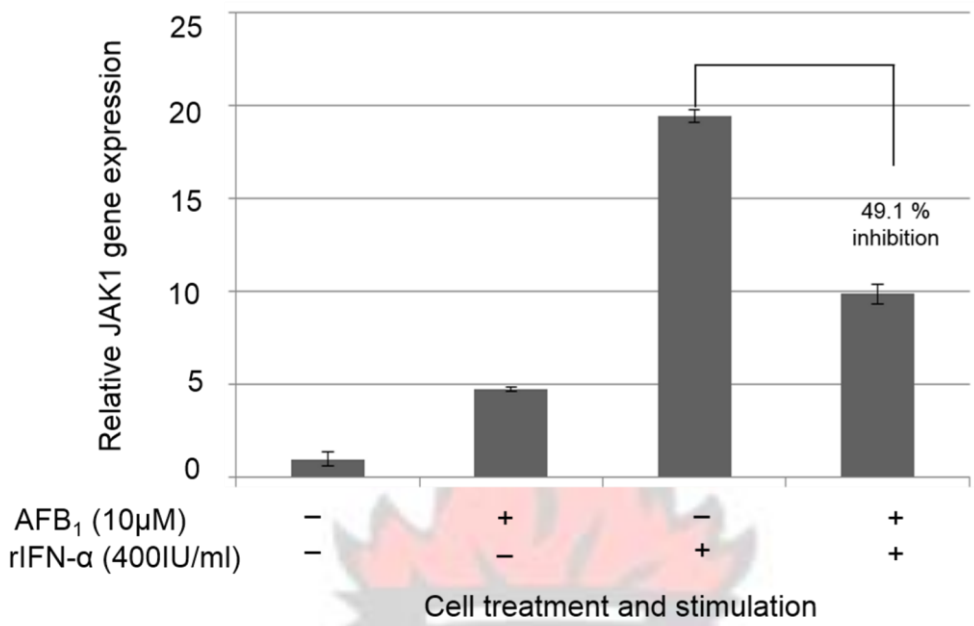

Fig. 5.1B: AFB_1 inhibits JAK 1 gene transcription. HepG2 cells were stimulated with or without IFN-α (400 IU/ml) and simultaneously treated with or without AFB_1 (10µM) for 24 hours followed by RT-qPCR analysis of *JAK1* transcription. The relative levels of JAK1 mRNA after normalization to GAPDH (endogenous control) was plotted. The *JAK 1* mRNA levels were determined by $\Delta\Delta C_T$. This is a representative data of three independent experiments each conducted in triplicate independent wells presented as mean and standard deviation, p-value ≤ 0.0001, Error bars = STD.

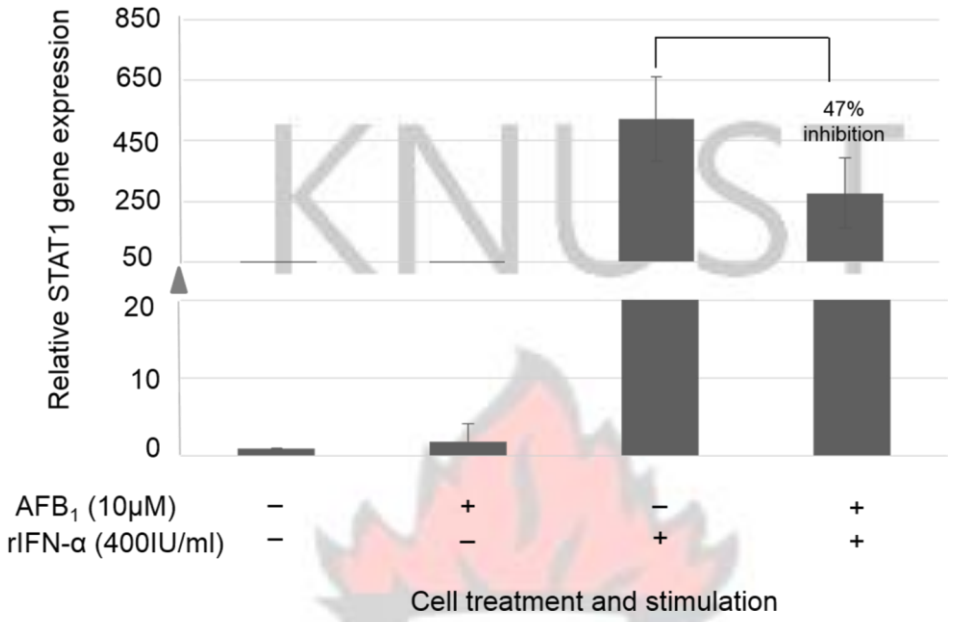

Fig. 5.1 C: AFB_1 inhibits STAT1 gene transcription. HepG2 cells were stimulated with or without IFN-α (400 IU/ml) and simultaneously treated with or without AFB_1 (10μM) for 24 hours followed by RT-qPCR analysis of *STAT1* transcription. The relative levels of STAT1 mRNA after normalization to GAPDH (endogenous control) was plotted. The *STAT1* mRNA levels were determined by $\Delta\Delta C_T$. This is a representative data of three independent experiments each conducted in triplicate independent wells presented as mean and standard deviation, p-value ≤ 0.03, Error bars = STD.

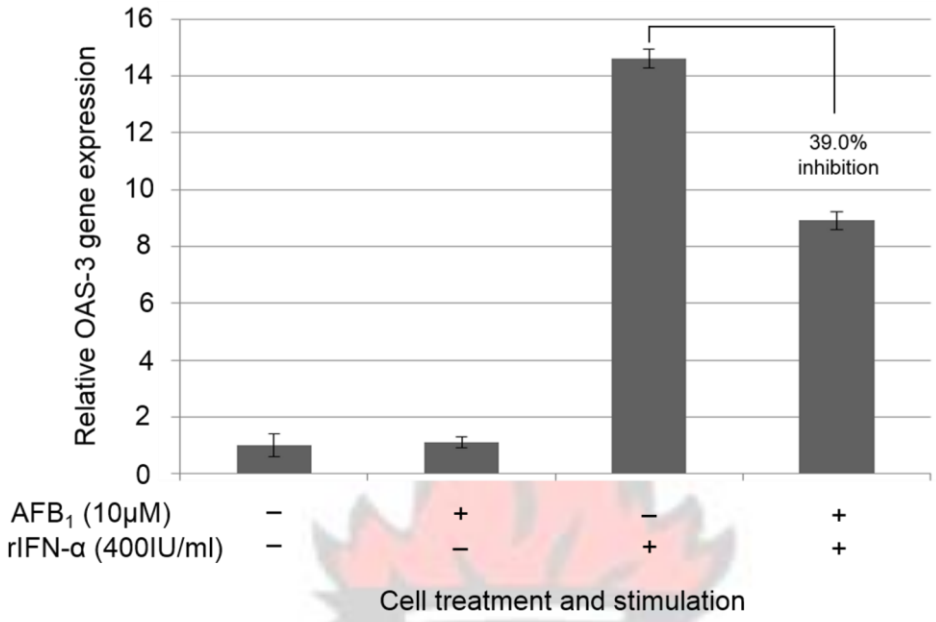

Fig. 5.1D: AFB_1 inhibits OAS 3 gene transcription. HepG2 cells were stimulated with or without IFN-α (400 IU/ml) and simultaneously treated with or without AFB_1 (10µM) for 24 hours followed by RT-qPCR analysis of *OAS3* transcription. The relative levels of OAS3 mRNA after normalization to GAPDH (endogenous control) was plotted. The *OAS3* mRNA levels were determined by $\Delta\Delta C_T$. This is a representative data of three independent experiments each conducted in triplicate independent wells presented as mean and standard deviation, p-value ≤ 0.05, Error bars = STD.

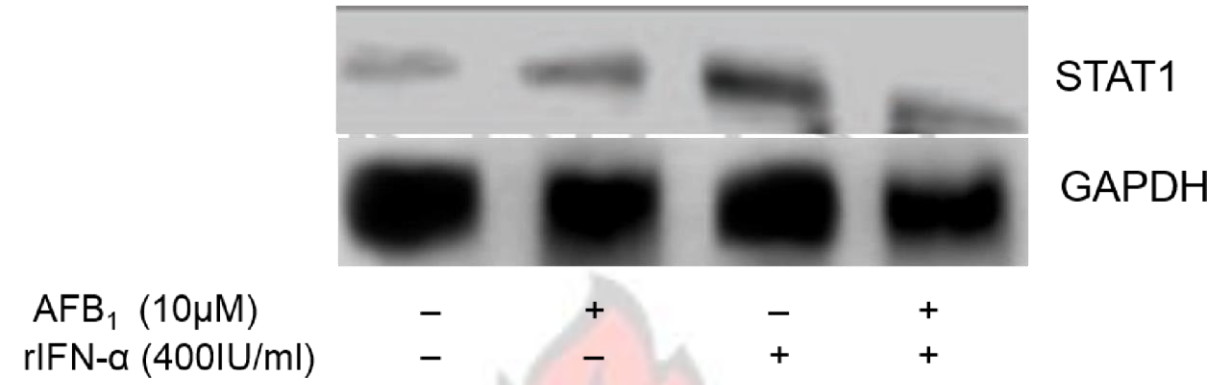

Fig. 5.1E: Western blot analysis of the levels of STAT1 proteins after stimulation and treatment of HepG2 cells with rIFN-α and AFB_1 respectively. This data is a representative of three experiment conducted independently. The protein bands from the three experiment showed the same pattern.

5.4 Discussion

Even though the mechanism by which AFB_1 affects the immune system is poorly understood some evidence available in literature suggest that AFB_1 may suppress the immune system by inhibiting DNA replication, transcription and protein synthesis (Jolly *et al*., 2008). For example AFB_1 and its metabolites have been reported to block RNA polymerase and ribosomal translocase and thus inhibit transcription (Eaton and Gallagher, 1994). In addition, aflatoxins have been reported to interfere with certain enzymes and substrates that are required for initiation, transcription and translation processes which are involved in the synthesis of proteins, reviewed in (Bbosa *et al*., 2013a).

Results from the RT-qPCR experiments demonstrated that AFB_1 suppressed the mRNA levels of *JAK1, STAT1* and *OAS3*. This finding is consistent with the results of Jiang *et al*. (2015) who investigated the effects of AFB_1 on the mRNA expression levels of IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ and TNF-α in the small intestines of broilers and reported that AFB_1 reduced the mRNA levels of IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ and TNF-α. In addition other researchers have reported that AFB_1 suppressed/inhibited the mRNA levels of IL-4, IL-6 and IL-10 from the peritoneal macrophages, splenic lymphocytes and macrophage cell lines (Bruneau *et al*., 2012, Dugyala and Sharma, 1996

, Marin *et al*., 2002). However Li *et al*. (2014) reported that AFB_1 increased the levels of IL-6, IFN-γ and TNF-α mRNA expression in the serum and spleen of broilers (Li *et al*., 2014).

The western blotting data showed that AFB_1 suppressed/inhibited the STAT1 protein levels a result which was consistent with the RT-qPCR data **(Fig. 5.1C**).The suppression of

STAT1 protein synthesis by AFB_1 observed in this study is consistent with the results of other researchers who have demonstrated that AFB_1 suppressed the protein levels of IL-4, IL-6 and IL-10 from the peritoneal macrophages, splenic lymphocytes and macrophage cell (Bruneau *et al*., 2012, Dugyala and Sharma, 1996 , Marin *et al*., 2002). However, findings from the study of Li et al. (2014) is at variance with this study. They reported that

AFB_1 increased the IL-6, IFN-γ and TNF-α protein levels in the serum and spleen of broilers Li *et al*. (2014).

STAT1 signaling pathway activated by type I IFNs has been thought about to be tumour suppressive (Dunn *et al*., 2006). The anti-tumour function of STAT1 is considered to be crucial at the onset of tumour development (Tymoszuk *et al*., 2014) and its role is to promote the eradication of cells that have become transformed by both the innate and adaptive immune system (Tymoszuk *et al*., 2014). The activation of STAT1 leads to the up-regulation of genes needed for processing and presentation of antigens in dendritic cells (Tymoszuk *et al*., 2014). Studies have shown that STAT1 prevent tumour development by promoting apoptosis and inhibiting cell proliferation in response to the presence of oncogenic cells (Kim and Lee, 2007). For example STAT1 has been reported to induce apoptosis by promoting the expression of Caspases, Fas and FasL (Ouchi *et al*., 2000, Lee *et al*., 2000). Taken all the evidence available in literature together, STAT1 is well known to play a crucial role in cancer immuno-surveillance. Therefore, the suppression/inhibition of STAT1 protein synthesis could possibly be another mechanism by which AFB_1 may cause cancer.

CHAPTER 6 THE BIOLOGICAL SIGNIFICANCE OF THE SUPPRESSION OF THE TYPE I IFN PATHWAY BY AFB_1

6.1 Introduction

The ability of IFNs to fight against viruses is essential for the survival of higher vertebrates against viral infection. The IFN-α/β which are members of the type I IFN family play crucial roles in conferring antiviral resistance in cells. Studies have shown that treatment of cells with IFN-α/β leads to the up-regulation of several genes and the upregulation of all those genes combine to establish antiviral state (reviewed in Randall and Goodbourn, 2008). For example, Clemens (2005) reported that mice that were deficient in IFN-α/β were highly susceptible to viral infections indicating that IFN-α/β are crucial in fighting against viruses.

After demonstrating that AFB_1 suppressed/inhibited the type I IFN pathway in HepG2 cells, it was hypothesized that AFB_1 would rescue viruses from the antiviral effects of the type I IFN response. If this hypothesis was found to be true then it could mean that AFB_1 could compromise the immune system of individuals exposed to AFB_1 making them more susceptible to viral infections. Therefore the study was conducted with the IFN sensitive chandipura virus as a model virus because: (i) Chandipura is a category II virus that causes mild disease to humans or is difficult to contract via aerosol in a laboratory setting, (ii) in adult humans chandipura virus is not associated with disease that is more severe than a mild refractory illness (Anukumar *et al*., 2013), (iii) the virus easily forms

visible plaques due to its high rate of replication, (iv) it is sensitive to IFN making it a useful tool for IFN bioassay (Berger and Zimmer, 2011).

6.1.1 Chandipura virus as a human pathogen

Chandipura virus which was isolated from the blood samples of patients from the Chandipura village located near Nagpur at the northern Maharashtra state in India (Bhatt and Rodriquez, 1967) has been associated with acute encephalitic illness in children in Andhra Pradesh in India (Rao *et al*., 2004). During the outbreak which occurred from JuneAugust 2003, 329 children were affected with 183 of them dying (Rao *et al*., 2004). Following that another outbreak of encephalitis was observed in children in Gujarat State in western India in 2004 in which 26 cases with 18 deaths were recorded (Chadha *et al*., 2005). In a study conducted by Tandale *et al*. (2008) to monitor acute encephalitis among children of North Telangana region of Andhra Pradesh between May 2005 and April 2006, chandipura virus aetiology was identified in 25 out of 52 cases recorded (Tandale *et al*., 2008).

Even though the virus has been isolated from sand flies in some West African countries like Nigeria and Senegal (Fontenille *et al*., 1994, Traore-Lamizana *et al*., 2001) suggesting a wide distribution, no human cases of infections caused by this virus has been reported outside India.

6.2 Methods

The methods that were employed in this section of the study were virus culture and plaque assay **(sections 3.8-3.8.1).**

6.2.1 Investigating the impact of AFB_1 on the replication of chandipura virus

Under **chapters 4 and 5,** it was demonstrated that AFB_1 suppressed/inhibited the type I IFN pathway. To elucidate the biological significance of this observation, an IFNsensitive virus was used to determine whether AFB_1 would rescue the virus from the antiviral effects of the type I IFN. Because of its sensitivity to IFN which makes it very useful tool for IFN bioassays (Berger and Zimmer, 2011), chandipura virus was used in this experiment.

The study was conducted by growing HepG2 cells as previously described **(section 3.2)**. Briefly monolayers of HepG2 cells were stimulated with or without IFN (up to 400 IU/ml) and simultaneously treated with or without 10 μM AFB_1. Twenty four hours later, the cells were infected with chandipura virus at MOI of 0.1 PFU/cell. After 1 hour of adsorption, fresh growth medium containing neither IFN nor AFB_1 was added to the cells and further incubated. The cell culture supernatants were harvested 24 hours later and the chandipura virus titer quantified by plaque assay **(section 3.8.1)**.

6.3 Results

The results from the virus culture and the plaque assay revealed that the virus titer was highest in cells which were neither stimulated with IFN nor treated with AFB_1. It was observed that AFB_1 killed the Chandipura viruses and that the rate of killing was exacerbated by IFN **(Fig. 6.1).**

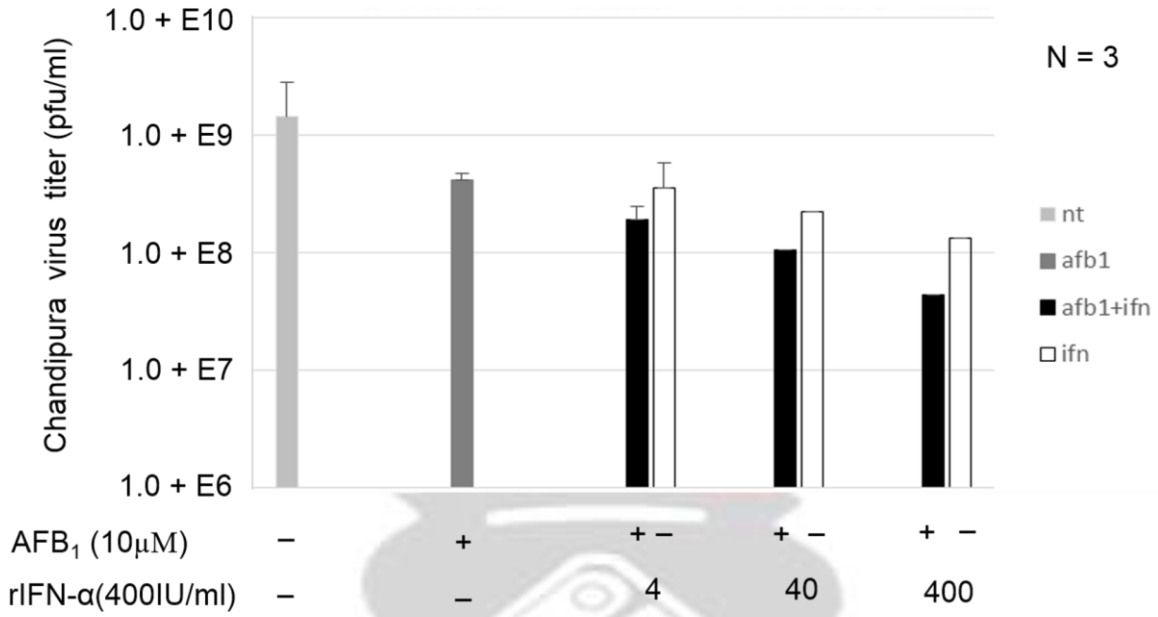

Fig. 6.1: AFB_1 inhibits the replication of Chandipura virus in HepG2 cells. Confluent monolayer of cells were stimulated with or without increasing concentrations of rIFN-α (4, 40 and 400 IU/ml) and simultaneously treated with or without AFB_1 (10 µM) for 24 hours. The cells were infected with chandipura virus at MOI of 0.1 and 24 hours later, the cell culture supernatants were harvested and the virus titer quantified by plaque assay using mouse fibroblast (L929) cells. After incubation for 3 days, plaques were identified by crystal violet staining and counted. Data are presented as mean and standard deviation of three independent experiments conducted in duplicate independent wells. NT: cells which were neither stimulated with IFN nor treated with AFB_1; AFB_1: cells which were treated with only AFB_1; AFB_1+IFN: cells which were stimulated with IFN and simultaneously treated with AFB_1; IFN: cells which were stimulated with IFN alone, Error bars = STD.

6.4 Discussion

The type I IFN pathway is well known for its antiviral property. Therefore after demonstrating at the transcription as well as protein synthesis level that AFB_1 inhibited/suppressed the type I IFN pathway, the expectation was that AFB_1 would ultimately suppress the antiviral pathway of the type I IFN and hence rescue viruses.

Quite a number of studies have utilized the replication of viruses to study the effects of different inhibitors or chemical groups on the antiviral effects of the type I IFN. For example Mutocheluh *et al*. (2011), utilized the replication of IFN sensitive encephalomyocarditis virus (EMCV) in vIRF-2 clone 3-9 and EV clone 5 cells to study the biological significance of vIRF-2 inhibition of the type I IFN pathway and reported that vIRF-2 rescued the EMCV from the antiviral effects of type I IFN (Mutocheluh *et al*.,

2011). In another study, Morrison and Racaniello (2009), also employed the replication of Enterovirus in human cervical carcinoma (HeLa S3), Vero and human hepatoma (Huh 7) cell lines to determine whether proteinase 2 A^{pro} was essential for Enterovirus Replication in type I IFN treated cells and reported that proteinase 2 A^{pro} rescued the Enterovirus from the antiviral effects of the type I IFN. However, proteinase 2 A^{pro} could not rescue the replication of vesicular stomatitis virus and EMCV in type I IFN pre-treated cells (Morrison and Racaniello, 2009).

Taken together the above information, the replication of chandipura virus was utilized in this study to investigate the biological significance of the AFB_1 inhibition of type I IFN pathway in HepG2 cells. Contrary to the initial expectation that AFB_1 would rescue the IFN sensitive virus from the antiviral effects of the type I IFN, results generated from

the virus work pointed to the fact that AFB_1 rather killed the IFN-sensitive chandipura virus and that the rate at which the viruses were killed was exacerbated by IFN. The results obtained from this study was similar to that of Lereau *et al*. (2012) who reported that AFB_1 decreased the replication of HBV in HepaRG cells after exposure of HBV to AFB_1 concentration of up to 5 μM (Lereau *et al*., 2012).

However, a study conducted by Barraud *et al*. (1999) to investigate the effects of AFB_1 exposure on the replication of duck hepatitis B virus in Pekin duck model during the initial stage of virus-AFB_1 interaction showed a significant increase in the titer of duck hepatitis B virus in the serum and liver of AFB_1 treated-ducks compared with dimethyl sulfoxide (DMSO) treated controls (Barraud *et al*., 1999). The increase in viral replication observed in the Pekin duck model was confirmed *in vitro* in primary duck hepatocytes (Barraud *et al*., 1999). In addition, Hahon *et al*. (1979) investigated the effects of AFB_1 exposure on the replication of influenza virus in LLC-MK_2 cells and reported that the levels of virus concentration attained in AFB_1 treated cells were two to four-fold higher compared with the untreated control. The enhanced influenza viral growth rate observed in AFB_1 treated cells in the study of Hahon et al. was attributed to the ability of AFB_1 to inhibit IFN production (Hahon *et al*., 1979).

Taken together, the results of Barraud *et al*. (1999) and Hahon *et al*. (1979) indicate that AFB_1 rescued some viruses from the antiviral effects of the type I IFN response. However, how AFB_1 inhibited the replication of chandipura virus in this study is unknown. Considering results from this study and that of Lereau *et al*. (2012), further studies may be required to unravel the exact mechanism by which AFB_1 kills some viruses. The reason

why AFB_1 might have affected the replication of chandipura virus in this study could be that the replication of chandipura virus may differ from that of other IFN sensitive viruses. Also, AFB_1 could be directly lethal to chandipura virus. It is also possible that AFB_1 may bind and block certain enzymes and substrates that are required for the replication of chandipura virus hence its ability to kill the virus.

CHAPTER 7 GENERAL DISCUSSION

7.1 General discussion

The discovery of aflatoxins in the 1960s sparked lots of studies into the effects of AFB_1 on the health and the immune status of individuals exposed to it. These studies led to the demonstration that AFB_1 causes HCC in humans and experimental animals by inducing AGG to AGT transversion mutations in the tumour suppression gene TP53. This mutation which is detected in most AFB_1 induced HCC leads to (i) inhibition of apoptosis, (ii) inhibition of p53 mediated transcription and (iii) stimulation of liver cell growth leading to cancer (Aguilar *et al*., 1993; Hsu *et al*., 1991).

However, the mechanism by which AFB_1 causes HCC may not be limited to p53 mutation alone and that AFB_1 may also induce cancers by deregulating other anticancer signalling pathways. For example Ubagai *et al*. (2010) demonstrated that AFB_1 may also induce tumourigenesis by deregulating the IGF-IR signalling pathway suggesting that AFB_1 may also induce tumourigenesis by deregulating other anticancer pathways such as the type I IFN signalling pathway. In this study the hypothesis that AFB_1 would suppress/inhibit the type I IFN signalling pathway and thus provide another mechanism by which AFB_1 may cause cancer was tested. In addition, the study was also aimed at investigating the biological significance of the suppression/inhibition of the type I IFN pathway.

The study was divided into three phases. The phase I was aimed at determining the working concentration of AFB_1 and also investigating the effects of AFB_1 on the type I

IFN pathway using the dual luciferase reporter gene assay. Initial results revealed that as the concentration of AFB_1 was increased the viability of the cells decreased. The decrease in cell viability suggested that AFB_1 could have probably affected certain physiological processes that were necessary for cell survival. For example, Al-Hammadi *et al*. (2014) reported that exposure of human lymphocytes to AFB_1 resulted in the impairment in cellular oxygen consumption, caspase activation and necrosis underscoring the immunosuppressive activity in humans exposed to this lethal mycotoxin.

It was also demonstrated that AFB_1 suppressed the type I IFN pathway in human liver derived cell line HepG2 cells. Since the type I IFN forms part of the innate immune system, the demonstration that AFB_1 suppresses the type I IFN pathway could suggest that the immune system of individuals exposed to AFB_1 could be compromised and thus predispose them to viral infections as well as cancers. In the HepG2 cells that were used as model system to mimic what happens in the liver *in vivo*, it was demonstrated that AFB_1 suppressed the mRNA expression levels of *JAK1*, *STAT1* and *OAS3*. In addition it was demonstrated that the suppression of the mRNA expression level of *STAT1* was accompanied by a corresponding decrease in STAT1 protein level **(Fig. 7.1).** The *JAK1* and *STAT1* transcriptional factors are components of the JAK-STAT signaling pathway while *OAS3* is a protein targeted by type I IFN signaling. The JAK-STAT pathway, via STAT1 is activated by IFN-α/β, growth factors etc. The STAT1 signaling pathway activated by IFN has tumour suppressive (Dunn *et al*., 2006) and also activates a myriad of genes that modulate immune functions such as the establishment of antiviral state and induction of apoptosis (Khodarev *et al*., 2012). Also the OAS3 is known to initiate cellular stress response that leads to apoptosis (Silverman, 2003) and hence aid in fighting cancers.

Therefore, the suppression of *STAT1* and *OAS3* mRNA expression in addition to the suppression of STAT1 protein synthesis will mean that the immune system will be greatly compromised and therefore predispose individuals to cancers and viral infections.

It is a well-known fact that the risk of HCC is amplified in the context of HBV and AFB_1 co-existing (Kew, 2003). Therefore, the ability of AFB_1 in enhancing the replication of viruses will support the fact that AFB_1 and viruses more specifically HBV co-operate in inducing HCC. In this study, the ability of AFB_1 in rescuing Chandipura virus was investigated. Even though the Chandipura virus used in this study has tropism for the cells of the nervous system (Anukumar *et al*., 2013), its sensitivity to IFN made it possible to used. It was however observed that AFB_1 was lethal and therefore killed the Chandipura virus, a finding which is in agreement with that of Lereau *et al*. (2012) who reported that AFB_1 inhibited the replication of HBV in HepaRG cells. The mechanism underlying the inability of AFB_1 in rescuing some viruses is not clear and that further studies will be required to unravel that.

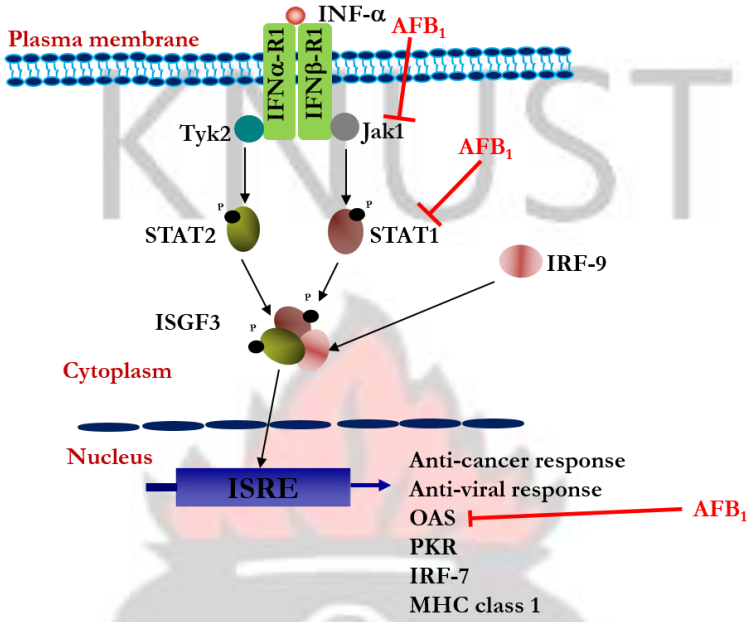

Fig 7.1: Proposed deregulation of type I IFN signaling pathway by AFB_1. The type I signaling pathway begins when ligand such as IFN-α/β binds to IFNAR1/2 which are associated with Tyk2 and JAK1 respectively. The interaction between IFN-α/β and the receptor results in phosphorylation and activation of Tyk2 and JAK1. The activated JAKs in turn recruit and phosphorylate STAT1 and 2 on tyrosine 701 (727 as well) and 690 respectively. The phosphorylated and activated STAT1 and 2 come together and form heterodimer. The dimerized STAT1 and 2 recruits IRF-9 and form the ISGF-3 transcription factor complex. The ISGF-3 enters the nucleus and interacts with ISRE which results in the transcription of IFN-inducible genes that switch on the anti-cancer as well as the anti-viral defense system. The current study has demonstrated that AFB_1 suppresses/inhibits the mRNA expression levels of JAK1, STAT1 and OAS3 (indicated by red font and straight lines crossed at one end). In addition protein expression level of STAT1 was also demonstrated to be supressed/inhibited by AFB_1.

7.2 Limitation(s) of the study

Some factors may have affected some aspect of the results in the current study therefore need to be addressed in future studies. For example, in order to investigate the ability of AFB_1 in rescuing viruses from the antiviral activities of the type I IFN pathway, an IFN sensitive Chandipura virus was used. The Chandipura virus is known to have tropism for the cells of the nervous system (Anukumar *et al*., 2013) and that it use on human liver derived cell line could have affected the result of the virus work. In place of the Chandipura virus, HBV which has tropism for liver cells could have been used. However the difficulty in procuring the virus from the foreign companies made it impossible for HBV to be used in the study. It is therefore recommended that other IFN-sensitive viruses such as EMCV and Dengue should be utilized in future IFN-bioassay works since they are more likely to give the expected results.

7.3 Conclusion(s)

Taken together, the current study has demonstrated that AFB_1 suppresses/inhibits the type I IFN response pathway by inhibiting the transcripts of *JAK1, STAT1* and *OAS3* and STAT1 protein which are the key signaling elements of the JAK-STAT-ISRE arm of the type I IFN pathway and that the inhibition of the type I IFN pathway could be another mechanism by which AFB_1 could cause HCC. The study showed that AFB_1 was lethal to chandipura virus suggesting that chandipura virus was not a suitable tool to be used to study the biological significance of the suppression/inhibition of the type I IFN response pathway by AFB_1.

7.4 Future perspectives

To better understand the role of AFB_1 in modulating the type I IFN response, the following recommendations have been suggested:

(i) The role of AFB_1 in modulating the type I IFN response in different cell lines must be investigated. It is a well-known fact that the liver plays a major role in the metabolism of many compounds and also represent an important target organ in systemic toxicity. This characteristic of liver explains why hepatic models are used in assessing the toxicity of many compounds *in vitro* (Davila *et al*., 1998). Even though the HepG2 cells used in this study are suitable *in vitro* model system, it is recommended that the study be repeated using other liver cell lines such as HepaRG and HuH7 cells so that the results does not become cell line specific.

(ii) Further studies are required to unravel the possible mechanism by which AFB_1 kills the viruses.

References

Aaronson, D. S. & Horvath, C. M. (2002). A road map for those who don't know JAK-STAT. *Science,* 296**,** 1653-5.

Aguilar, F., Hussain, S. P. & Cerutti, P. (1993). Aflatoxin B1 induces the transversion of G→T in codon 249 of the p53 tumor suppressor gene in human hepatocytes. *Proc. Natl. Acad. Sci. USA,* 90:8586–8590.

Al-Hammadi, S., Marzouqi, F., Al-Mansouri, A., Shahin, A., Al-Shamsi, M., Mensah-Brown, E. & Abdul-Kader, S. (2014). The Cytotoxicity of Aflatoxin B1 in Human Lymphocytes. *Sultan Qaboos University Med J,* Vol. 14, Iss. 1, pp. e65-71.

Allain, J. P., Candotti, D., Giachetti, C., Shyamala, V., Soldam, K., Sarkodie, F. & Phelpi, B. (2003). The risk of hepatitis B virus infection by transfusion in Kumasi, Ghana. *Blood* 101(6):2419-2425.

Allione, A., Bernabei, P., Bosticardo, M., Ariotti, S., Forni, G. & Novelli, F. (1999). Nitric oxide suppresses human T lymphocyte proliferation through IFN-gamma-dependent and IFNgamma- independent induction of apoptosis. *J Immunol,* 163:4182-91.

Anukumar, B., Amirthalingam, B. G., Shelke, V. N., Gunjikar, R. & Shewale, P. (2013). Neuroinvasion of Chandipura virus mediates pathogenesis in experimentally infected mice. *Int J Clin Exp Pathol,* 6(7): 1272–1281.

Atanasiu, P. & Chany, C. (1960). Effect of interferon originating in malignant cells on experimental infection of the newborn hamster with polyoma virus. *C R Seances Soc Biol Fil,* 25:1687–1689.

Au, W. C., Moore, P. A., LaFleur, D. W., Tombal, B. & Pitha, P. M. (1998). Characterization of the interferon regulatory factor-7 and its potential role in the transcription activation of interferon A genes. *J Biol Chem,* 273, **,** 29210–29217.

Autrup, J. L., Schmidt, J. & Autrup, H. (1993). Exposure to aflatoxin B1 in animal-feed production plant workers. *Environ. Health. Perspect,* 99:195-197.

Awuah, R. T. & Kpodo, K. A. (1996). High incidence of Aspergillus flavus and aflatoxins in stored groundnut in Ghana and the use of a microbial assay to assess the inhibitory effects of plant extracts on aflatoxin synthesis. *Mycopathologia* 134(2): 109-114.

Aziz, T. A., Aziz, M. A. & et al (2005). Interferon-alpha gene therapy prevents aflatoxin and carbon tetrachloride promoted hepatic carcinogenesis in rats. *Int. J. Mol. Med,* 15(1): 21-

26.

Azziz-Baumgartner, E., Lindblade, K., Gieseker, K., Rogers, H. S., Kieszak, E. & et al (2005). Case-control study of an acute aflatoxicosis outbreak, Kenya, 2004. *Environ. Health. Perspect*, 113:1779-83.

Baccala, R., Kono, D. H. & Theofilopoulos, A. N. (2005). Interferons as pathogenic effectors in autoimmunity. *Immunol Rev* 204:9–26.

Banninger, G. & Reich, N. C. (2004). STAT2 nuclear trafficking. *J. Biol. Chem,* 279, 39199–39206.

Barraud, L., Guerret, S., Chevallier, M., Borel, C., Jamard, C., Trepo, C. & et al (1999). Enhanced Duck Hepatitis B Virus Gene Expression Following Aflatoxin B1 Exposure. *Hepatology* 29:1317-1323.

Bbosa, G. S., David Kitya, D., Lubega, A., Ogwal-Okeng, J., Anokbonggo, W. W. & Kyegombe, D. B. (2013a). Re- view of the biological and health effects of aflatoxins on body organs and body systems: Aflatoxins-Recent ad- vances and future prospects. *Intechopen Publisher,* 12, 239-265. .

Bbosa, G. S., Kitya, D., Odda, J. & Ogwal-Okeng, J. (2013b). Aflatoxins metabolism, effects on epigenetic mechanisms and their role in carcinogenesis. *Health,* Vol.5, No.10A, 14-34

Bekisz, J., Baron, S., Balinsky, C., Morrow, A. & Zoon, K. C. (2010). Anti-proliferative properties of type I and type II Interferon. *Pharmaceuticals,* 3**,** 994-1015.

Belardelli, F., Ferrantini, M., Proietti, E. & Kirkwood, J. M. (2002). Interferon-alpha in tumor immunity and immunotherapy. *Cytokine Growth Factor Rev* 13: 119-134.

Berger, R. M. & Zimmer, G. (2011). A vesicular stomatitis virus replicon-based bioassay for the rapid and sensitive determination of multi-species type I interferon. *PLoS ONE* 6, e25858.

Bhat, N. K., Emeh, J. K., Niranjan, B. G. & Avadhani, N. G. (1982). Inhibition of mitochondrial protein synthesis during early stages of aflatoxin b-induced hepatocarcino- genesis. *Cancer Research,* 42, 1876-1880.

Bhatt, P. N. & Rodriquez, F. M. (1967). Chandipura: A new Arbovirus isolated in India from patients with febrile illness. *Indian Journal of Medical Research,* 55(12): 1295-1305**,** .

Bhattacharya, S., Eckner, R., Grossman, S., Oldread, E., Arany, Z., D'Andrea, A. & Livingston, D. M. (1996). Cooperation of Stat2 and p300/CBP in signalling induced by interferon-a.

Nature, 383**,** 344–347.

Blount, W. P. (1961). Turkey "X" disease. *J. Br. Turk. Fed,* 9**,** 52-57.

Bosch, F. X., Ribes, J., Cleries, R. & Diaz, M. (2005). Epidemiology of hepatocellular carcinoma. *Clin Liver Dis*, 9**,** 191-211.

Bressac, B., Kew, M., Wands, J. & Ozturk, M. (1991). Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. *Nature,* 350**,,** 429-431.

Bretagnol, F. & et al (2008). The effect of sterilization processes on the bioadhesive properties and surface chemistry of a plasma-polymerized polyethylene glycol film: XPS characterization and L929 cell proliferation tests. *Acta Biomater* vol. 4, pp. 1745-51.

Bruneau, J. C., Stack, E., O'Kennedy, R. & Loscher, C. E. (2012). Aflatoxins B1, B2 and G1 modulate cytokine secretion and cell surface marker expression in J774A. 1 murine macrophages. *Toxicol. In Vitro,* 26, 686–693.

Casey, S. C., Amedei, A., Aquilano, K., Azmid, A. S., Benencia, F., Bhaktaf, D. & et al (2015). Cancer prevention and therapy through the modulation of the tumor microenvironment. *Seminars in Cancer Biology* 35:S199–S223.

CDC (2004). Outbreak of aflatoxin poisoning in eastern and central provinces, Kenya, JanuaryJuly 2004 *MMWR. Morb. Mortal. Wkly Rep,* 53 (34): 790-3.

Chadha, M. S., Arankalle, V. A., Jadi, R. S., Joshi, M. V., Thakare, J. P., Mahadev, P. V. & Mishra, A. C. (2005). An Outbreak of Chandipura Virus Encephalitis in the Eastern Districts of Gujarat State, India. *American Journal of Tropical Medicine and Hygiene* 73 (3): 566–570.

Chemin, I., Ohgaki, H., Chisari, F. V. & Wild, C. P. (1999). Altered expression of hepatic carcinogen metabolizing enzymes with liver injury in HBV transgenic mouse lineages expressing various amounts of hepatitis B surface antigen. *Liver,* 19, 81–87.

Chen, C. J., Yu, M. W. & Liaw, Y. F. (1997). Epidemiological characteristics and risk factors of hepatocellular carcinoma. *J. Gastroenterol. Hepatol,* 12: S294–S308.

Chen, H., Chang, M. H., Ni, Y. H., Hsu, H. Y., Lee, P. I., Lee, C. Y. & et al (1996). Seroepidemiology of hepatitis B virus infection in children: ten years of mass vaccination in Taiwan. *JAMA* 276(11)**,** 906–908.

Cheng, Y. H., Shen, T. F., Pang, V. F. & Chen, B. J. (2002). Effect of Aflatoxin B1 on the Function of Peritoneal Macrophage from Mule Duck. *Asian-Aust. J. Anim. Sci,* Vol. 15, No. 3: 438-444.

References

Chin, Y. E., Kitagawa, M., Kuida, K., Flavell, R. A. & Fu, X. Y. (1997). Activation of the STAT signaling pathway can cause expression of caspase 1 and apoptosis. *Mol Cell Biol,* 17:5328-37.

Civas, A., Island, M. L., Genin, P., Morin, P. & Navarro, S. (2002). Regulation of virus-induced interferon-A genes. . *Biochimie* 84**,** 643–654.

Clemens, M. J. (2005). Translational control in virus infected cells:models for cellular stress responses. *Semin. Cell. Dev. Biol,* 16, 13-20.

Clifford, J. L., Yang, X., Walch, E., Wang, M. & Lippman, S. M. (2003). Dominant negative signal transducer and activator of transcription 2 (STAT2) protein: stable expression blocks interferon α action in skin squamous cell carcinoma cells. *Mol Cancer Ther* 2:453–9.

Cohen, B. & Parkin, J. (2001). An overview of the immune system. *Lancet* 357 (9270): 1777–89.

Colamonici, O. R., Porterfield, B., Domanski, P., Constantinescu, S. & Pfeffer, L. M. (1994). Complementation of the interferon alpha response in resistant cells by expression of the cloned subunit of the interferon alpha receptor. A central role of this subunit in interferon alpha signaling. *J. Biol. Chem,* 269:9598–9602.

Colombo, M. P. & Trinchieri, G. (2002). Interleukin-12 in anti-tumor immunity and immunotherapy. *Cytokine Growth Factor Rev* 13:155–168.

Cullen, J. M. & Newberne, P. M. (1994). *Acute hepatotoxicity of aflatoxins. In: Eaton DL, Groopman JD (Eds.). The toxicology of aflatoxins*.

Darnell Jr, J. E., Kerr, I. M. & Stark, G. R. (1994). Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* 264**,** 1415–21.

Dauffy, J., Mouchiroud, G. & Bourette, R. P. (2006). The interferon-inducible gene, Ifi204, is transcriptionally activated in response to M-CSF, and its expression favors macrophage differentiation in myeloid progenitor cells. *J Leukoc Biol* 79:173–83.

Davila, J. C., Rodriguez, R. J., Melchert, R. B. & Acosta, D. J. (1998). Predictive value of in vitro model systems in toxicology. *Annu. Rev. Pharmacol. Toxicol,* 38, 63–96. de Weerd, N. A., Samarajiwa, S. A. & Hertzog, P. J. (2007). Type I interferon receptors:

biochemistry and biological functions. *J Biol Chem* 282 (28)**,** 20053–20057.

Diamond, M. S., Kinder, M., Matsushita, H., Mashayekhi, M., Dunn, G. P., Archambault, J. M. & et al (2011). Type I interferon is selectively required by dendritic cells for immune rejection of tumors. *J. Exp. Med,* 208: 1989–2003.

Domanski, P. & Colamonici, O. R. (1996). The type-I interferon receptor; The long and short of it. *Cytokine Growth Factor Rev*, 7: 143–151.

Donnelly, R. P. & Kotenko, S. V. (2010). Interferon-lambda: a new addition to an old family. *J Interferon Cytokine Res* 30:555–64.

Dragan, A. I., Hargreaves, V. V., Makeyeva, E. N. & Privalov, P. L. (2007). Mechanisms of activation of interferon regulator factor 3: the role of C-terminal domain phosphorylation in IRF-3 dimerization and DNA binding. *Nucleic Acids Res,* 35, 3525–3534.

Du, Y. P., Peng, J. S., Sun, A., Zang, Z. H., Ling, W. H. & Zhu, H. L. (2009). Assessment of the effect of betaine on p16 and c-myc DNA methylation and mRNA expression in a chemical induced rat liver cancer model. *BMC Cancer,* 9:261

Dugyala, R. R. & Sharma, R. P. (1996). The effect of aflatoxin B1 on cytokine mRNA and corresponding protein levels in peritoneal macrophages and splenic lymphocytes. *International Journal of Immunopharmacology,* 18, 599-608.

Dulbecco, R. & Vogt, M. (1953). Some problems of animal virology as studied by the plaque technique. *Cold Spring Harbor Symp. Quant. Biol,* 18, 273-279.

Dunn, G. P., Koebel, C. M. & Schreiber, R. D. (2006). Interferons, immunity and cancer immunoediting. *Nat. Rev. Immunol*, 6:836–848.

Dutta, P. & Li, W. X. (2013). Role of the JAKSTAT Signalling Pathway in Cancer. *In: eLS. John Wiley & Sons, Ltd: Chichester*.

Earle, W. R. & et al (1943). Production of malignancy in vitro. IV. The mouse fibroblast cultures and changes seen in the living cells. *J. Natl. Cancer Inst. 4: 165-212,* 4: 165-212.

Eaton, D. L. & Gallagher, E. P. (1994). Mechanisms of aflatoxin carcinogenesis. *Annual Review of Pharmacology and Toxicology,* 34, 135-172.

Eaton, D. L. & Groopman, J. D. (1994). *The toxicology of aflatoxins: Human Health, Verterinary and Agricultural Significance,* San Diego: Academic.

References

Edds, G. T., Nair, K. P. C. & Simpson, C. F. (1973). Effect of aflatoxin B1 on resistance in poultry against cecal coccidiosis and Marek's disease. *American Journal of Veterinary Research* 34, 819-826.

Egwuagu, C. E., Li, W., Yu, C. R. & et al (2006). Interferon-gamma induces regression of epithelial cell carcinoma: critical roles of IRF-1 and ICSBP transcription factors. *Oncogene* 25(26)**,** 3670-3679.

Elahi, A., Zhang, L., Yeatman, T. J. & et al (2008). HPP1-mediated tumor suppression requires activation of STAT1 pathways. *Int J Cancer* 122(7) 1567-1572.

Elinav, E., Nowarski, R., Thaiss, C. A., Hu, B., Jin, C. & Flavell, R. A. (2013). Inflammationinduced cancer: crosstalk between tumours, immune cells and microorganisms *Nat. Rev. Cancer*, 13:759–771.

Faria, G. & et al (2009). Chlorhexidine-induced apoptosis or necrosis in L929 fibroblasts: A role for endoplasmic reticulum stress. *Toxicol Appl Pharmacol*, vol. 234, pp. 256-65.

Feng, G. S., Chong, K., Kumar, A. & Williams, B. R. (1992). Identification of double-stranded RNA-binding domains in the interferon-induced double-stranded RNA-activated p68 kinase. Proc. Natl. Acad. Sci. U.S.A.,.

Fontenille, D., Traore-Lamizana, M., Trouillet, J., Leclerc, A., Mondo, M., Ba, Y., Digoutte, J. P. & G, Z. H. (1994). First Isolations of Arboviruses from Phlebotomine Sand Flies in West Africa. *American Journal of Tropical Medicine and Hygiene* 50 (5): 570–574

Forouharmehr, A., Harkinezhad, T. & Qasemi-Panahi, B. (2013). Evaluation of STAT5A Gene Expression in Aflatoxin B1 Treated Bovine Mammary Epithelial Cells. *Advanced Pharmaceutical Bulletin*, 3(2), 461-464.

Fragale, A., Marsili, G. & Battistini, A. (2013). Genetic and Epigenetic Regulation of Interferon Regulatory Factor Expression: Implications in Human Malignancies. *J Genet Syndr Gene Ther* 4:11.

Frahm, T., Hauser, H. & Koster, M. (2006). IFN-type-I-mediated signaling is regulated by modulation of STAT2 nuclear export. *J. Cell. Sci*, 119, 1092–1104.

Fu, X. Y., Schindler, C., Improta, T., Aebersold, R. & Darnell Jr, J. E. (1992). The proteins of ISGF-3, the interferon alpha-induced transcriptional activator, define a gene family involved in signal transduction. *Proc Natl Acad Sci USA* 89:7840–3.

References

Fuertes, M. B., Kacha, A. K., Kline, J., Woo, S. R., Kranz, D. M., Murphy, K. M. & F, G. T. (2011). Host type I IFN signals are required for antitumor CD8+ T cell responses through CD8alpha+ dendritic cells. *J. Exp. Med,* 208: 2005–2016.

Fuertes, M. B., Woo, S. R., Burnett, B., Fu, Y. X. & Gajewski, T. F. (2013). Type I interferon response and innate immune sensing of cancer. *Trends Immunol,* 34, 67–73.

Gajewski, T. F., Schreiber, H. & Fu.Y.X (2013). Innate and adaptive immune cells in the tumor microenvironment. *Nat. Immunol,* 14: 1014–1022.

Gallagher, E. P., Wienkers, L. C., Stapleton, P. L., Kunze, K. L. & Eaton, D. L. (1994). Role of human microsomal and human complementary DNA-expressed cytochromes P4501A2 and P4503A4 in the bioactivation of aflatoxin B1. *Cancer Res,* 54:101-108.

Gamero, A. M., Potla, R., Wegrzyn, J., Szelag, M., Edling, A. E., Shimoda, K. & et al (2006). Activation of Tyk2 and Stat3 is required for the apoptotic actions of interferon-beta in primary pro-B cells. *J. Biol. Chem,* 281:16238–16244.

Ghosh, S. K., Desai, M. R., Pandya, G. L. & Venkaiah, K. (1997). Airborne aflatoxin in the grain processing industries in India. *Am. Ind. Hyg. Assoc. J,* 58(8): 583-586.

Gnonlonfin, G. J., Hell, K., Fandohan, P. & Siame, A. B. (2008). Mycoflora and natural occurrence of aflatoxins and fumonisin B1 in cassava and yam chips from Benin, West Africa. *Int. J. Food Microbiol,* 122(1–2):140–147.

Gong, Y., Hounsa, A. & Egal, S. (2004). Post weaning exposure to aflatoxin results in impaired child growth: a longitudinal study in Benin, West Africa *Environmental Health Perspective,* 112: 1334-38.

Gong, Y., Turner, P. C., Hall, A. J. & Wild, C. P. (2008). Aflatoxin exposure and impaired child growth in West Africa: An unexplored international public health burden? In: Mycotoxins: Detection Methods, Management, Public Health and Agricultural Trade, (Leslie JF, Bandyopadhyay R, Visconti A, eds) *Oxfordshire, UK: CAB International,* 53-65.

Gong, Y. Y., Egal, S., Hounsa, A., Turner, P. C., Hall, A. J., Cardwell, K. F. & Wild, C. P. (2003). "Determinants of aflatoxin exposure in young children from Benin and Togo, West Africa: the critical role of weaning". . *Int. J. Epidemiol. ,* 32(4): 556-62.

References

Gouas, D., Shi, H. & Hainaut, P. (2009). The aflatoxin-induced TP53 mutation at codon 249 (R249S): biomarker of exposure, early detection and target for therapy. *Cancer Lett* 286, 29–37.

Gresser, I. (1970). Interferon and cancer: therapeutic prospects *Rev. Eur. Etud. Clin. Biol*, 15, 23-27.

Grivennikov, S. I., Greten, F. R. & Karin, M. (2010). Immunity, inflammation, and cancer. *Cell* 140:883–899.

Groopman, J. D. & Kensler, T. W. (2005). Role of metabolism and viruses in aflatoxin-induced liver cancer. *Toxicol. Appl. Pharmacol*, 206, 131–137.

Groopman, J. D., Kensler, T. W. & Wild, C. P. (2008). Protective interventions to prevent aflatoxin-induced carcinogenesis in developing countries. *Ann. Rev. Public Health*, 29:187–203.

Guillouzo, A., Corlu, A., Aninat, C., Glaise, D., Morel, F. & Guguen-Guillouzo, C. (2007). The human hepatoma HepaRG cells: a highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. *Chem Biol Interact* 168**,** 66–73.

Guirnalda, P., Wood, L., Goenka, R. & et al (2013). Interferon gamma-induced intratumoral expression of CXCL9 alters the local distribution of T cells following immunotherapy with Listeria monocytogenes. *Oncoimmunology* 2:e25752.

Guo, Y., Xiao, P., Lei, S., Deng, F., Xiao, G. G. & et al (2008). How is mRNA expression predictive for protein expression? A correlation study on human circulating monocytes. *Acta Biochim Biophys Sin* 40: 426–436

Hagberg, N. & Ronnblom, L. (2015). Systemic Lupus Erythematosus – A Disease with A Dysregulated Type I Interferon System. *Scandinavian Journal of Immunology*, 82, 199–207.

Hahon, N., Booth, J., A & Stewart, J. D. (1979). Aflatoxin inhibition of viral interferon induction. *Antimicrob Agents Chemother*, 16(3): 277-282.

Hall, A. J. & Wild, C. P. (1994). Epidemiology of aflatoxin-related disease. The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance. D. L Ealton and J.D. Groopman. *San Diego, CA, Academic Press, Inc***,** 233-258.

Hamid, A. S., Tesfamariam, I. G., Zhang, Y. & Zhang, Z. G. (2013). Aflatoxin B1-induced hepatocellular carcinoma in developing countries: Geographical distribution, mechanism of action and prevention (Review). *Oncology letters*, 5: 1087-1092.

References

Hamilton, P. B. & Harris, J. R. (1971). Interaction of aflatoxicosis with *Candida albicans* infections and other stresses in chickens *Poultry Science* 50, 906-912.

Hayden, M. S. & Ghosh, S. (2004). Signaling to NF-kB. *Genes Dev*, 18, 2195-2224.

Henry, S. H., Bosch, F. X., Troxell, T. C. & Bolger, P. M. (1999). Policy forum: Public health. Reducing liver cancer-global control of aflatoxin. *Science*, 286 (5449):2453–2454.

Hollstein, M. & Hainaut, P. (2010). Massively regulated genes: the example of TP53 *J. Pathol*, 220, 164–173.

Honda, K., Takaoka, A. & Taniguchi, T. (2006a). Type I Inteferon Gene Induction by the Interferon Regulatory Factor Family of Transcription Factors. *Immunity*, Volume 25, Issue 3, p349–360.

Honda, K. & Taniguchi, T. (2006). IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors *Nat Rev Immunol*, 6, 644–658.

Hong, Y., Muller, U. R. & Lai, F. (2003). Discriminating two classes of toxicants through expression analysis of HepG2 cells with DNA arrays. *Toxicol. In Vitro*, 17**,** 85–92.

Hsu, I. C., Metcalf, R. A., Sun, T., Welsh, J. A., Wang, N. J. & Harris, C. C. (1991). Mutational hotspot in the p53 gene in human hepatocellular carcinomas. *Nature* 350**,** 427–428.

Huang, B., Qi, Z. T., Xu, Z. & Nie, P. (2010). Global characterization of interferon regulatory factor (IRF) genes in vertebrates: Glimpse of the diversification in evolution. *BMC immunology* 11: 22

IARC (1987). Aflatoxins: In Overall Evaluations of Carcinogenicity. *IARC Monographs on the Evaluation of the Carcinogenic Risks of Chemicals to Humans*, suppl.7. Lyon, France: International Agency for Research on Cancer. pp. 245-395.

IARC (1993). Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins. *IARC Press,Lyon*.

IARC (1995). IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol. 62,Wood Dust and Formaldehyde, Lyon, IARCPress, pp. 217–362.

IARC (2002). Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. *IARC Monogr Eval Carcinog Risks Hum* 82**,** 171–300.

IARC (2013). Latest world cancer statistics. *press release*, Lyon,France

Ilkovitch, D., Handel-Fernandez, M. E., Herbert, L. M. & Lopez, D. M. (2008). Antitumor

References

Effects of Mucin 1/sec Involves the Modulation of Urokinase-Type Plasminogen Activator and Signal Transducer and Activator of Transcription 1 Expression in Tumor Cells. *American Journal of Cancer***,** 1931-1940.

Indraccolo, S. (2010). Interferon-alpha as angiogenesis inhibitor: learning from tumor models. *Autoimmunity,* 43: 244–247.

Isaacs, A. & Lindenmann, J. (1957). Virus interference. I. The interferon. *Proc R Soc London, Ser B* 147**,** 258-267.

Ito, T., Yang, M. & May, W. S. (1999). RAX, a cellular activator for double-stranded RNAdependent protein kinase during stress signaling. *J. Biol.Chem,* 274 (22), 15427-15432.

Jablonska, J., Leschner, S., Westphal, K., Lienenklaus, S. & Weiss, S. (2010). Neutrophils responsive to endogenous IFN-beta regulate tumor angiogenesis and growth in a mouse tumor model. *J. Clin. Invest* 120:1151–1164.

Jemal, A., Bray, F., Center, M. M. & et al (2011). Global cancer statistics. *CA Cancer J Clin,* 61**,** 69.

Jia, L., Wang, X. W. & Harris, C. C. (1999). Hepatitis B virus X protein inhibits nucleotide excision repair. *Int J Cancer* 80 875–879.

Jiang, M., Peng, X., Fang, J., Cui, H., Yu, Z. & Chen, Z. (2015). Effects of Aflatoxin B1 on TCell Subsets and mRNA Expression of Cytokines in the Intestine of Broilers. *Int. J. Mol. Sci,* 16, 6945-6959.

Jiang, Y., Jolly, P., Ellis, W. O., Wang, J. S., Phillips, T. D. & Williams, J. H. (2005). Aflatoxin B_1 albumin adduct levels and cellular immune status in Ghanaians. *Int Immunol,* 17(6): 807-814.

Joint FAO/WHO Expert Committee on Food Additives (JECFA) (1998). Safety evaluation of certain food additives and contaminants, WHO Food Additives Series 40, Aflatoxins. In: The forty-ninth meeting of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization.

Jolly, P., Jiang, Y., Ellis, W. O., Wang, J. S., Afriyie-Gyawu, E. & Phillips, T. D., et al. (2008). *Modulation of the human immune system by aflatoxin. In: Mycotoxins: Detection Methods, Management, Public Health and Agricultural Trade (Leslie JFBR, Visconti A, ed).* , Oxfordshire, UK: CAB International, 41-52.

References

Kanebratt, K. P. & Andersson, T. B. (2008). Evaluation of HepaRG cells as an in vitro model for human drug metabolism studies. *Drug Metab Dispos,* 36**,** 1444–1452.

Kessler, D. S., Levy, D. E. & Darnell Jr, J. E. (1988). Two interferons induced nuclear factors bind a single promoter element in interferon-stimulated genes. *Proc Natl Acad Sci USA* 85**,** 8521-8525.

Kessler, D. S., Veals, S. A., Fu, X. Y. & Levy, D. E. (1990). Interferonalpha regulates nuclear translocation and DNA-binding affinity of ISGF3, a multimeric transcriptional activator. *Genes Dev* 4:1753-65.

Kew, M. C. (2003). Synergistic interaction between aflatoxin B1 and hepatitis B virus in hepatocarcinogenesis. *Liver Int,* 23**,** 405–409.

Kew, M. C. (2008). *Hepatocellular carcinoma. Molecular biology and genetics. In: Gastrointestinal Oncology. 2nd. Ed. Kelsen DP, Daly J. M, Kem, S. E. Levin, B., Tepper, J.E., Van Cutsem E (eds.),* Philadelphia: Lippincott, Williams and Wilkins; pp. 405-18. Khodarev, N. N., Roizman, B. & Weichselbaum, R. R. (2012). Molecular pathways: interferon/stat1 pathway: role in the tumor resistance to genotoxic stress and aggressive growth. *Clin Cancer Res* 18(11):3015–3021.

Killip, M. J., Fodor, E. & Randall, R. E. (2015). Influenza virus activation of the interferon system. *Virus Research* 209;11–22.

Kim, H. S. & Lee, M. S. (2007). STAT1 as a key modulator of cell death. *Cell Signal* 19(3):454–465.

Klover, P. J., Muller, W. J., Robinson, G. W., Pfeiffer, R. M., Yamaji, D. & Hennighausen, L. (2010). Loss of STAT1 from mouse mammary epithelium results in an increased neuinduced tumor burden. *Neoplasia* 12(11):899-905.

Knasmuller, S., Parzefall, W., Sanyal, R., Ecker, S., Schwab, C., Uhl, M. & et al (1998). Use of metabolically competent human hepatoma cells for the detection of mutagens and antimutagens. *Mutat. Res,* 402**,** 185–202.

Kominsky, S. L., Hobeika, A. C., Lake, F. A., Torres, B. A. & Johnson, H. M. (2000). Downregulation of neu/HER-2 by interferon-gamma in prostate cancer cells. *Cancer Res,* 60:3904-8.

Kortylewski, M. & Yu, H. (2008). Role of Stat3 in suppressing anti-tumor immunity *CurrOpin Immunol,* 20:228–33.

References

Kpodo, K. A., Thrane, U. & Hald, B. (2000). *Fusaria* and fumonisins in maize from Ghana and their co-occurence with aflatoxins. *International Journal of Food Microbiology,* 61.

Kumar, K. P., McBride, K. M., Weaver, B. K., Dingwall, C. & Reich, N. C. (2000). Regulated nuclear-cytoplasmic localization of interferon regulatory factor 3, a subunit of doublestranded RNA-activated factor 1. *Mol Cell Biol*, 20, 4159–4168.

Kumi, J., Mitchell, N. J., Asare, G. A., Dotse, E., Kwaa, F., Phillips, T. D. & Ankrah, N.-A. (2014). Aflatoxins and Fumonisins Contamination of Home-Made Food (Weanimix) from Cereal-Legume Blends for Children. *Ghana Med J,* Sep; 48(3): 121–126.

Ladep, N. G., Lesi, O. A., Mark, P., Lemoine, M., Onyekwere, C., Afihene, M., Crossey, M. M. E. a. & Taylor-Robinson, S. D. (2014). Problem of hepatocellular carcinoma in West Africa. *World J Hepatol*, Nov 27; 6(11): 783–792.

Lampson, G. P., Tytell, A. A., Nemes, M. M. & Hilleman, M. R. (1963). Purification and characterization of chick embryo interferon *Proc. Soc. exp. BioL Med,* 112, 468.

Laryea, D. O., Awuah, B., Amoako, Y. A., Osei-Bonsu, E., Dogbe, J., Larsen-Reindorf, R. & et al (2014). Cancer incidence in Ghana, 2012: evidence from a population-based cancer registry. *BMC Cancer*, 14: 362.

Lee, C. K., Rao, D. T., Gertner, R., Gimeno, R., Frey, A. B. & Levy, D. E. (2000). Distinct requirements for IFNs and STAT1 in NK cell function *J Immunol* 165**,** 3571–7.

Lembo, M., Sacchi, C., Zappador, C. & et al (1998). Inhibition of cell proliferation by the interferon-inducible 204 gene, a member of the Ifi 200 cluster. *Oncogene*, 16:1543–51.

Lereau, M., Gouas, D., Villar, S., Besaratinia, A., Hautefeuille, A., Berthillon, P. & et al (2012). Interactions between hepatitis B virus and aflatoxin B1: effects on p53 induction in HepaRG cells. *Journal of General Virology* 93**,** 640–650.

Li, Y., Ma, Q. G., Zhao, L. H., Wei, H., Duan, G. X., Zhang, J. Y. & Ji, C. (2014). Effects of lipoic acid on immune function, the antioxidant defense system, and inflammation-related genes expression of broiler chickens fed aflatoxin contaminated diets. *Int. J. Mol. Sci,* 15, 5649–5662.

Lin, R., Genin, P., Mamane, Y. & Hiscott, J. (2000). Selective DNA binding and association with the CREB binding protein coactivator contribute to differential activation of alpha/beta interferon genes by interferon regulatory factors 3 and 7. *Mol Cell Biol*, 20, 6342–6353.

References

Lin, R., Heylbroeck, C., Pitha, P. M. & Hiscott, J. (1998). Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential, and proteasome-mediated degradation. *Mol. Cell. Biol*, 18:2986–2996.

Liu, Y. & Wu, F. (2010). Global Burden of Aflatoxin-Induced Hepatocellular Carcinoma: A Risk Assessment. *Environ Health Perspect*, 118**,** 818-24.

Livak, K. J. & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods*, 25, 402–408.

Lye, M. S., Ghazali, A. A., Mohan, J., Alwin, N. & Nair, R. C. (1995). An outbreak of acute hepatic encephalopathy due to severe aflatoxicosis in Malaysia. . *Am J Trop Med Hyg.* , 53:68–72.

Mamane, Y., Heylbroeck, C., Genin, P., Algarte, M., Servant, M. J., LePage, C. & et al (1999). Interferon regulatory factors: the next generation. *Gene*, 237**,** pp. 1–14.

Marie, I., Durbin, J. E. & Levy, D. E. (1998). Differential viral induction of distinct interferonalpha genes by positive feedback through interferon regulatory factor-7. *EMBO J*, 17 pp.

6660–6669.

Marin, D., Taranu, I., Bunaciu, R., Pascale, F., Tudor, D., Avram, N. & et al (2002). Changes in performance, blood parameters, humoral and cellular immune responses in weanling piglets exposed to low doses of aflatoxin. *J. Anim. Sci*, 80, 1250–1257.

Marroqui, L., Dos Santos, R. S., Floyel, T., Grieco, F. A., Santin, I., Op de Beeck, A. & et al (2015). TYK2, a candidate gene for type 1 diabetes, modulates apoptosis and the innate immune response in human pancreatic beta-cells. *Diabetes* http://dx.doi.org/10.2337/db15-0362. pii: db150362 (Epub ahead of print).

Martin, J. & Dufour, J. F. (2008). Tumor suppressor and hepatocellular carcinoma. *World J. Gastroenterol*, 14:1720–1733.

Matsuyama, T., Kimura, T., Kitagawa, M., Pfeffer, K., Kawakami, T., Watanabe, N. & et al (1993). Targeted disruption of IRF-1 or IRF-2 results in abnormal type I IFN gene induction and aberrant lymphocyte development. *Cell*, 75**,** pp. 83–97.

Michael, G. Y., Thaxton, P. & Hamilton, P. B. (1973). Impairment of the reticuloendothelial system of chickens during aflatoxicosis. *Poult Sci*, 52(3)1206–1207.

References

Miller, D. M. & Wilson, D. M. (1994). *Veterinary diseases related to aflatoxins. The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance. D.L Ealton and J.D. Groopman,* San Diego, CA, Academic Press, Inc:347-364.

Miyamoto, M., Fujita, T., Kimura, Y., Maruyama, M., Harada, H., Sudo, Y., Miyata, T. & Taniguchi, T. (1988). Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-beta gene regulatory elements. *Cell,* 54**,** pp. 903–913.

Mogensen, K. E., Lewerenz, M., Reboul, J., Lutfalla, G. & Uze, G. (1999). The type I interferon receptor: structure, function, and evolution of a family business. *J Interferon Cytokine Res,* 19**,** 1069-98.

Mori, M., Yoneyama, M., Ito, T., Takahashi, K., Inagaki, F. & Fujita, T. (2004). Identification of Ser-386 of interferon regulatory factor 3 as critical target for inducible phosphorylation that determines activation. *J. Biol. Chem,* 279**,** pp. 9698–9702.

Morin, P., Braganca, J., Bandu, M. T., Lin, R., Hiscott, J., Doly, J. & Civas, A. (2002). Preferential binding sites for interferon regulatory factors 3 and 7 involved in interferonA gene transcription. *J Mol Biol,* 316, 1009–1022.

Morrison, J. M. & Racaniello, V. R. (2009). Proteinase $2A^{pro}$ Is Essential for Enterovirus Replication in Type I Interferon-Treated Cells. *Journal of Virology,* 83(9):4412–4422.

Muller, U., Steinhoff, U., Reis, L. F., Hemmi, S., Pavlovic, J., Zinkerna-gel, R. M. & Aguet, M. (1994a). Functional role of type I and type Coordinates and structure factors have been deposited in the Pro- II interferons in antiviral defense. . *Science,* 264, 1918–1921.

Muller, U., Steinhoff, U., Reis, L. F., Hemmi, S., Pavlovic, J., Zinkernagel, R. M. & Aguet, M. (1994b). Functional role of type I and type II interferons in antiviral defense. *Science,* 264, 1918–1921.

Muller, U., Steinhoff, U., Reis, L. F. L., Hemmi, S. & Pavlovic, J., et al. (1994). Functional role of type I and type II interferons in antiviral defense. . *Science; ,* 264:**,** 1918-1921.

Mutocheluh, M., Hindle, L., Aresté, C., Chanas, S. A., Butler, L. M., Lowry, K. & et al (2011). Kaposi's sarcoma-associated herpesvirus viral interferon regulatory factor-2 inhibits type 1 interferon signalling by targeting interferon-stimulated gene factor-3. *J Gen Virol,* 92(Pt 10):2394-8.

National Research Council (NRC) (2008). Science and Decisions: Advancing Risk Assessment. *Washington, DC:National Academy Press*.

References

Nordenstedt, H., White, D. L. & H.B, E.-S. (2010). The changing pattern of epidemiology in hepatocellular carcinoma. *Dig Liver Dis,* 42 Suppl 3:S206–S214.

Nordin, M. & et al (1991). Effects of exposure period of acetylsalicylic acid, paracetamol and isopropanol on L929 cytotoxicity. *Toxicol In Vitro,* vol. 5, pp. 449-50.

Novick, D., Cohen, B. & Rubinstein, M. (1994). The human interferon alpha/beta receptor: characterization and molecular cloning. *Cell,* 77**,** 391-400.

NRC (1983). *Risk Assessment in the Federal Government: Managing the Process,* Washington, DC: National Academy Press.

Nunez, R. (1999). Revision of the functional analysis and structural features of immortalized dendritic cell lines derived from mice lacking both type I and type II interferon receptors. *Immunology Letters*, 68, 173–186.

O'Shea, J. J., Holland, S. M. & Staudt, L. M. (2013). JAKs and STATs in Immunity, Immunodeficiency, and Cancer. *N Engl J Med* 368:161-70.

Ouchi, T., Lee, S. W., Ouchi, M., Aaronson, S. A. & Horvath. C, M. (2000). Collaboration of signal transducer and activator of transcription 1 (STAT1) and BRCA1 in differential regulation of IFN-gamma target genes. *Proc Natl Acad Sci U S A,* 97, 10, 5208-5213.

Oyelami, O. A., Maxwell, S. M., Adelusola, K. A., Aladekoma, T. A. & Oyelese, A. O. (1996). Aflatoxins in the autopsy brain tissue of children in Nigeria. *Mycopathologia,* 132, 35–38.

Ozturk, M. (1991). p53 mutation in hepatocellular carcinoma after aflatoxin exposure. *Lancet,* 338**,** 1356–1359.

Padovan, E., Spagnoli, G. C., Ferrantini, M. & et al (2002). IFNalpha2a induces IP-10/CXCL10 and MIG/CXCL9 production in monocyte-derived dendritic cells and enhances their capacity to attract and stimulate CD81effector T cells. *J LeukocBiol* 71:669–76.

Panne, D., Maniatis, T. & Harrison, S. C. (2007). An atomic model of the interferon-b enhanceosome. *Cell,* 129, 1111-1123.

Park, C., Li, S., Cha, E. & Schindler, C. (2000). Immune response in Stat2 knockout mice. *Immunity,* 13:795–804.

Patel, C. V., Handy, I., Goldsmith, T. & Patel, R. C. (2000). PACT, a stress-modulated cellular activator of interferon-induced doublestranded RNA-activated protein kinase, PKR. *J Biol Chem* 275, 37993–37998.

References

Paucker, K., Cantell, K. & Henle, W. (1962). Quantitative studies on viral interference in suspended L cells: III. Effect of interfering viruses and interferon on the growth rate of cells. *Virology* 17,324-334.

Paun, A. & Pitha, P. M. (2007). The IRF family, revisited. *Biochimie,* 89, 744–753.

Pensa, S., Regis, G., Boselli, D., Novelli, F. & Poli, V. (2008). STAT1 and STAT3 in tumorigenesis: two sides of the same coin. In: Stephanou A, ed. JAK-STAT Pathway in Disease. *Austin: Landes Bioscience* 100-121.

Pier, A. C. & Heddleston, K. L. (1970). The effect of aflatoxin on immunity in turkeys. I. Impairment of actively acquired resistance to bacterial challenge. *Avian Dis,* 14(4)**,** 797–809.

Pier, A. C., Heddleston, K. L., Cysewski, S. J. & Patterson, J. M. (1972). Effect of aflatoxin on immunity in turkeys. II. Reversal of impaired resistance to bacterial infection by passive transfer of plasma. *Avian Dis,* 16(2)**,** 381–387.

Platanias, L. C. (2005). Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol,* 5(5)**,** 375-86.

Plymoth, A., Viviani, S. & Hainaut, P. (2009). Control of hepatocellular carcinoma through Hepatitis B vaccination in areas of high endemicity: perspectives for global liver cancer prevention. *Cancer Lett,* 286(1).

Potla, R., Koeck, T., Wegrzyn, J., Cherukuri, S., Shimoda, K., Baker, D. P. & et al (2006). Tyk2 tyrosine kinase expression is required for the maintenance of mitochondrial respiration in primary pro-B lymphocytes *Mol. Cell Biol,* 26: 8562–8571.

Precious, B., Young, D. F., Andrejeva, L., Goodbourn, S. & Randall, R. E. (2005). In vitro and in vivo specificity of ubiquitination and degradation of STAT1 and STAT2 by the V proteins of the paramyxoviruses simian virus 5 and human parainfluenza virus type 2. *J Gen Virol,* 86, 151–158.

Probst, C., Njapau, H. & Cotty, P. (2007). Outbreak of an Acute Aflatoxicocosis in Kenya in 2004: Identification of the Causal Agent *Applied and Environmental Microbiology***,** p 2762-2764.

Prokunina-Olsson, L., Muchmore, B., Tang, W. & et al (2013). A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. *Nat Genet,* 45:164–71.

References

Qian, G., Tang, L., Guo, X., Wang, F., Massey, M. E., Su, J. & et al (2014). Aflatoxin B1 modulates the expression of phenotypic markers and cytokines by splenic lymphocytes of male F344 rats. *J. Appl. Toxicol,* 34, 241–249.

Qian, G. S., Ross, R. K., Yu, M. C., Yuan, J. M., Gao, Y. T., Henderson, B. E. & et al (1994) "A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in Shanghai, People's Republic of China. *Cancer Epidemiology." Biomarkers and Prevention,* 3**,** 3-10.

Randall, R. E. & Goodbourn, S. (2008). Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. *J Gen Virol,* 89(Pt 1):1-47. Rao, B. L., Basu, A., Wairagkar, N. S., Gore, M. M., Arankalle, V. A., Thakare, J. P. & et al (2004). A Large Outbreak of Acute Encephalitis with High Fatality Rate in Children in Andhra Pradesh, India, in 2003, Associated with Chandipura Virus. *Lancet* 364 (9437): 869–874.

Rasooly, R., Hernlem, B. & Friedman, M. (2013). Low Levels of Aflatoxin B1, Ricin, and Milk Enhance Recombinant Protein Production in Mammalian Cells. *PLoS One,* 8(8): e71682.

Raven, J. F., Williams, V., Wang, S., Tremblay, M. L., Muller, W. J., Durbin, J. E. & Koromilas, A. E. (2011). Stat1 is a suppressor of ErbB2/Neu-mediated cellular transformation and mouse mammary gland tumor formation. *Cell Cycle* 10(5):794–804.

Reich, N. C. & Liu, L. (2006). Tracking STAT nuclear traffic. *Nat Rev Immunol,* 6(8):602–12.

Richard, J. L. & Thurston, J. R. (1975). Effects of aflatoxin on phagocytosis of Aspergillus fumigatus spores by rabbit alveolar macrophages. *Appl. Microbiol,* 30**,** 44-47.

Rodriguez, T., Mendez, R. & et al (2007). Distinct mechanisms of loss of IFN-gamma mediated HLA class I inducibility in two melanoma cell lines. *BMC Cancer* 7: 34.

Roelofs, B. & et al (2006). Acute activation of glucose uptake by glucose deprivation in L929 fibroblast cells. *Biochimie,* vol. 88, pp. 1941-6.

Romero-Weaver, A. L., Wang, H. W., Steen, H. C., Scarzello, A. J., Hall, V. L. & Sheikh, F. (2010). Resistance to IFN-alpha-induced apoptosis is linked to a loss of STAT2. *Mol Cancer Res,* 8:80–92.

Ross, R. K., Yuan, J. M., Yu, M. C., Wogan, G. N., Qian, G. S. & et al (1992). Original article. Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma. *Lancet,* 339**,** 943–46.

Rossano, F., Ortega De Ulna, L., Booming, E., Cusumano, V., Lois, E. & Captain, M. R. (1999).

References

Secondary metabolites of Aspergillus exert immunobiological effects on human monocytes *Research in Microbiology* 150:13–19.

Sanford, K. K. & et al (1948). The growth in vitro of single isolated tissue cells. *J. Natl. Cancer Inst,* 9: 229-246.

Sarasin, A. R., Smith, C. A. & Hanawalt, P. C. (1977). Repair of DNA in human cells after treatment with activated aflatoxin B1. *Cancer Res,* 37**,** 1786–1793.

Sarma, P. S., Shiu, G., Baron, S. & Huebner, R. J. (1969). Inhibitory effect of interferon on murine sarcoma and leukaemia virus infection in vitro. *Nature* 223**,** 845-846.

Sato, M., Hata, N., Asagiri, M., Nakaya, T., Taniguchi, T. & Tanaka, N. (1998b). Positive feedback regulation of type I IFN genes by the IFN-inducible transcription factor IRF-7. *FEBS Lett,* 441**,** 106–110.

Savel, H., Forsyth, B., Schaeffer, W. & Cardella, T. (1970). Effect of aflatoxin B1 upon phytohemagglutinin-transformed human lymphocytes. *Proc Soc Exp Biol Med,* 134(4)**,** 1112–1115.

Savitsky, D., Tamura, T., Yanai, H. & Taniguchi, T. (2010). Regulation of immunity and oncogenesis by the IRF transcription factor family. *Cancer Immunol Immunother,* 59:489-510.

Schindler, C., Levy, D. E. & Decker, T. (2007). JAK-STAT signaling: from interferons tocytokines. *J Biol Chem* 282:20059–63.

Schindler, C., Shuai, K., Prezioso, V. R. & Darnell Jr, J. E. (1992). Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. *Science* 257:809–13.

Sen, G. C. (2001). Viruses and interferons. *Annu. Rev. Microbiol*, 55, 255–281.

Serrano, M. C. & et al (2005). Transitory oxidative stress in L929 fibroblasts cultured on poly(epsilon-caprolactone) films. *Biomaterials,* vol. 26, pp. 5827-34.

Servant, M. J., Grandvaux, N., TenOever, B. R., Duguay, D., Lin, R. & Hiscott, J. (2003). Identification of the minimal phosphoacceptor site required for in vivo activation of interferon regulatory factor 3 in response to virus and double-stranded RNA. *J. Biol. Chem,* 278**,** 9441–9447.

Shephard, G. S. (2008). Risk assessment of aflatoxins in food in Africa. Food Additives & Contaminants: Part A. *Chemistry, Analysis, Control, and Exposure & Risk Assessment,* 25(10)**,** 1246 – 1256.

References

Shimoda, H. K., Shide, K., Kameda, T., Matsunaga, T. & Shimoda, K. (2010). Tyrosine kinase 2 interacts with the proapoptotic protein Siva-1 and augments its apoptotic functions. *Biochem. Biophys. Res. Commun,* 400: 252–257.

Shimoda, K., Kamesaki, K., Numata, A., Aoki, K., Matsuda, T., Oritani, K. & et al (2002). Cutting edge: tyk2 is required for the induction and nuclear translocation of Daxx which regulates IFN-alpha-induced suppression of B lymphocyte formation. *J. Immunol,* 169: 4707–4711.

Silverman, R. H. (2003). Implications for RNase L in Prostate Cancer Biology. *Biochemistry,* 42: 1805-1812.

Silverman, R. H. (2007). Viral encounters with 2',5'-oligoadenylate synthetase and RNase L during the interferon antiviral response. *J Virol* 81(23)**,** 12720-12729.

Silverman, R. H., Cayley, P. J., Knight, M., Gilbert, C. S. & Kerr, I. M. (1982). Control of the ppp(a2'p)nA system in HeLa cells. Effects of interferon and virus infection. *Eur. J. Biochem,* 124**,** 131-138.

Smela, M. E., Hamm, M. L., Henderson, P. T., Harris, C. M., Harris, T. M. & Essigmann, J. M. (2002). The aflatoxin B1 formamidopyrimidine adduct plays a major role in causing the types of mutations observed in human hepatocellular carcinoma. *Proc Natl Acad Sci USA,* 99**,** 6655–6660.

Sommereyns, C., Paul, S., Staeheli, P. & Michiels, T. (2008). IFN-lambda (IFN-k) is expressed in a tissue-dependent fashion and primarily acts on epithelial cells in vivo. *PLoS Pathog* 4:e1000017.

Somogyi, A. & Beck, H. (1993). Nurturing and breast-feeding: exposure to chemicals in breast milk. *Environ Health Perspect,* 101(Suppl 2)**,** 45-52.

Song, L., Rawal, B., Nemeth, J. A. & E.B, H. (2011). JAK1 Activates STAT3 Activity in NonSmall–Cell Lung Cancer Cells and IL-6 Neutralizing Antibodies Can Suppress JAK1STAT3 Signaling. *Mol Cancer Ther,* 10(3); 481–94.

Spaapen, R. M., Leung, M. Y. K., Fuertes, M. B., Kline, J. P., Zhang, L., Zheng, Y. & et al (2014). Therapeutic Activity of High-Dose Intratumoral IFN-b Requires Direct Effect on the Tumor Vasculature. *Immunol Rev,* 193:4254-4260.

Spranger, S., Spaapen, R. M., Zha, Y., Williams, J., Meng, Y., Ha, T. T. & Gajewski, T. F. (2013). Up-Regulation of PD-L1, IDO, and Tregs in the Melanoma Tumor Microenvironment Is Driven by CD8+ T Cells. *Sci Transl Med* 5(200):200ra116.

References

Stancato, L. F., David, M., Carter-Su, C., Larner, A. C. & Pratt, W. B. (1996). Preassociation of STAT1 with STAT2 and STAT3 in separate signalling complexes prior to cytokine stimulation. *J Biol Chem*, 271, 4134–4137.

Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H. & Schreiber, R. D. (1998). How cells respond to interferons. *Annu. Rev. Biochem*, 67 227-264.

Stephanou, A. & Latchman, D. S. (2003). STAT-1: a novel regulator of apoptosis. *Int J Exp Pathol* 84(6)**,** 239-244.

Strosnider, H., Azziz-Baumgartner, E., Banziger, M., Bhat, R. V., Breiman, R., Brune, M. & et al (2006). Workgroup Report: Public Health Strategies for Reducing Aflatoxin Exposure in Developing Countries. *Environmental Health Perspectives*, 114**,** 1989-1903.

Sudakin, D. L. (2003). Dietary aflatoxin exposure and chemoprevention of cancer: a clinical review. *J Toxicol Clin Toxicol*, 41**,** 195-204.

Tajkarimi, M., Shojaee-Aliabadi, F., Salah Nejad, M., Pursoltani, H., Motallebi, A. A. & Mahdavi, H. (2007). Seasonal study of aflatoxin M1 contamination in milk in five regions in Iran. *Int J Food Microbiol*, 116(3)**,** 346–349.

Takaoka, A., Hayakawa, S., Yanai, H. & et al (2003). Integration of interferon-alpha/beta signalling to p53 responses in tumour suppression and antiviral defence. *Nature*, 424 (6948)**,** 516–23.

Takaoka, A., Yanai, H., Kondo, S., Duncan, G., Negishi, H., Mizutani, T. & et al (2005). Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. *Nature*, 434**,** 243–249.

Tamura, T., Yanai, H., Savitsky, D. & Taniguchi, T. (2008). The IRF family transcription factors in immunity and oncogenesis. *Annu Rev Immunol* 26:535-84.

Tandale, B. V., Tikute, S. S., Arankalle, V. A., Sathe, P. S., Joshi, M. V., Ranadive, S. N. & et al (2008). Chandipura virus: a major cause of acute encephalitis in children in North Telangana, Andhra Pradesh. *India. J Med Virol*, 80 : 118-24.

Tang, X., Gao, J. S., Guan, Y. J., McLane, K. E., Yuan, Z. L., Ramratnam, B. & Chin, Y. E. (2007). Acetylation-dependent signal transduction for type I interferon receptor. *Cell*, 131, 93–105.

References

Taniguchi, T. & et al (2006). Hydrogen peroxide-induced arachidonic acid release in L929 cells; roles of Src, protein kinase C and cytosolic phospholipase A2alpha. *Eur J Pharmacol*, vol. 546, pp. 1-10.

Taniguchi, T., Ogasawara, K., Takaoka, A. & Tanakam, N. (2001). IRF family of transcription factors as regulators of host defense. *Annu. Rev. Immunol,* 19**,** 623–655.

Tay, C. H., Yu, L. Y., Kumar, V., Mason, L., Ortaldo, J. R. & Welsh, R. M. (1999). The role of LY49 NK cell subsets in the regulation of murine cytomegalovirus infections. *Journal of Immunology*, 162, 718–726.

Theofilopoulos, A. N., Baccala, R., Beutler, B. & Kono, D. H. (2005). Type I interferons (alpha/beta) in immunity and autoimmunity. *Annu Rev Immunol,* 23**,** 307-36.

Thurston, J. R., Deyoe, B. L., Baetz, A. L., Richard, J. L. & Booth, G. D. (1974). Effect of aflatoxin on serum proteins, complement activity, and the antibody response to Brucella abortus in guinea pigs. *Am J Vet Res,* 35(8)**,** 1097–1100.

Traore-Lamizana, M., Fontenille, D., Diallo, M., Ba, Y., Mondo, M. & Adam, F. (2001). Arbovirus surveillance from 1990 to 1995 in the Barkedji area (Ferlo) of Senegal, a possible natural focus of Rift Valley fever virus. *J Med Entomol* 38 : 480-92.

Tsuno, T., Mejido, J., Zhao, T., Schmeisser, H., Morrow, A. & et al (2009). IRF9 is a key factor for eliciting the antiproliferative activity of IFN-alpha. *J Immunother,* 32: 803-816.

Turner, P. C., Moore, S. E., Hall, A. J., Prentice, A. M. & Wild, C. P. (2003). Modification of immune function through exposure to dietary aflatoxin in Gambian children. *Environ Health Perspect,* 111(2)**,** 217-20.

Turpeinen, M., Tolonen, A., Chesne, C., Guillouzo, A., Uusitalo, J. & Pelkonen, O. (2009). Functional expression, inhibition and induction of CYP enzymes in HepaRG cells. *Toxicol. In Vitro,* 23**,** 748–753.

Tymoszuk, P., Charoentong, P., Hackl, H., Spilka, R., Müller-Holzner, E., Trajanoski, Z., Obrist, P. & et al (2014). High STAT1 mRNA levels but not its tyrosine phosphorylation are associated with macrophage infiltration and bad prognosis in breast cancer. *BMC Cancer* 14:257

Tymoszuk, P., Charoentong, P., Hackl, H., Spilka, R., Müller-Holzner, E., Trajanoski, Z., Obrist, P., Revillion, F., Peyrat, J. P., Fiegl, H. a. & Doppler, W. (2014). High STAT1 mRNA levels but not its tyrosine phosphorylation are associated with macrophage infiltration and bad prognosis in breast cancer. *BMC Cancer* 14:257

References

Ubagai, T., Kikuchi, T., Fukusato, T. & Ono, Y. (2010). Aflatoxin B1 modulates the insulin-like growth factor-2 dependent signaling axis. *Toxicol in vitro,* 24: 783-789.

Ubel, C., Mousset, S., Trufa, D., Sirbu, H. & Finotto, S. (2013). Establishing the role of tyrosine kinase 2 in cancer. *Oncoimmunology,* 2 e22840.

van Boxel-Dezaire, A. H., Rani, M. R. & Stark, G. R. (2006). Complex modulation of cell typespecific signaling in response to type I infections. *Immunity,* 25**,** 361-372.

van Delft, J. H., van Agen, E., van Breda, S. G., Herwijnen, M. H., Staal, Y. C. & Kleinjans, J. C. (2005). Comparison of supervised clustering methods to discriminate genotoxic from non-genotoxic carcinogens by gene expression profiling. *Mutat. Res,* 575 17–33.

Veals, S. A., Schindler, C., Leonard, D., Fu, X. Y., Aebersold, R., Darnell, J. E. J. & Levy, D. E. (1992). Subunit of an alpha-interferon-responsive transcription factor is related to interferon regulatory factor and Myb families of DNA-binding proteins. *Mol. Cell. Biol,* 12**,** 3315–3324.

Verma, R. J. (2004). Aflatoxin cause DNA damage. *Inter- national Journal of Human Genetics,* 4, 231-236.

Wang, J., Pham-Mitchell, N., Schindler, C. & et al (2003). Dysregulated Sonic hedgehog signaling and medulloblastoma consequent to IFN-alphastimulated STAT2-independent production of IFN-gamma in the brain. *J Clin Invest* 112:535–43.

Waters, R., Jones, C. J., Martin, E. A., Yang, A. L. & Jones, N. J. (1992). The repair of large DNA adducts in mammalian cells. *Mutat Res,* 273**,** 145–155.

Weisz, A., Marx, P., Sharf, R., Appella, E., Driggers, P. H., Ozato, K. & Levi, B. Z. (1992). Human interferon consensus sequence binding protein is a negative regulator of enhancer elements common to interferon-inducible genes. *J Biol Chem,* 267(35):25589-96.

Wild, C. P. & Gong, Y. Y. (2010). Mycotoxins and human disease: a largely ignored global health issue. *Carcinogenesis,* 31: 71-82.

Wild, C. P. & Hall, A. J. (2000). Primary prevention of hepatocellular carcinoma in developing countries *Mutat Res Rev,* 462(2–3)**,** 381–393.

Wilkening, S., Stahl, F. & Bader, A. (2003). Comparison of primary human hepatocytes and hepatoma cell line HepG2 with regard to their biotransformation properties. *Drug Metab. Dispos,* 31**,** 1035–1042.

References

Williams, B. R. (1991). Transcriptional regulation of interferon-stimulated genes. *Eur J Biochem,* 200**,** 1-11.

Williams, J. H., T. D. Phillips, P. E. Jolly, J. K. Stiles, C. M. Jolly and D. Aggarwal. 2004. Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *The American Journal of Clinical Nutrition,* **80**(5), pp.1106-1122.

Wiredu, E. K. & Armah, H. B. (2006). Cancer mortality patterns in Ghana: a 10-year review of autopsies and hospital mortality. *BMC Public Health,* 6:159–65.

Wogan, G. N. (1992). Aflatoxins as risk factors for hepatocellular carcinoma in humans. *Cancer Res.,* Apr 1;52(7 Suppl):2114s-2118s.

Wogan, G. N., Paglialunga, S. & Newberne, P. M. (1974). Carcinogenic effects of low dietary levels of aflatoxin B1 in rats. *Food Cosmet. Toxicol,* 12:681–85**,** .

Wu, F. (2010). The global burden of disease caused by foodborne aflatoxin. WHO Commissioned Report, Foodborne Disease Burden Epidemiology Reference Group (FERG).

Wu, F. & Khlangwiset, P. (2010). Health economic impacts and cost-effectiveness of aflatoxin reduction strategies in Africa: case studies in biocontrol and postharvest interventions. *Food Addit Contam: ,* 27:**,** 496-509.

Wullaert, A., Heyninck, K., Janssens, S. & Beyaert, R. (2006). Ubiquitin: tool and target for intracellular NF-kB inhibitors. *Trends Immunol,* 27, 533–540.

Yanai, H., Negishi, H. & Taniguchi, T. (2012). The IRF family of transcription factors Inception, impact and implications in oncogenesis. *OncoImmunology* 1:8, 1376–1386.

Yeh, F. S., Yu, M. C., Mo, C. C., Luo, S., Tong, M. J. & Henderson, B. J. (1989). Hepatitis B virus, aflatoxins, and hepatocellular carcinoma in southern Guangxi, China. *Cancer Res,* 49**,** 2506-2509.

Yin, Z., Dai, J., Deng, J. & et al (2012). Type III IFNs are produced by and stimulate human plasmacytoid dendritic cells. *J Immunol,* 189:2735–45.

Yoneyama, M., Suhara, W., Fukuhara, Y., Fukuda, M., Nishida, E. & Fujita, T. (1998). Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. *EMBO J,* 17**,** 1087–1095.

Yue, C., Xu, J., Estioko, M. D. T., Kotredes, K. P., Lopez-Otalora, Y., Hilliard, B. A. & et al (2015). Host STAT2/type I interferon axis controls tumor growth. *Int. J. Cancer,* 00, 00–

00.

Zarba, A., Wild, C. P., Hall, P. J., Montesano, R., Hudson, G. J. & Groopman, J. D. (1992). Aflatoxin M1in human breast milk from Gambia, West Africa, quantified by combined monoclonal antibody immunoaffinity chromatography and HPLC. *Carcinogenesis*, 13, 891-894.

Zhou, D., Huang, C., Lin, Z., Zhan, S., Kong, L., C, F. & et al (2014). Macrophage polarizationand function with emphasis on the evolving roles of coordinated regulationof cellular signaling pathways. *Cell Signal* 26:192–7.

Zitvogel, L., Galluzzi, L., Kepp, O., Smyth, M. J. & Kroemer, G. (2015). Type I interferons in anticancer immunity. *Nat. Rev. Immunol,* 15 405–414.

Appendices

Appendix A:

Table 1: Reagents and chemicals used

Reagents / chemicals	Composition	Supplier /country
Aflatoxin B1 (AFB1)	Aflatoxin B1	Sigma Aldrich, Germany
Dulbecco's Modified Eagle's Medium with high glucose (DMEM)	4.5g/l glucose	Sigma Aldrich, Germany
Human interferon-alpha 2b (rIFNα2b)	Recombinant human interferonalpha 2	PBL Biomedical Laboratories, USA
100X MEM non-essential amino acids	Non-essential amino acids	Sigma Aldrich, Germany
100X Halt™ phosphatase inhibitor cocktail	Sodium fluoride, sodium orthovanadate, sodium pyrophosphate, βglycerophosphate	Thermo Scientific, USA
100X Halt Protease inhibitor cocktail	AEBSF, Aprotinin, Bestatin, E64, Leupeptin, Pepstatin, EDTA	Thermo Scientific, USA
30% Acrylamide / bis-acrylmide Solution	30 % w/v acrylamide; 0.8% w/v bis-acrylamide	Bio-Rad, USA
Absolute methanol (analytical grade)	methanol	Chem-Lab NV, Belgium
Absolute ethanol (analytical grade)	ethanol	Fisher Scientific, UK
5X reaction mixture (PCR) for cDNA synthesis	Reaction buffer, dNTPs Oligo $(dT)_{18}$ and random hexamer primers	Thermo Scientific, Germany
Maxima enzyme Mix	Maxima Reverse transcriptase and Thermo Scientific RiboLock RNase inhibitor	Thermo Scientific, Germany
Coomassie brilliant blue staining solution	0.05% w/v Coomasie Brilliant Blue, 40% v/v ethanol, 50% v/v water, 10% acetic acid	Prepared in the Lab
Quick start™ Bradford 1X Dye Reagent	Coomassie Brilliant Blue G-250 dye methanol and phosphoric acid	Bio-Rad, USA
Glycine	Glycine	Bio-Rad, USA
Tris	Tris	Bio-Rad, USA
10% Sodium dodecyl sulphate (SDS)	SDS pH 7.2	Bio-Rad, USA
Sodium chloride	Sodium chloride	VWR, Belgium
Ammonium per sulphate (APS)	Ammonium per sulphate	Sigma Aldrich, Germany

Appendices

0.4% Trypan Blue solution	Trypan blue dye prepared in 0.081% sodium chloride and o.06% potassium phosphate	Sigma Aldrich, Germany
Tetramethylethylenediamine (TEMED)	Tetramethylethylenediamine (TEMED)	Sigma Aldrich, Germany
Dual Luciferase Reporter Assay Kit	Luciferase assay reagent II(Beetle Luciferin); Stop & Glo reagent (Coelenterazine)	Promega, USA
Penstrep	Penicillin & streptomycin	Gibco by life technologies, UK
Foetal Bovine serum	Growth factors, hormones, protein etc.	Sigma Aldrich, Germany
Protein markers	Pre-stained protein markers	Thermo Scientific, Germany
Pierce ECL Western blotting substrate	Detection reagent 1:Luminol/enhancer 2: Peroxide solution	Thermo Scientific, Germany
2-β-mercaptoethanol	2-β-mercaptoethanol	Bio-Rad, USA
Maxima Enzyme mix with ds DNase for cDNA synthesis	Maxima Reverse Transcriptase, Thermo Scientific RiboLock RNase inhibitor, ds DNase	Thermo Scientific, Germany
5X reaction Mix for cDNA synthesis	Reaction buffer, dNTPs, oligo $(dT)_{18}$ and random hexamer primers	Thermo Scientific, Germany
Nuclease free water	water	Thermo Scientific, Germany
0.5M Ethylenediaminetetraacetic acid (EDTA)	EDTA.2H2O; NaOH, pH 8.0	Thermo Scientific, Germany
2X Maxima Probe/ROX qPCR Master mix	Tag DNA polymerase, dNTPs, PCR buffer (Potassium Chloride, Ammonium Sulphate and Magnesium chloride)	Thermo Scientific, Germany
1X 0.25% w/v Trypsin EDTA	0.25% Trypsin, 0.025mM Phenol Red,0.02% EDTA and inorganic salts	Gibco by Life Technologies, UK
CellTiter 96® AQueous One Solution	Tetrazolium compound, phenazine ethosulfate	Promega, USA
Ampicillin	Ampicillin	Thermo scientific, Germany
Luria Bertani broth	10g tryptone; 5g yeast extract; 10 g NaCl; 15g agar; topped up with distilled water to 1L mark	Sigma Aldrich, USA

Luria Bertani agar	10g tryptone; 5g yeast extract; 10 g NaCl; 15g agar; topped up with distilled water to 1L mark	Sigma Aldrich, USA
Ultra-Pure ™Agarose	Agar	Invitrogen, Spain
Pre-diluted protein standards	Bovine serum albumin	Bio-Rad, USA
Crystal Violet stain	0.5g crystal violet, 20ml of absolute ethanol, 0.9g NaCl, 100ml of 40% formaldehyde solution, 880 ml of distilled water to make 1litre	Prepared in the laboratory

Appendix B:

Table 1: Commonly used buffer and other solutions

Buffer solution	Composition	Supplier
Buffer P1 (resuspension buffer)	50mM Tris-Cl, PH 8.0 10mM EDTA, 100 µg/ml RNase A	QIAGEN®, USA
Buffer P2 (lysis buffer)	200mM NaOH. 1% SDS (w/v)	QIAGEN®, USA
Buffer P3 (neutralization buffer)	3.0 M potassium acetate, pH 5.5	QIAGEN®, USA
Buffer QBT (equilibrium buffer)	750 mM NaCl, 50mM MOPS, pH 7.0; 15% isopropanol (v/v); 0.15% Triton X-100 (v/v)	QIAGEN®, USA
Buffer QC (wash buffer)	1.0M NaCl, 50mM MOPS, pH 7.0 15% isopropanol (v/v)	QIAGEN®, USA
Buffer QF (elution buffer)	1.25 M NaCl, 50mM Tris-Cl, pH 8.5; 15% isopropanol (v/v)	QIAGEN®, USA
Buffer QN (elution buffer)	1.6 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v)	QIAGEN®, USA
TE	10mM Tris-Cl, pH 8.0; 1mM EDTA	QIAGEN®, USA
Lysis buffer for RNA purification	Proteinase, Tritirachium album serine, Guanidine thiocyanate supplemented with βmercaptoethanol before use	Thermo Scientific, Germany
Wash buffer 1	96-100% ethanol, Tris-EDTA	Thermo Scientific, Germany
Wash buffer 2	ethanol, Tris-EDTA	Thermo Scientific, Germany
Phosphate buffered saline	8g/L NaCl, 0.2g/L KCl, 1.15g/L Disodium hydrogen phosphate, 0.2g/L potassium dihydrogen phosphate	Oxoid Limited, UK

Pierce RIPA buffer	25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS	Thermo Scientific, Germany
Stacking gel buffer	0.5M Tris-HCl, pH 6.8	Bio-Rad, USA
Resolving gel buffer	1.5M Tris-HCl, pH 8.8	Bio-Rad, USA
10X Electrophoresis / Running buffer	Tris base 30.24 g, Glycine 142.5 g, add distilled water up to 800 ml mark, adjust pH to 8.4 with HCl and add distilled water to 1L mark	Prepared at the laboratory
1X Electrophoresis/Running buffer	100 ml 10X electrophoresis buffer, 1ml of SDS (0.1%) 900 ml dH2O	Prepared at the laboratory
10X Transfer buffer 1 L	Tris base 30.24 g, Glycine 142.5 g, add distilled water up to 800 ml mark, adjust pH to	Prepared at the laboratory
	8.4 with HCl and add distilled water to 1L mark	
1X Transfer buffer 1 L	100 ml 10X transfer buffer, 200 ml methanol (20%), 700 ml distilled water	Prepared at the laboratory
10 X Tris Buffered Saline	60.6 g Tris base, 87.6 g NaCl, add distilled water up to 800 ml mark, adjust pH to 7.6 with HCl and add distilled water to 1L mark	Prepared at the laboratory
1X Tris Buffered saline with Tween 20 (TBST)	100 ml of 10X Tris Buffered Saline, 900 ml of distilled water, 1ml of Tween 20 (0.1%)	Prepared at the laboratory
2X Loading sample buffer 10 ml	1 M Tris-HCl, pH 6.8, 4 ml 10% SDS, 2 ml glycerol, 2.5 ml β-mercaptoethanol, 500 µl bromophenol blue (1%)	Bio-Rad, USA
Stripping buffer	2% SDS, 62.5mM Tris-HCl pH6.8, 100mM Betamercaptoethanol	Prepared at the laboratory
Blocking buffer	5% w/v non-fat marvel dried skimmed milk in 1X Tris Buffered Saline with Tween-20	Prepared at the laboratory
50 X Tris acetate EDTA electrophoresis buffer	40mM Tris, 20mM acetic acid, 1mM M EDTA, pH 8.0	Thermo Scientific, Germany

1 X Tris acetate EDTA electrophoresis buffer	20 ml of 50 X Tris acetate EDTA electrophoresis buffer, 980 ml of distilled water	Prepared at the laboratory
6 X gel loading buffer	0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol	Thermo Scientific, Germany
5X Passive lysis Buffer	50 mM Tris/Cl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% (v/v) Nonident P-40	Promega, USA
1X passive lysis Buffer	1 part of 5X passive lysis to 4 parts of distilled water	Prepared at the laboratory

Appendix C:

Table 1: Plasmids used

Name of plasmids/stock concentration	Final concentration	Property
pISRE-luc (1000ng)	500 ng/well (96-well plate)	luciferase reporter plasmid containing tandem repeats of the *ISG56* ISRE driving the expression of a firefly Luciferase reporter gene.
pRLSV-40 (1ng)	1 ng/well (96-well plate)	Contains a constitutively active CMV promoter driving the expression of a *Renilla* luciferase reporter gene.

Table 2: Transfection complex preparation chart

Plasmid Stock Conc.	Amount of DNA per well	Vol. in µl per well	Total Amount for 14-wells in µl
Dilute the plasmid DNA in 1.5 ml eppendorf tube as follows			

pISRE-luc (1000 ng)	500 ng	0.5	7.0
pRLSV40 (1ng)	1ng	1.0	14.0
Serum free medium	-	25	350
Gently mix the diluted DN A and incubate at room temperature for 5 min utes			
Dilute the Lipofectamine 2 000 in another 1.5 ml eppendorf tube as follows			
Lipofectamine 2000	-	1.5	21
Serum free medium	-	25	350
Gently mix the diluted Lipofectamine 2000 and incubate at room temperature for 5 minutes			
After 5 minutes of incubation, gently mix the diluted DNA and Lipofectamine and incubate at room temperature for 20 minutes			

Appendix D

Table 1: RT-qPCR reaction set-up

Components	Vol.(µl) per tube	Final conc.(µM) in PCR mixture	Total volume (µl) required for 18 reactions
Nuclease free H2O	6		108
Stat1 forward primer	0.75	0.3	13.5
Stat1 reverse primer	0.75	0.3	13.5
Stat1 probe	0.5	0.2	9
GAPDH forward primer	0.75	0.3	13.5
GAPDH reverse primer	0.75	0.3	13.5
GAPDH probe	0.5	0.2	9
Rox master mix (2x)	12.5	4mM MgCl2	225
Total	22.5		
Add 2.5µl of the cDNA template to each tube			

Table 2A: GAPDH optimization reaction set-up (0.3 µM forward primer x 0.3 µM reverse primer)

Components	Vol.(µl) per tube	Final conc. (µM) in PCR mixture	Total volume (µl) required for 4 reactions
nuclease free H2O	8		32
GAPDH forward primer	0.75	0.3	3
GAPDH reverse primer	0.75	0.3	3
GAPDH probe	0.5	0.2	2
Rox master mix (2x)	12.5	4mM MgCl2	50
Total	22.5		
Add 2.5µl of the cDNA template to each tube			

Table 2B: GAPDH optimization reaction set-up (0.2 µM forward primer x 0.2 µM reverse primer)

Components	Vol.(µl) per tube	Final conc.(µM) in PCR mixture	Total volume (µl) required for 4 reactions
Nuclease free H2O	8.5		34
GAPDH forward primer	0.5	0.2	2
GAPDH reverse primer	0.5	0.2	2
GAPDH probe	0.5	0.2	2
Rox master mix (2x)	12.5	4mM MgCl2	50
Total	22.5		
Add 2.5µl of the cDNA template to each tube			

Table 2C: GAPDH optimization reaction set-up (0.1 µM forward primer x 0.1 µM reverse primer)

Components	Vol.(µl) per tube	Final conc. (µM) in PCR mixture	Total volume (µl) required for 4 reactions
Nuclease free H2O	9		36
GAPDH forward primer	0.25	0.1	1
GAPDH reverse primer	0.25	0.1	1
GAPDH probe	0.5	0.2	2
Rox master mix (2x)	12.5	4mM MgCl2	50
Total	22.5		
Add 2.5µl of the cDNA template to each tube			

Table 2D: GAPDH primer optimization

Target	Primer combinations	Threshold Cycle (Ct)	C(t) Mean
GAPDH	0.3 µM Forward primer x 0.3µ M Reverse primer	24.18	24.18
GAPDH	0.3 µM Forward primer x 0.3µ M Reverse primer	24.13	24.13
GAPDH	0.2 µM Forward primer x 0.2 µ M Reverse primer	24.50	24.50
GAPDH	0.2 µM Forward primer x 0.2 µ M Reverse primer	24.64	24.64

GAPDH	0.1 µM Forward primer x 0.1 µ M Reverse primer	25.10	25.10
GAPDH	0.1 µM Forward primer x 0.1 µ M Reverse primer	24.12	24.12

Appendix E: Antibodies

Table 1: Primary antibody

Antibody	**Host**	**Supplier**	**Dilution**

STAT1	Rabbit	Thermo Scientific	1:1000
GAPDH	Rabbit	Thermo Scientific	1:2500

Table 2: Secondary Antibody

Antibody	**Host**	**Supplier**	**Dilution**
Anti-Rabbit HRP	Goat	Thermo Scientific	1:5000

Appendices

Appendices