Basis for the role of NF-κB in Inflammation & Cancer

A DISSERTATION

SUBMITTED TO THE DEPARTMENT OF MEDICINE OF **IMPERIAL COLLEGE LONDON** IN FULFILMENT OF THE REQUIRMENTS FOR THE DEGREE OF **DOCTOR OF PHILOSOPHY**

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Dedicated to my family

Abstract

The 19th-century pathologist Virchow noted an association between inflammation and cancer. This initial observation has been supported by recent epidemiological data, which has lead to the estimation that 20% of cancers are linked to chronic infections and persistent inflammation. Primary examples are gastric cancer, hepatocellular carcinoma (HCC), viral hepatitis and colitis- associated cancer. The NF-κB signalling pathway has particular relevance for several liver diseases including hepatitis, liver fibrosis and hepatocellular carcinoma. Constitutive NF-κB activation is a hallmark for many cancers and a major link between inflammation and cancer is provided by NF-κB transcription factor. Inhibition of apoptosis is perhaps the most obvious way through which NF-κB signalling promotes the development of cancer. Numerous NF-κB target genes, such as *Gadd45β,* prevent apoptosis and have been shown to be required for liver regeneration post-partial hepatectomy. The precise mechanism by which NF-κB regulates the inflammatory mechanisms that drive tumourigenesis, however are poorly understood. In order to elucidate these mechanisms, we propose to examine the role of *Gadd45β* in the NF-κB mediated link between inflammation and cancer. This study reports that *Gadd45β* deficient mice are more resistant to diethylnitrosamine (DEN) induced hepatocellular carcinogenesis. *Gadd45β* knockout (KO) mice developed HCC approximately four-fold lower than in control mice. Tumour number and maximum tumour size were also markedly reduced in KO mice. To further investigate in which cell types

Gadd45β exerts its tumourigenic action we used bone marrow chimeric mice. Results have demonstrated that *Gadd45β* exerts its tumourigenic action primarily within the bone marrow derived cells but not in hepatocytes specifically during tumour progression stage than in tumour initiation stage. Furthermore, bone marrow-derived macrophages from KO mice preferentially polarised more towards macrophage M1 like phenotype that promotes anti-tumour immunity and inflammation.

Thus, here we report a previously unidentified mechanism by which NF-κB linked inflammation to cancer and identified *Gadd45β* as a putative mediator of the NF-κB's tumourigenic activity.

Declaration

I, Anil Kumar Thotakura, declare that this research study was carried out at Department of Medicine, Imperial College London. This PhD thesis is of my own work, and all the indicated results and discussion are drawn to the best of my knowledge. This work has not been submitted in any form for another degree at Imperial College London or any other institution. All the animal research reported in this thesis has been conducted ethically. The Information derived from the published work has been acknowledged in the references section. Information derived from collaboration work of others has listed below.

Results section 4.1 and 4.2:

Initial DEN injection experiments and short term effects of DEN was done in collaboration with Dr. Francesca Zazzeroni's laboratory at Department of Biotechnological and Applied Clinical Sciences, University of L'Aquila, Via Vetoio, Coppito 2, 67100 L'Aquila, Italy.

Result section 4.4:

Histological assessment of MCA-203 tumour samples was done in collaboration with

Fabio PASQUALINI at Humanitas research hospital, Italy.

Results section 4.5 and 4.6:

Real-time PCR analysis data of BM-derived macrophages was done in collaboration with Dr. Jason Bennett at Centre for Cell Signaling and Inflammation, Department of Medicine, Imperial College London.

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Anil Kumar Thotakura

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Table of contents

Abstract……3

Declaration……5

1. Introduction……27

1.1 Overview of NF-κB pathways……28

1.2 IKK complex……32

1.2.1 Biological functions of IKK complex……34

1.3 Role of NF-κB within the liver……38

1.3.1 Role of NF-κB in embryonic liver development……41

1.3.2 Role of NF- κB in promoting liver regeneration……43

1.4 Role of NF-κB Inflammation and tumourigenesis……44

1.5 The control of PCD by NF-κB……52

1.6 Gadd45beta……56

1.6.1 Role of *Gadd45β* in autoimmunity……58

1.6.1.1 Role of *Gadd45β* in experimental autoimmune encephalomyelitis……59

1.6.1.2 Role of *Gadd45β* in rheumatoid arthritis……61

1.6.1.3 Role of *Gadd45β* in type 1 diabetes ……63

1.6.2 Role of *Gadd45β* in liver regeneration……65

1.6.3 Role of *Gadd45β* in autophagy……66

1.6.4 Role of *Gadd45β* in haematopoietic cells……68

1.6.5 Role of *Gadd45β* in cardiomyocytes……71

1.7 Macrophage……74

1.7.1 Macrophage activation…… 76
1.7.2 Macrophages in cancer……79
1.7.3 Tumour Associated Macrophages (TAM) ……79
1.7.4 Strategies to target TAMs……82
1.7.4.1 Inhibiting macrophage recruitment……83.
1.7.4.2 Suppressing TAMs survival……86
1.7.4.2.1 T-Cell mediated macrophage depletion……88
1.7.4.3 Enhancing tumouricidal activity of TAMs……88
1.7.4.4 Blocking tumour promoting activity of TAMs……91

1.8 Hepatocellular carcinoma……93

2 Aims and Objectives…..……96

3 Material and Methods……99
3.1 Materials……100
3.2 Methods……107
3.2.1 Mice……107
3.2.2 Tumour induction……107
3.2.3 Tumour analysis……108
3.2.4 Hematoxylin and eosin stain……108
3.2.5 Short –term DEN injection……109
3.2.6 TUNEL assay……110
3.2.7 BrdU assay……110
3.2.8 RNA isolation……110
3.2.9 RT-PCR conversion of mRNA……111
3.2.10 qRT-PCR……111

3.2.11 Western blot……112
3.2.11.1 Protein isolation……112
3.2.11.2 Protein quantification……113
3.2.11.3 Sodium dodecyl sulfate- polyacrylamide gel electrophoresis……113
3.2.11.4 Western blotting……114
3.2.12 Adoptive transfer and carcinogenesis……115
3.2.12.1 Bone Marrow chimeras……115
3.2.12.2 Protocol: A…… 115
3.2.12.3 Flow-Cytometer (FACS) ……116
3.2.12.4 Macrophage depletion and F4/80 staining……117
3.2.12.5 Immunofluorescence……119
3.2.12.6 Tumour Analysis……120
3.2.12.7 Protocol: B……121
3.2.12.8 Tumour Analysis……121
3.2.13 Freezing and thawing of cells……122
3.2.14 Cell culture medium……122
3.2.15 Allograft Model……123
3.2.16 Generation of BM-derived macrophages……124
3.2.17 Macrophage tumour cell co-culture…… 125
3.2.18 Macrophage polarisation…… 126
3.2.19 Statistical analysis……126

4 Results……127

4.1 *Gadd45β* is required for DEN-induced hepatocellular carcinogenesis……..128

4.2 Effects of loss of *Gadd45β* in acute DEN injected mice……136

4.2.1 Loss of *Gadd45β* has no effect on hepatocyte survival shortly after DEN injection……136

4.2.2 Loss of *Gadd45β* attenuates DEN induced hepatocyte proliferation……139

4.2.3 Loss of *Gadd45β* attenuates DEN induced mitogen factor production……142

4.2.4 Loss of *Gadd45β* has no effect on hepatocyte MAP kinase signalling in the livers of DEN treated mice……147

4.3 Loss of *Gadd45β* in haematopoietic cells reduces the DEN induced hepatocelluar carcinogenesis……151

4.4 Reduced MCA-203 tumour growth in *Gadd45β* knockout mice……174

4.5 The loss of *Gadd45β* promotes M1 Macrophage differentiation *in vitro*……182

4.6 The loss of *Gadd45β* promotes M1 macrophage polarisation upon cytokine stimulation *in vitro*……188

5 Discussion……195

5.1 *Gadd45β* promotes DEN induced HCC development……198

5.2 *Gadd45β* acts within proinflammatory cells during tumour progression……203

5.3 *Gadd45β* promotes MCA-203 tumour progression by restraining tumouricidal cells ……207

5.4 *Gadd45β* plays an important role in macrophage differentiation and polarization *in vitro*……210

6 Future works……217
6.1 Developing conditional *Gadd45β*$^{-/-}$ mice……218
6.2 Mechanisms for *Gadd45β* macrophage function. ……219
6.3 Targeting TAMs with *Gadd45β* inhibitors ……219

7 References……221

List of Figure

Introduction

Figure. 1. NF-κB activation pathways

Figure 2. Various roles of NF-κB

Figure 3. Diethylnitrosamine induced HCC model

Figure 4. Cell-type dependent functions of NF-κB

Figure 5. *Gadd45β* mediated inhibition of JNK induced cell death

Figure 6. Timeline: Advancement in macrophage polarisation research

Figure 7. Monocyte differentiation into mature macrophage depends on cytokine milieu

Results

Figure 1: The schematic representation of DEN induced HCC experimental protocol.

Figure 2: Reduced DEN induced tumour development in *Gadd45β*$^{-/-}$ mice livers.

Figure 3: Reduced HCC growth in *Gadd45β*$^{-/-}$ mice.

Figure 4: Increased hepatocyte cell death after DEN injection.

Figure 5: BrdU incorporation assay.

Figure 6: Decreased expression of pro-inflammatory factors shortly after DEN injection.

Figure: 7 Loss of *Gadd45β* augment Kupffer cell death after DEN treatment.

Figure 8: Biochemical characterisation of liver after acute DEN injection.

Figure 9: Schematic diagram of bone marrow chimeric experimental "Protocol A".

Figure 10: Bone marrow reconstitution.

Figure 11: Macrophage depletion by clodronate liposome injection.

Figure 12: Successful macrophage replacement in liver by donor bone marrow-derived cells.

Figure 13: Loss of *Gadd45β* in haematopoietic compartment cells reduces the DEN induced HCC.

Figure 14: The loss of *Gadd45β* in haematopoietic cells reduces DEN induced HCC formation.

Figure 15: lung metastasis in the chimeric mice from experimental protocol A.

Figure 16: The loss of *Gadd45β* in haematopoietic cells reduces the DEN induced HCC formation.

Figure 17: Schematic diagram of bone marrow chimeric experimental "protocol B".

Figure 18: Bone marrow reconstitution.

Figure 19: Macrophage replacement in liver by donor bone marrow-derived cells.

Figure 20: The loss of *Gadd45β* in haematopoietic cells during tumour progression reduced the DEN induced HCC.

Figure 21: The loss of *Gadd45β* in haematopoietic cells during tumour progression reduces the DEN induced HCC formation.

Figure 22: The reduced tumour growth in *Gadd45β*$^{-/-}$ mice.

Figure 23: Histological examination of the inflammatory infiltrates of MCA-203 tumours isolated from *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ mice.

Figure 24: Quantitative real-time PCR analysis of gene expression in a macrophage co-culture system.

Figure 25: Quantitative real-time PCR analysis of gene expression in a macrophage co-culture system.

Figure 26: Cytokine stimulation promotes the expression of macrophage lineage-specific cytokines.

Figure 27: The *In vitro* polarisation of bone marrow-derived macrophage towards M1 phenotype.

Figure 28: The *In vitro* polarisation of bone marrow-derived macrophage towards M2 phenotype.

List of Publications

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Tornatore L, **Thotakura AK**, Bennett J, Moretti M, Franzoso G. (2012) The nuclear factor kappa B signalling pathway: integrating metabolism with inflammation. ***Trends Cell Biol.*** Nov; 22(11): 557-66.

Moretti M, Bennett J, Tornatore L, **Thotakura AK**, Franzoso G. (2012) Cancer: NF-κB regulates energy metabolism. ***Int J Biochem Cell Biol.*** Dec; 44(12): 2238-43.

Mauro C, Leow SC, Anso E, Rocha S, **Thotakura AK**, Tornatore L, Moretti M, De Smaele E, Beg A A, Tergaonkar V, Chandel NS, Franzoso G. (2011) NF-κB controls energy homeostasis and metabolic adaptation by upregulating mitochondrial respiration. ***Nature Cell Biology.*** Aug 28; 13(10): 1272-9.

List of abbreviations

AICD Activation-induced cell death

ALT Alanine aminotransaminase

AMPK AMP-activated protein kinase

AOM Azoxymethane

AP-1 Activator protein 1

APC/C Anaphase-Promoting Complex

APF-1 ATP-dependent proteolysis factor 1

Atg Autophagy related genes

ATP Adenosine triphosphate

BAFF-R B-cell-activating factor receptor

Bak Bcl2 antagonist/killer

Bax Bcl2-associated X protein

Bcl-xL- B-cell lymphoma-extra large

BM Bone marrow

BMDM Bone marrow-derived macrophages

BMP Bone morphogenetic proteins

BrdU Bromodeoxyuridine

CAC Colitis associated colon cancer

CCL Chemokine (C-C motif) ligand

CCL4 Carbon tetrachloride

CD Cluster of differentiation

cDNA Complementary DNA

cFLIP Cellular FLICE-inhibitory protein

CHI3l3 Mouse Chitinase 3like 3

CIA Collagen-induced arthritis

cIAP Cellular inhibitor of apoptosis protein

CNS Central nervous system

ConA Concanavalin A

COX-2 Cyclooxygenase-2

CREB cAMP response element-binding protein

CSF Colony stimulating factor

CXCL Chemokine (C-X-C motif) ligand

DEN Diethylnitrosamine

DMEM Dulbecco's modified Eagle's medium

DNA Deoxyribonucleic acid

DNR Daunorubicin

DSS Dextran sulfate sodium

E Embryonic

EAE Experimental autoimmune encephalitis

ECL Enhanced chemiluminescence

ECM Extracellular matrix

EDTA Ethylenediaminetetraacetic acid

EGF Endothelial growth factor

EGFP Enhanced green fluorescent protein

EMT Epithelial-mesenchymal transition

FACS Fluorescence activated cell sorting

FBS Fetal bovine serum

FDA Food and drug administration

FGF Fibroblast growth factor

Fip3 Family-interacting protein 3

FITC Fluorescein isothiocyanate

FIZZ1 Found in inflammatory zone-1

FR Folate receptor

G Gauge

Gadd45 Growth arrest DNA damage-inducible gene 45

Gadd45β Growth arrest DNA damage-inducible gene 45beta

GAPDH Glyceraldehyde 3-phosphate dehydrogenase

GM-CSF Granulocyte macrophage colony-stimulating factor

HBV Hepatitis B virus

HCC Hepatocellular carcinoma

HCV Hepatitis C virus

HGF Hepatocyte growth factor

HIF Hypoxia-inducible factor

HO-1 Heme-oxygenase-1

HRP Horseradish-peroxidase

HSC Haematopoietic stem cell

IAPs Inhibitors of apoptosis

i.p. Intraperitoneal

i.v. Intravenous

IBD Inflammatory bowel disease

IECs Intestinal epithelial cells

IF Immunofluorescence

IFN Interferon

Ig Immunoglobulin

IHC Immunohistochemistry

IκB I kappa B

IKK I kappa B kinase

IL Interleukin

iNOS Inducible nitric oxide synthase

IRF Interferon regulatory factor

JNK c-Jun N-terminal kinase

kDa Kilo Dalton

Kg Kilogram

KGF Keratinocyte growth factor

KO Knockout

LPS lipopolysaccharide

LT lymphotoxin

Luc Luciferase

M-CSF Macrophage colony-stimulating factor

M1 Classically activated macrophage

M2 Alternatively activated macrophage

MAPK Mitogen-activated protein kinase

MCP Monocyte chemoattractant protein

MDR2 Multiple drug resistance gene

MEFs Mouse embryonic fibroblasts

MEKK MAP kinase kinase kinase

Mg Milligram

MHC Major histocompatibility complex

MI Myocardial infarction

MKK7 MAP kinase kinase 7

mm Millimeter

MMP Matrix metalloproteinase

MOG Myelin oligodendrocyte glycoprotein

mRNA Messenger RNA

MS Multiple sclerosis

MyD88 Myeloid differentiation factor 88

NADPH Nicotinamide adenine dinucleotide phosphate-oxidase

NASH Non-alcoholic steatohepatitis

NEMO NF-κB essential modulator

NO Nitric oxide

NOS Nitric oxide synthase

PAMP Pathogen associated molecular pattern

PBS Phosphate buffered solution

PCD Programmed cell death

PCR Polymerase chain reaction

PFA Paraformaldehyde

PH Partial hepatectomy

PMA phorbol 12-myristate 13-acetate

PPAR Peroxisome proliferator-activated receptor

RA Retinoic acid

RA Rheumatoid arthritis

RANTES Regulated on activation, normal T cell expressed and secreted

RBC Red blood cells

RHD Rel-Homology domain

RIP1 Receptor interacting protein 1

RNA Ribonucleic acid

ROS Reactive oxygen species

RPM Revolutions per minute

RPMI Roswell park memorial institute-1640 medium

RT-PCR Reverse transcriptase polymerase chain reaction

RUNX2 Runt-related transcription factor 2

SDS-PAGE Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEM Standard error of the mean

SOD Superoxide dismutase

STAT Signal transducer and activator of transcription

TAM Tumour-associated macrophage

TCR T Cell Receptor

TGF Transforming growth factor

TH1 T helper 1

TH17 T helper 17

TH2 T helper 2

TLR Toll-like receptor

TNF Tumour necrosis factor

TNF-R Tumour necrosis factor receptor

TRADD TNF-R1-associated death domain

TRAF TNF-receptor associated factor

Treg Regulatory T cell

TUNEL Terminal deoxynucleotidyl transferase dUTP nick end labeling

UV Ultraviolet

V Volte

VEGF Vascular endothelial growth factor

WB Western blot

WHO World health organization

WT Wild-type

XIAP X-linked inhibitor of apoptosis

1:Introduction

1.1Overview of NF-κB pathway

Since its discovery more than 25 years ago, NF-κB has served as a paradigm for inducible transcription factors because of its broad physiological and medical effects, and it has gained an enormous research interest (Figure 2) (Oeckinghaus *et al.,* 2011; Hayden and Ghosh, 2012). NF-κB family of transcription factor consists of five members, NF-κB1 (p50/p105), NF-κB2 (p52/p100), Rel-A (p65), Rel (c-Rel) and Rel-B. It is characterised by the presence of a conserved 300 amino acid Rel-Homology domain (RHD) that is located towards the N- terminus of the protein, that mediates DNA binding, dimerization, and interaction with the inhibitory proteins of the IκB family (Hayden and Ghosh, 2004; Papa *et al.,* 2006). NF-κB dimers are retained in the cytoplasm in their inactive state through binding to IκBs. A wide range of stimuli is capable of activating NF-κB from the cytoplasmic pools by inducing the sequential phosphorylation and proteolysis of IκB inhibitors by the IκB kinase (IKK) complex and the ubiquitin/proteasome pathway, respectively (Hayden and Ghosh, 2004; Gilmore, 2006). Subsequently freed NF-κB dimers translocate to the nucleus and bind to specific sequences called κB sites, where they induce the transcription of a co-ordinate array of target genes. Including those regulating inflammation, immunity, and cell survival (Kumar *et al.,* 2004; Courtois and Gilmore, 2006; Baker *et al.,* 2011). There are eight established IκB family members in mammalian cells: IκBα, IκBβ, IκBε, IκBζ, BCL-3, IκB-NS and the precursor proteins p100 and p105 (Hayden and

Ghosh, 2004). IKK complex is composed of three major components; IKK1 (IKKα), IKK2 (IKKβ), and the NF-κB essential modulator (NEMO) (IKKγ) (Chen *et al.,* 1996; DiDonato *et al.,* 1997; Yamaoka *et al.,* 1998). To date, there are two main pathways for NF-κB activation has been reported (Pahl, 1999; Scheidereit, 2006). The two major distinct NF-κB activation pathways are the classical and the alternative pathway (Bonizzi and Karin 2004). Activation of NF-κB is normally through either of these pathways (Figure 1). However, few recent studies have suggested IKK independent pathways of NF-κB. An important feature of this IKK independent pathway is phosphorylation of IkBα at sites other than Ser32 and Ser36 (Perkin, 2006). Another study has also reported a Casein kinase 2 (CK2) mediated phosphorylation and IkB degradation after exposure to short range UV light (Kato *et al.,* 2003).

Firstly, the canonical or classical pathway is triggered in response to proinflammatory cytokines (TNF-α and IL-1), microbial products, and stress. Canonical pathway depends on IKKβ and leads primarily to the activation of p50/Rel-A and p50/c-Rel dimers by IκB phosphorylation (Hayden and Ghosh, 2004; Claudio *et al.,* 2006). Most of our knowledge relating to the pro-carcinogenic function of NF-κB is through the classic pathway that is triggered by proinflammatory cytokines. Classical NF-κB activation pathway leads to the transcription of a variety of genes, including those that encode

mediators of inflammation, cytokines, chemokines, proteases and inhibitors of apoptosis. It has been proposed that classical NF-κB activation pathway might link inflammation to cancer (Karin *et al.,* 2002).

Secondly, the non-canonical pathway or alternative pathway is engaged in certain members of the tumour necrosis factor receptor (TNF-R) family, such as lymphotoxins (LT) β and BAFF receptor. This pathway depends on IKKα and results in activation of p52/Rel-B complexes (Hayden and Ghosh, 2004; Claudio *et al.,* 2006). Neither IKKβ nor NEMO loss effect the alternative pathway, although the alternative pathway is very much less characterised than the canonical pathway. Its role in the development of lymphoid organs responsible for the generation of B and T lymphocytes has been very well documented (Dejardin *et al.,* 2002; Li and Verma, 2002; Rescigno *et al.,* 1998).

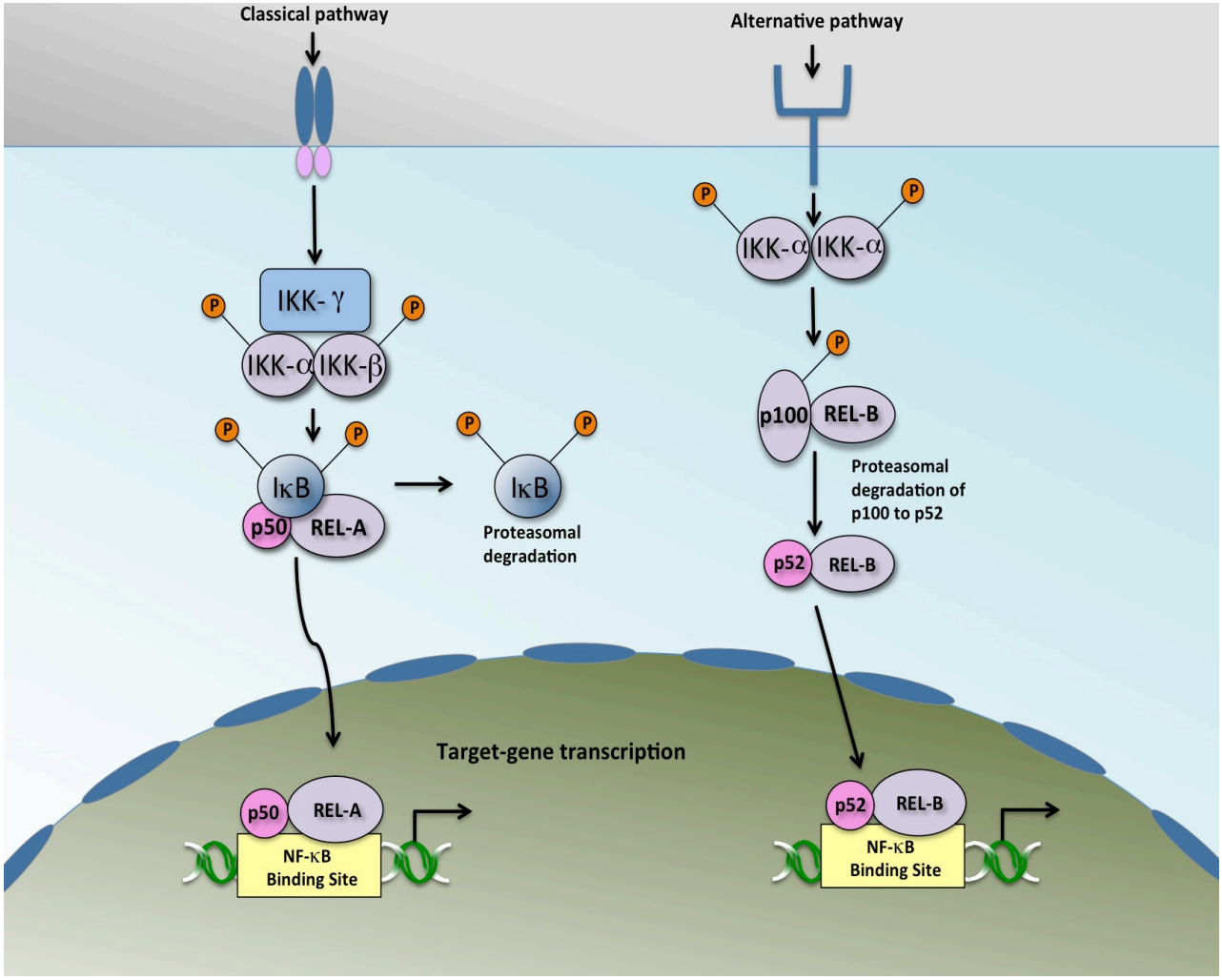


Figure. 1. NF-κB activation pathways. Classical NF-κB activation pathway is triggered through TNF-R and involves in the activation of genes that regulate cell survival, inflammation, and innate immunity. Alternative NF-κB activation pathway is triggered by CD40, BAFF-R and involves in lymphoid organ development. (Adapted from Karin and Greten, 2005).

1.2 IKK complex

NF-κB is a major transcription factor that is immediately activated after exposure to a variety of stimuli. The IκB kinase (IKK) complex is the primary regulator of the NF-κB signalling pathway and the activation of IKK complex is essential for the activation of NF-κB. The initial biochemical experiments that were undertaken to study the cytosolic IκB kinase activity led to the purification of high molecular weight (700-900kDa) kinase complex from unstimulated cells (Mercurio *et al.,* 1997; Chen *et al.,* 1996; Didonato *et al.,* 1997). This kinase complex was capable of specifically phosphorylating IκBα on the serine 32 and 36 residues. Purification of these complexes suggested that the presence of two catalytically active kinases IKKα, IKKβ, and a regulatory subunit NEMO (IKKγ) (Makris *et al.,* 2002). The IKK protein complexes exist in a complex of 1:1:2 ratios, supporting the notion that one IKKα and IKKβ heterodimer is bound to a homo dimer of NEMO (Hayden and Ghosh, 2008; Scheidereit, 2006; Hacker and Karin, 2006). The very first component of this high molecular weight complex was shown to be a CHUK, a 745 amino acid protein serine/threonine kinase that that has been renamed as IKKα (Regnier *et al.,* 1997). Very shortly after the discovery of IKKα, IKKβ was discovered based on the sequence homology and biochemical purification. IKKγ, also known as NEMO, IKKAP1, and Fip3, is the regulatory

subunit of IKK complex (Makris *et al.,* 2002). IKKα and IKKβ have been shown to possess 52% overall sequence identity and 65% identity within their catalytic domain (Mercurio *et al.,* 1997; Woronicz *et al.,* 1997; Zandi *et al.,* 1997). In both *in vitro* and *in vivo,* IKKα and IKKβ preferentially formed heterodimers, and studies have indicated that IKKα/IKKβ heterodimers have higher catalytic activity than either of their homo-dimers. Experiments using purified recombinant IKKα and IKKβ demonstrated that each subunit protein can directly phosphorylate IκB that is bound to NF-κB (Hayden and Ghosh, 2004). Phosphorylation of IκB by IKKα and IKKβ is more efficient when IκB bounds to NF-κB than the phosphorylation of free IκB. These studies explain how cytoplasmic free IκB, which is also an NF-κB target gene, can accumulate even in the presence of active IKK and thus contribute to the termination of NF-κB activation. IKKα and IKKβ are capable of phosphorylating IκB family members at multiple sites and are capable of phosphorylating IκBα at serine 32 and 36 and IκBβ at serine 19 and serine 23 (Zandi *et al.,* 1997). The non-catalytic IKK subunit NEMO is a glutamine-rich 48kDa protein that lacks a catalytic domain, and it is not related to IKKα and IKKβ subunits of IKK complex. NEMO also contains a c-terminal Zinc finger domain and a leucine zipper and an N-terminal and C-terminal coiled –coil domain (Rothwarf and Karin, 1999). The kinase activity of NEMO also has substrates outside the IκB family most notably p65.

Thus, so far IKKα, IKKβ, and IKKγ are the major components that have been demonstrated to be a part of IKK complex.

1.2.1 Biological functions of IKK complex

To study the basis for the biological role of IKK subunits, knockout models were generated (Li *et al.,* 1999a; Li *et al.,* 1999b; Li *et al.,* 2000). Knockout mouse models for any of the IKK complex subunits lead to failure of neonatal or embryonic development. The lethal phenotype due to liver apoptosis observed in IKKβ$^{-/-}$ mice was very similar although more severe than that observed in the p65/Rel-A$^{-/-}$ mice (Li *et al.,* 1999; Beg *et al.,* 1995; Li *et al.,* 1999). IKKβ$^{-/-}$ mice die during embryonic day E13, and this lethality is due to the massive liver apoptosis induced by TNF signalling during embryogenesis. As a consequence of the loss of IKKβ gene product, IKKβ$^{-/-}$ mice appeared to suffer severe liver degeneration at the gestation stage. Histological examination of wild-type and IKKβ$^{-/-}$ embryos revealed that there was a massive apoptosis. The extensive apoptosis in IKKβ$^{-/-}$ embryos might have played a significant role in lethality in IKKβ$^{-/-}$ embryos (Li *et al.,* 1999; Tanaka *et al.,* 1999; Li *et al.,* 1999). The central role of IKKβ induced NF-κB activity by TNF-α in liver development was supported by the finding that this lethal

phenotype in IKKβ$^{-/-}$ was rescued by crossing them with TNF-R$^{-/-}$ knockout mice. Thereby suggesting that IKKβ is essential for NF-κB signalling in order to rescue developing liver cells from TNF-mediated apoptosis (Li *et al.,* 1999a). This TNF-mediated phenotype is also seen in mice with IKKβ deleted in their skin (Pasparakis *et al.,* 2002). However, in mature hepatocytes loss of IKKβ did not lead to hepatocyte death (Luedde *et al.,* 2005). In contrast to IKKβ$^{-/-}$ mice, IKKα$^{-/-}$ mice are born and survive until a month after birth but they die after because of multiple morphological defects, particularly in their epidermal and skeletal development. IKKα has been shown to phosphorylate IκBβ preferentially at the serine 23 residues. Unlike IKKβ$^{-/-}$ mice, IKKα$^{-/-}$ mice did not show any hepatocyte apoptosis. Cells derived from IKKα$^{-/-}$ mice have reduced NF-κB activation in response to IL-1 or TNF, but the IKKβ & NEMO complex is still capable of phosphorylating IκBα and IκBβ *in vitro* (Hu *et al.,* 2001; Li *et al.,* 1999a; Li *et al.,* 1999). After careful examination of the IKKα$^{-/-}$ new-born pups, they have shiny and sticky skin with a thicker and undifferentiated epidermis. The pups lacked whiskers, and their limbs have bud morphology (Takeda *et al.,* 1999). Subsequent analyses have showed that, these defects are NF-κB independent. The phenotype of IKKα$^{-/-}$ mice was rescued by knocking in IKKα, in which the activation sites are mutated, thus indicating that the epidermal development is independent of its kinase activity (Hu *et al.,* 1999). These observations suggest that IKKα regulates the

alternative NF-κB pathway that has been shown to be essential for lymphoid organogenesis. However, IKKα also has a role in inflammation, unlike IKKβ, it has a negative regulatory effect on NF-κB activity in macrophage and inflammation. IKKα contributes to the suppression of NF-κB activity by accelerating both the turnover of NF-κB subunit and their removal from proinflammatory cytokine gene promoter (Lawrence *et al.,* 2005). Further studies have demonstrated that, by deletion of IKKα in mice there is an enhanced inflammation (Li *et al.,* 2005). Hence, both the IKK complex catalytic subunits have an opposite, but complementary roles that are needed to maintain a control of inflammation and innate immunity.

Like IKKβ, NEMO is also required for signalling through the canonical pathway, knockout studies have demonstrated that the C-terminal domain is needed for the activation. Similar to IKKβ$^{-/-}$, NEMO$^{-/-}$ mice die during embryonic day E12.5-E13 because of massive liver apoptosis (Rudolph *et al.,* 2000). Mouse embryonic fibroblasts (MEFs) isolated from these mice have shown a complete block in NF-κB activation by TNF, LPS, or IL-1 stimulation. These MEFs are highly sensitive to TNF, implying that the liver apoptosis observed in NEMO$^{-/-}$ mice could be due to the lack of NF-κB mediated protection from endogenous TNF-α induced cytotoxicity. These results have indicated that NEMO$^{-/-}$ mice displayed a more dramatic phenotype than

deletion of any other catalytically active IKK subunits. Thus, results from the knockout models suggested that only IKKβ and NEMO subunits participated in the signalling to NF-κB in response to proinflammatory cytokines (Makris *et al.,* 2000; Makris *et al.,* 2002).

Given the critical role of NF-κB signalling in the inflammatory disease and malignant disease, defining the precise mechanism involved in the process of activation and termination of NF-κB signalling is essential for developing new diagnostic and therapeutic tools.

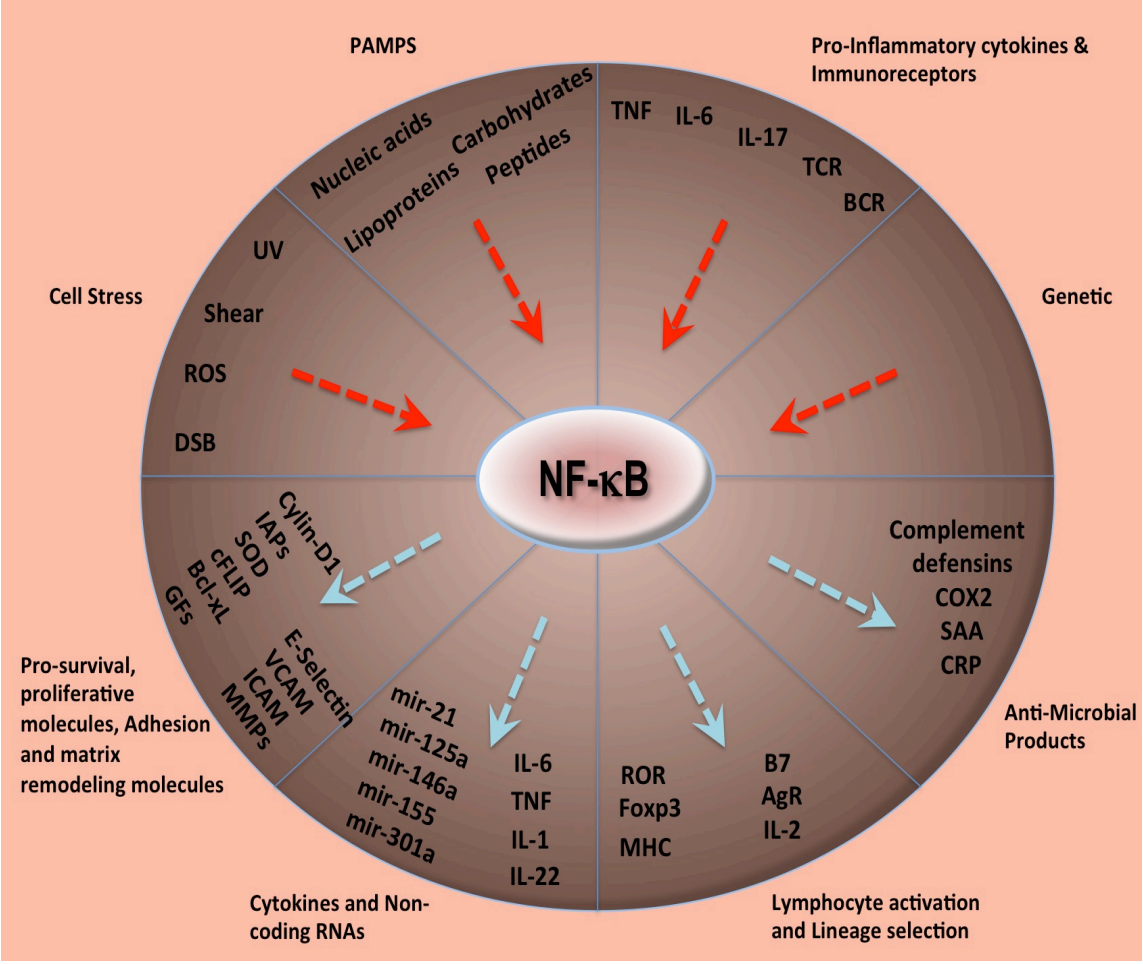


Figure 2. Various roles of NF-κB

1.3 Role of NF-κB within the liver

The liver plays an essential role in the degradation of exogenous and endogenous chemicals such as cholesterol, steroid hormones, fatty acids, and proteins that could otherwise harm the body. All the exogenous and endogenous chemicals degraded by the liver are eliminated through bile or

urine. This essential process of detoxification by the liver makes it susceptible to the toxic effects. Indeed, a number of these toxins cause liver injury that ultimately affects the body in several ways (Antoine *et al.,* 2008).

Hepatocytes are the major parenchymal cells in the liver. The common consequence of toxin-induced liver injury is the death of these major parenchymal cells (Malhi and Gores, 2008) that eventually lead to a broad range of liver diseases, including viral and alcoholic hepatitis, ischemia/reperfusion liver injury and fulminant hepatic failure (Guicciardi and Gores, 2005). However, hepatocytes are well equipped to face this toxin-induced challenge and possess an extraordinary ability to counter the damaging effects. Hepatocyte injury activates a specific programme of gene expression that is capable of antagonising the programmed cell death pathways thereby protecting hepatocytes from cell death and also protecting liver integrity (Wullaert *et al.,* 2007; Sun and Karin, 2008). However, when the process of antagonising PCD fails, a continued hepatocyte death causes compensatory proliferation of surviving hepatocytes, aimed at maintaining the overall liver mass. The continuous process of hepatocyte cell death and subsequent compensatory proliferation eventually promotes the development and progression of hepatocellular carcinoma (HCC) (Karin, 2006). It had become increasingly evident that when the balance between

cell survival and cell death in the liver is tipped to one or the other the road to liver diseases are wide open (Wullaert *et al.,* 2007).

NF-κB signalling has been shown to plays a vital role in the liver physiology and development (Hayden and Ghosh, 2004; Dutta *et al.,* 2006). Hepatocytes have a remarkable proliferative capacity but are largely quiescent. Liver regeneration after PH provided a fundamental paradigm for the liver response to injury (Fausto *et al.,* 2006; Michalopoulos K, 2007). NF-κB is one of the transcription factors that protects cells from undergoing apoptosis in a wide range of cell types, including hepatocytes (Fausto *et al.,* 2006) through the expression and activation of critical genes that encode anti-apoptotic proteins (Dutta *et al.,* 2006; Wullaert *et al.,* 2007). There is still a controversy as to in which liver cell type NF-κB primarily carries out its function during liver regeneration after PH. Few studies have reported that PH induces NF-κB activation primarily within Kupffer cells that stimulate hepatocyte cell growth (Chaisson *et al.,* 2002; Abshagen *et al.,* 2007).

Tumour development involves an imbalance between cell proliferation and cell death (Karin, 2006; Wullaert *et al.,* 2007). The evidence for the participation of NF-κB in liver carcinogenesis, indeed, has recently become more apparent. Studies in animal models underscore the fundamental role of the NF-κB pathway in the regulation of PCD and proliferation in hepatocytes

that when deregulated may contribute to the onset of HCC. Furthermore, it was also shown that NF-κB protects transformed hepatocytes from PCD through the upregulation of pro-survival genes. Suggesting that NF-κB is a tumour-promoting factor in hepatocytes (Pikarsky *et al.,* 2004).

Like NF-κB, the p38α MAPK has also been suggested to suppress cancer development in the liver. It was shown that by using distinct mechanisms, NF-κB and p38α co-operate to prevent ROS accumulation that promoted the necrotic cell death during this process (Sakurai *et al.,* 2008). Given that HCC develops in the setting of inflammation associated with the challenge of hepatitis virus and chronic alcoholism, it would be appropriate in the future to obtain further data on these cases. Another important challenge will be to delineate the mechanisms and the critical NF-κB controlled checkpoints that control hepatocyte death and proliferation in HCC.

1.3.1 Role of NF-κB in embryonic liver development

NF-κB transcription factor orchestrates expression of a large number of genes that are involved in growth, differentiation and development. It has also been shown that NF-κB signalling plays a significant role in liver physiology and development (Hayden and Ghosh, 2004; Dutta *et al*., 2006). Using mice deficient of Rel-A, provides very first evidence for the role of NF-

κB in liver homoeostasis. Rel-A deficiency led to embryonic lethality at mid-gestation. Liver sections from these embryos displayed a massive liver degeneration and areas of tissue loss. These findings suggested that hepatocytes were being specifically targeted, and this lethality is associated with a massive induction of PCD only in the liver (Beg *et al.,* 1995; Beg and Baltimore, 1996). The breakdown of liver architectures may have closely preceded death of embryos. However, at day 14 liver sections of Rel-A deficient embryos were indistinguishable from those of wild-type, suggesting that Rel-A is not required for early liver development (Beg *et al.,* 1995). Further studies have shown that Rel-A is not important for normal liver development, however, Rel-A has a vital protective function against TNF-R1 induced PCD. In support of these findings, mice lacking Rel-A and TNF-R1 survived during embryonic development. At birth, double mutant mice have normal liver morphology, but soon after birth most animals developed acute hepatitis and die around ten days of age (Rosenfeld *et al.,* 2000). The lethality phenotype showed in embryos of Rel-A $^{-/-}$ mice has also been detected in embryos that lacking either IKKβ or IKKγ (Li *et al.,* 1999; Makris *et al.,* 2000; Rosenfeld *et al.,* 2000). This phenotype of Rel-A$^{-/-}$ is very similar to IKKβ$^{-/-}$ mice, since the rescue of the phenotype is induced by the inactivation of TNF-R1 gene. These results suggested that hepatocyte apoptosis in both Rel-A and IKKβ$^{-/-}$ mice is caused by TNF-α induced cell death in the absence of NF-κB activation (Beg *et al.,* 1995; Beg and Baltimore, 1996).

1.3.2 Role of NF- κB in promoting liver regeneration

In a healthy liver, hepatocytes have a remarkable proliferative capacity; NF-κB transcription factor protects hepatocytes from undergoing apoptosis (Fausto *et al.,* 2006). NF-κB is rapidly activated after partial hepatectomy (PH), once activated, NF-κB protects hepatocytes from cell death and promotes their proliferation; this induction of NF-κB is through TNF-α. Furthermore, TNF-α is induced during liver regeneration and blocking TNF-α by using anti-TNF-α antibody inhibited liver regeneration after PH (Akerman *et al.,* 1992). Supporting this, in TNF-R1 knockout mice, NF-κB activity was impaired after PH (Yamada *et al.,* 1997). Moreover, by using IκBα super-repressor or by using pharmacological inhibitors of NF-κB activity resulted in the induction of massive apoptosis (Iimuro *et al.,* 1998; Cressman *et al.,* 1994; Plumpe *et al.,* 2000). The cell type that was responsible for this function of NF-κB during liver regeneration is not very precisely known. It has been reported that PH induces NF-κB activation primarily in Kupffer cells (Chaisson *et al.,* 2002; Abshagen *et al.,* 2007). However, NF-κB activation in hepatocytes during liver regeneration has also been reported. Mechanistically, NF-κB in hepatocytes induces protective genes that may antagonise TNF-α mediated PCD (Maeda *et al.,* 2005; Luedde *et al.,* 2007). Taken together, NF-κB plays an important role in liver regeneration both in parenchymal cells (hepatocytes) and non- parenchymal cells.

1.4 Role of NF-κB in Inflammation and tumourigenesis

NF-κB transcription factors are the central coordinators of innate and adaptive immunity and inflammation (Hayden and Ghosh, 2004; Claudio *et al.,* 2006; Papa *et al.,* 2006). NF-κB also controls cell survival, tumourigenesis and chemoresistance to cancer through its antagonistic action on programmed cell death (PCD) induced by the proinflammatory cytokine TNF-α (Dutta *et al.,* 2006). Recent studies have shed new light on the basis of this pivotal role of NF-κB in carcinogenesis (Greten *et al.,* 2004; Maeda *et al.,* 2005; Pikarsky *et al.,* 2004). Galen and later Rudolf Virchow who noticed an infiltration of leukocytes into malignant tissues first uncovered a casual connection between inflammation and cancer (Coussens and Werb, 2002; Virchow, 1881). Suggesting that cancers may originate from the regions of chronic inflammation. However, the exact mechanisms linking inflammation and tumourigenesis are still not very well understood.

NF-κB is activated by inflammatory stimuli, and constitutive activation of NF-κB has been associated with various cancers, suggesting that NF-κB might provide a link between inflammation and cancer. One common epigenetic event in cancer is inflammation, and studies have shown that chronic infections and inflammation account for 15-20% of all the cancer death in humans (Kuper *et al.,* 2000). To define the role of NF-κB in inflammation

driven cancers, the use of mouse models in which tumour induction depends on inflammation have been used thus mimicking inflammation driven cancers in humans.

The first such model was a mouse model for colitis-associated colon cancer (CAC) (Greten *et al.,* 2004). As its name indicates a colorectal cancer, a type of colon cancer that appears in patients suffering from ulcerative colitis, a chronic inflammatory bowel disease (IBD). CAC is a classic inflammation driven cancer that resultes in response to chronic inflammation (Greten *et al.,* 2004). In this mouse model, using Azoxymethane (AOM) injection, combined with a repeated administration of the irritant dextran sulfate sodium (DSS) caused colonic inflammation. This study demonstrated that IKKβ driven NF-κB activation in intestinal epithelial cells (IECs) is essential for the development of colonic adenomas (Greten *et al.,* 2004). The oncogenic role of NF-κB in IECs appears to be mediated through its anti-apoptotic function, which is mediated through the induction of anti-apoptotic gene Bcl-xL. Thus preventing apoptotic elimination of premalignant cells (Greten *et al.,* 2004). The proliferative function of NF-κB is indirect and is mediated through IL-6 and related cytokines production by myeloid cells that lead to the activation of transcription factor STAT3 in IECs (Greten *et al.,* 2004). Subsequent work has demonstrated that the effect of immune cells on CAC growth is also

mediated through the proinflammatory cytokines IL-6, IL-1, TNF-α and IL-1β (Becker *et al.,* 2004; Bollrath *et al.,* 2009; Grivenikov *et al.,* 2009). The ablation of STAT3 in IEC inhibited CAC development, affecting both cell survival and cell proliferation (Grivennikov *et al.,* 2009). These studies provided a critical support for the important tumour promoting function of NF-κB in preventing apoptotic elimination of pre-neoplastic IEC by activating anti-apoptotic genes. Whereas, NF-κB in lamina propria inflammatory cells induced the production of growth factors that promoted the proliferation of pre-neoplastic cells.

Another interesting example of an inflammation-linked cancer is the hepatocellular carcinoma (HCC), the most common form of liver cancer in humans. HCC for instance, often can result from cirrhosis or chronic infection with HBV and HCV viruses (Karin, 2006; Kim *et al.,* 2006). One mouse model in which spontaneous HCC development is dependent on chronic liver inflammation is the Mdr2$^{-/-}$ knockout mouse model (Pikarsky *et al.,* 2004). The absence of the MDR2 transporter leads to accumulation of bile acids and phospholipids within the hepatocytes that resulted in a low-grade liver inflammation and hepatosteatosis in these mice that eventually led to the development of HCC (Allen *et al.,* 2011). In this study, it has been reported that hepatocytes adjacent to the regions of inflammation express high levels

of nuclear NF-κB. Furthermore, inhibition of NF-κB activation in hepatocytes of Mdr2$^{-/-}$ mice resulted in a reduced HCC development. The exact mechanism how bile acids and phospholipid accumulation lead to NF-κB activation in this model is not clear, but it seems to be dependent on the production of TNF-α by non-parenchymal cells. Treatment of these mice with a neutralising anti-TNF-α antibody blocked the activation of NF-κB in hepatocytes that lead to decreased expression of NF-κB dependent anti-apoptotic genes. In this model, the major mechanism by which NF-κB was suggested to exert its tumour-promoting role was through the suppression of apoptosis (Pikarsky *et al.,* 2004).

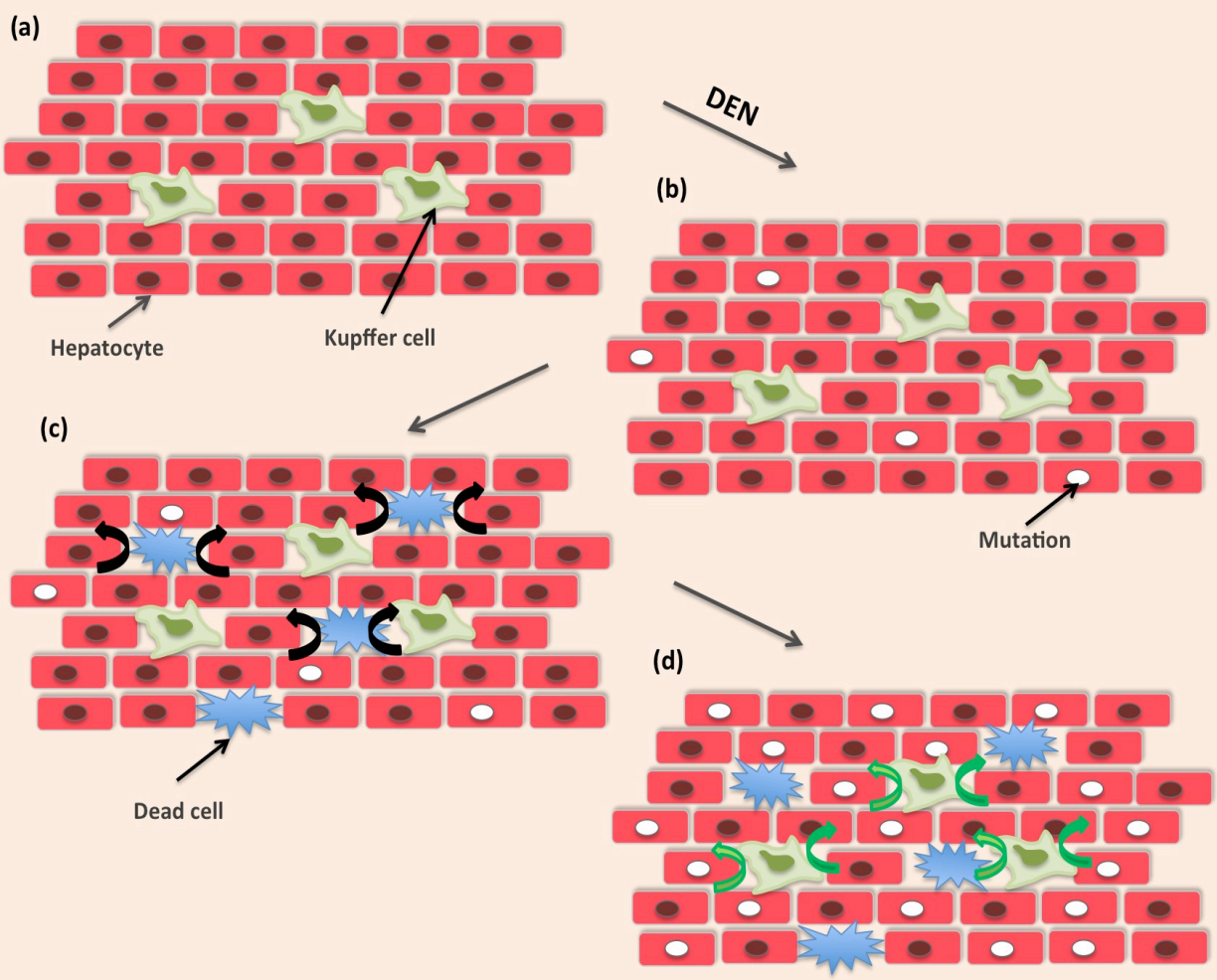


Figure 3. Diethylnitrosamine induced HCC model: (a) Normal liver tissue with hepatocytes and Kupffer cells; (b) DEN exposure cause mutation in hepatocytes; (c) combined with hepatocyte cell death and subsequent activation of Kuppfer cells (d). Kupffer cells once activated produce factors that induce the quiescent hepatocyte division and transmit oncogenic mutation, eventually leading to HCC development.

A completely different scenario applies to the role of NF-κB in HCC development in mice when injected with the procarcinogen diethylnitrosamine (DEN). DEN undergoes metabolic activation in zone 3 hepatocytes, and it is a complete carcinogen when injected into mice that are two weeks old (Verna *et al.,* 1996). DEN administration into mice lacking IKKβ (NF-κB inhibition by deletion of IKKβ) in their hepatocytes resulted in increased liver tumour formation (Maeda *et al.,* 2005; Sakurai *et al.,* 2006; He and Karin, 2011). Deletion of IKKβ in both hepatocyte and haematopoietic derived cells inhibited this process. These results supported a model whereby lower activation of NF-κB augment hepatocyte death in the form of necrosis, thereby releasing products that induce the secretion of proinflammatory cytokines (i.e. TNF-α) by adjacent myeloid cells that resulted in the stimulation of compensatory proliferation of surviving and DNA-damaged hepatocytes (Figure 3) (Maeda *et al.,* 2005; Sakurai *et al.,* 2008). The compensatory proliferation of NF-κB-deficient hepatocytes in response to DEN treatment plays a key role in the development of liver cancer (Koch *et al.,* 2009). DEN induced HCC requires NF-κB activation in Kupffer cells (macrophages in liver) that produce IL-6 (Naugler *et al.,* 2007) followed by the activation of STAT3 in hepatocytes (He *et al.,* 2010). Notably, it was shown that a crucial link between inflammation and carcinogenesis depends on NF-κB and is mediated by at least two mechanisms (Figure 4). 1) Within the pre-cancerous/cancerous cells, NF-κB upregulates pro-survival

genes that enable cells to evade PCD and propagate their malignant phenotype 2) In non-parenchymal cells, NF-κB induces proinflammatory cytokine production that potently stimulate cancer cell growth. Indeed, constitutively activated NF-κB is most commonly found in many tumours and certain other solid cancers.

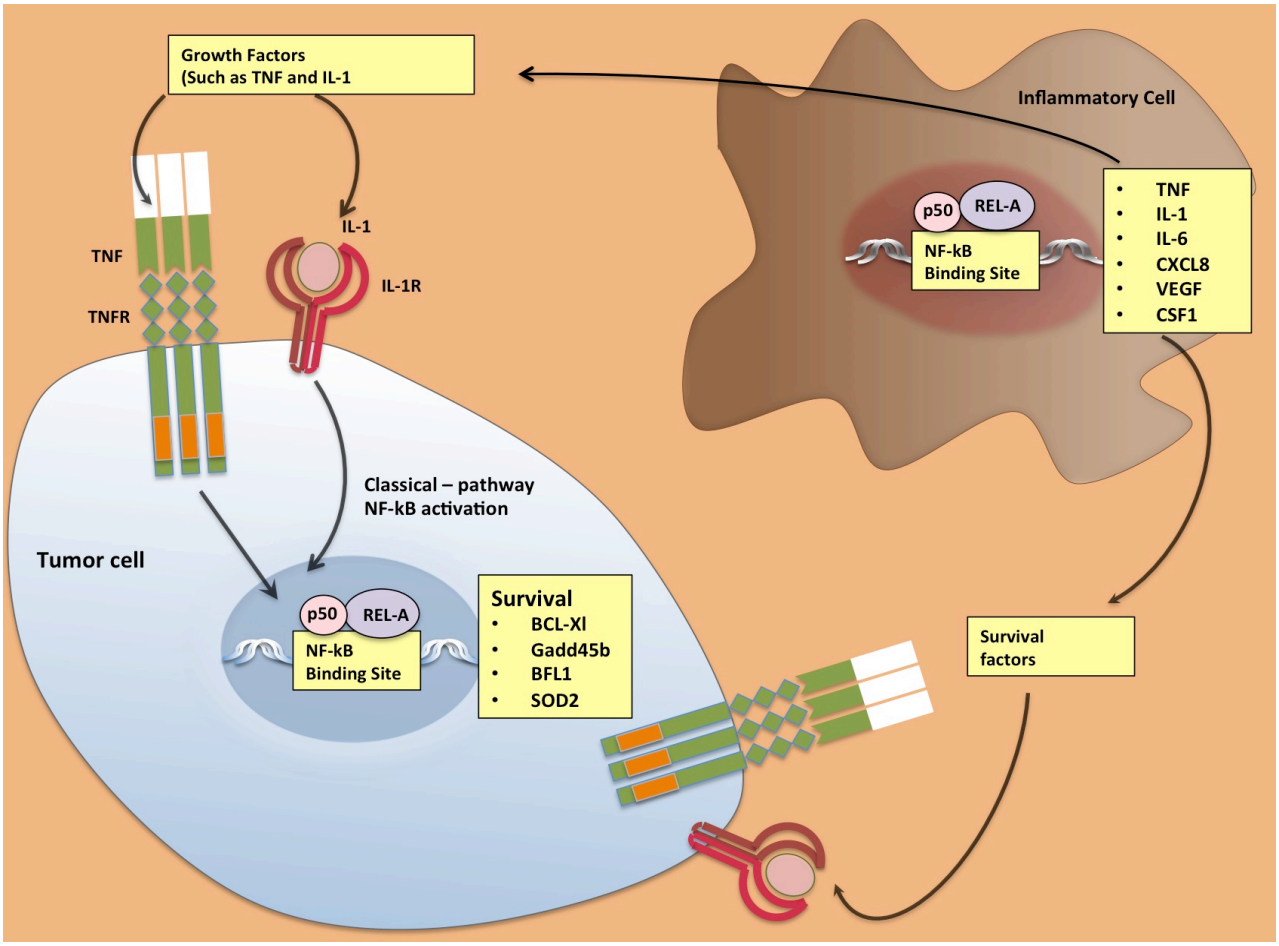


Figure 4. Cell-type dependent functions of NF-κB: NF-κB activation in tumour cells and inflammatory cells increase tumour growth and progression through activation of different genes. In tumour cells, NF-κB activation leads to the upregulation of survival genes to ensure the proliferation of tumour cells. In inflammatory cells, activation of NF-κB leads to

the production of cytokines and survival factors that affect the tumour stroma. (Adapted from Karin, Nat review, 2005).

In addition to its role in tumourigenesis, elevated NF-κB activity has been shown to hamper tumour cell killing inflicted by the radiation and chemotherapeutic drugs and in doing so NF-κB activity promotes resistance to the anti-cancer therapy (Pikarsky *et al.,* 2004). In addition to its tumour-promoting role, NF-κB may also participate in tumour initiation. For example, activation of NF-κB in macrophages and neutrophils promote the production of reactive oxygen species (ROS) through the induction of NO synthase and other enzymes. The ROS produced in these cells diffuse to cause DNA damage and carcinogenic lesions in the surrounding cells (Behrend L *et al.,* 2003; Pelicano *et al.,* 2004; Karin, 2002).

Accordingly, owing to its role in various cancers, NF-κB targeting drugs (proteasome inhibitors, glucocorticoids) are increasingly used to treat human malignancies. These drugs however have serious side effects, especially upon prolonged use, most notably immunosuppressive effects (Karin, 2006). Thus, a preferable therapeutic approach would be to block the critical NF-κB downstream target genes that promote cancer cell survival, rather than blocking NF-κB itself. These genes however remain unknown.

1.5 The control of programmed cell death (PCD) by NF-κB

Programmed cell death (PCD) was observed more than 170 years ago, and it has a fundamental role in homoeostasis and animal development (Glucksmann, 1951). Aberrant regulation of PCD may lead to large a number of human diseases, including immunological disorders, neurodegeneration, and cancers (Fuchs and Steller, 2011). The basis for the protective activity of NF-κB in programmed cell death is best understood in the context of stimulation via TNF-R1 (Dutta *et al.,* 2006). Although, signalling through this receptor can trigger PCD, cells normally survive this signalling due to activation of NF-κB. The protective action of NF-κB involves a suppression of sustained activation of the JNK cascade a major MAPK cascade in eukaryotes. In some systems, NF-κB afforded inhibition of this cascade depends on a blockade of formation of the reactive oxygen species (ROS) that promotes JNK activation downstream of TNF-Rs (Papa *et al.,* 2004).

To understand the mechanism that control TNF-R1 inflicted PCD and tumourigenesis, much was focused on the identification of pro-survival genes that are subject to NF-κB control. Using an unbiased screen, *Gadd45β/Myd118*, a member of the Gadd45 family of inducible factor was identified as a pivotal effector of the NF-κB afforded inhibition of JNK signalling and PCD (De Smaele *et al.,* 2001). Its expression abrogates TNF-α induced JNK activation and PCD in NF-κB proficient cells. *Gadd45β* mediated

blockade of TNF-α induced JNK signalling involves a direct targeting of the MAPK kinase (MAPKK), MKK7/JNKK2, a specific and essential inducer of JNK downstream of TNF-Rs (Papa *et al.,* 2004; Papa *et al.,* 2006). *Gadd45β* binds tightly to MKK7, blocking its catalytic activity (Figure 5). This blockade is specific; since *Gadd45β* has no apparent effect on other MAPK-pathway kinases participating in TNF-R1 induced JNK activation (Papa *et al.,* 2004; Papa *et al.,* 2006; Tornatore *et al.,* 2014). Indeed, *Gadd45β* seemingly engaged the ATP-binding site and critical helix C-proximal region of MKK7, preventing the access of the kinase to the ATP binding site thereby inducing conformational changes incompatible with catalytic function. The *Gadd45β-MKK7* interaction is a crucial molecular link between the NF-κB and JNK pathways, and consequently is a potential target for anti-inflammatory and anti-cancer therapies. Indeed the importance of this link is underscored by the findings that cell-permeable peptides (P1) specifically disrupting the *Gadd45β-MKK7* complex thereby hindering the NF-κB protective activity against TNF- α induced PCD. Furthermore, silencing of MKK7 halts this PCD in NF-κB null cells, whereas knocking-out *Gadd45β* promotes it in NF-κB proficient cells (Papa *et al.,* 2004; Papa *et al.,* 2006). Whether JNK inhibition represents a protective mechanism mediated by NF-κB during tumourigenesis and whether this inhibition also involves *Gadd45β* upregulation, however, remains unknown.

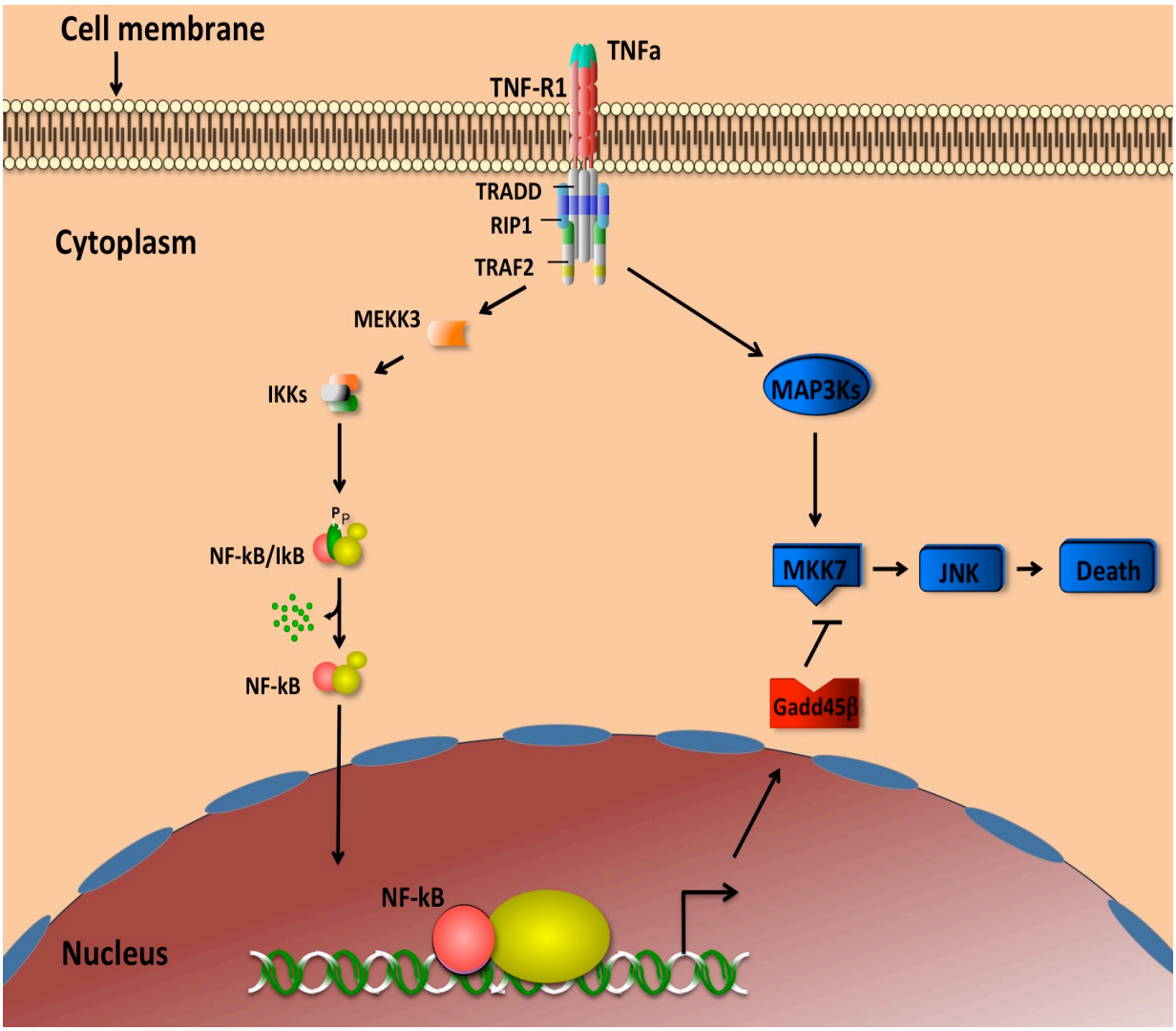


Figure 5. *Gadd45β* mediated inhibition of JNK induced cell death.

NF-κB dependent induction of *Gadd45β* and subsequent inhibition of JNK induced cell death by blocking MKK7 upstream of JNK.

Owing to NF-κB's central role in oncogenesis, there has been enormous interest in developing drugs targeting the NF-κB pathway in cancer and other disorders (Baud and Karin, 2009; Papa *et al.,* 2006; Courtois and Gilmore, 2006). NF-κB-targeting drugs (e.g. proteasome inhibitors), however, have failed to achieve long-lasting disease remission in cancer patients, due to low therapeutic index and adverse side effects, reflecting the pleiotropic functions of the NF-κB pathway (McConkey and Zhu, 2008; Nencioni *et al.,* 2007; Laubach *et al.,* 2009). Similar concerns exist for IKK inhibitors (Greten *et al.,* 2007). A deeper knowledge of the mechanisms regulating the NF-κB signalling and the roles of its downstream target genes in tumourigenic functions are therefore required not only to further our understanding of cancer, but also to develop better approaches for the improved cancer therapies.

1.6 Gadd45beta

To understand the mechanism controlling TNF-R1 inflicted PCD and tumourigenesis, we focused on the identification of pro-survival genes that are subject to NF-κB control. Using an unbiased screen *Gadd45β* was identified (De Smaele *et al.,* 2001). Growth arrest and DNA-damage-inducible beta, also known as *Gadd45β* is a part of a family of genes comprising of Gadd45α, *Gadd45β,* and Gadd45γ (Abdollahi *et al.,* 1991; Zhang *et al.,* 1999; Nakayama *et al.,* 1999). *Gadd45β* is an acidic protein of about 18 kDa in size, without any enzymatic activity, and its function is only through interacting with its protein partners (Tornatore *et al.,* 2008). *Gadd45β* proteins have been implicated in several biological functions including growth control and DNA repair leading to cellular homeostasis. *Gadd45β* is also involved in stress responses, cell cycle regulation, oncogenesis (Luo *et al.,* 2011), autoimmune disease, T-cell differentiation and activation. (Yang *et al.,* 2000; Vairapandi *et al.,* 2000; Zhan *et al.,* 1999; Wang *et al.,* 1999; Smith *et al.,* 2000). *Gadd45β* was initially identified as a gene that is readily induced by IL-6 and IL-1 cytokines that are produced by murine myeloid leukaemia cells upon induction of terminal differentiation (Abdollahi *et al.,* 1991). Subsequent studies have shown that *Gadd45β* may also be induced by TGF-β, TNF-α and IL-18 as well as PMA and ionomycin, genotoxic and oxidative stress

(Takekawa and Saito, 1998; Zhang *et al.,* 1999; De Smaele *et al.,* 2001; Yang *et al.,* 2001).

Recent studies have indicated that, *Gadd45β* promoter is under the direct control of NF-κB transcription factor (Jin *et al.,* 2002). *Gadd45β* contains three binding sites for NF-κB, and all of these sites are required for optimal transcriptional activation (Zazzeroni *et al.,* 2003). Further studies dissected the precise role of *Gadd45β* in promoting cell survival and found that, after induction of NF-κB, a selective inactivation of JNK was observed in MEFs and other cell types. This inhibition was an upstream effect by binding *Gadd45β* tightly to MKK7/JNKK2 a selective and an essential inducer of JNK (Papa *et al.,* 2007; Papa *et al*., 2004). The basis for this ability to block MKK7 is still unknown, because, unlike other Gadd45 proteins, *Gadd45β* lacks enzymatic activity and phosphatase activity (Liebermann and Hoffman, 2002; Papa *et al.,* 2004). *Gadd45β* function is mainly through protein-protein interaction, and these interactions were cell type specific. For example, in haematopoietic cells, JNK inactivation by *Gadd45β* is through binding to MKK4 instead of MKK7 (Gupta *et al.,* 2005; Gupta *et al.,* 2006). These observations led to the conclusion that, *Gadd45β* acts as an anti-apoptotic factor by exerting JNK inactivation. In support to its differential activation and function, several studies have reported that *Gadd45β* may act as an active pro-apoptotic factor

(Takekawa *et al.,* 1998; Vairapandi *et al*., 2000; Engelmann *et al.,* 2008).

The role of *Gadd45β* in apoptosis is often debated. Few studies have shown that *Gadd45β* expression prevents apoptosis (De Smaele *et al.,* 2001; Papa *et al.,* 2004; Zazzeroni *et al.,* 2003) while others have shown that *Gadd45β* expression promotes apoptosis (Takekawa and Saito, 1998). In contrast, few recent studies have shown no effect of *Gadd45β's* expression in cell survival and apoptosis (Amanullah *et al.,* 2003; Zerbini *et al.,* 2004). Despite its ambiguous role in apoptosis, *Gadd45β* expression has been implicated in various pathologies as described.

1.6.1 Role of *Gadd45β* in autoimmunity

Autoimmunity is largely controlled by a delicate balance between effector T helper cell proliferation and their programmed cell death, loss of this balance would lead to an increased accumulation of self-destructive T helper cells. In $CD4^+$ T- cells, *Gadd45β* is readily induced by TCR signalling or by stimulating with proinflammatory cytokines such as IL-12, IL-18, IL-6, IL-12, and TNF-α. Anti-inflammatory cytokines like tumour growth factor (TGF)-β stimulation has also been shown to induce *Gadd45β* expression (Yang *et al.,* 2001; Lu *et al.,* 2001; De Smaele *et al.,* 2001; Takekawa *et al.,* 2002). Indeed, *Gadd45β* has

been shown to be required for AICD in T_H1 effector cells (Liu *et al.,* 2005), thereby limiting the survival of $CD4^+$ helper T cells in response to TCRs or cytokine stimulation. Mechanistically, *Gadd45β* deletion has shown to reduce sustained but not transient activation of p38 and eventually lead to reduced IFN-γ production in T_H1 cells. In addition to the defects in IFN-γ production by T_H1 cells, *Gadd45β* deficiency also resulted in defective function of naïve $CD4^+$T cells (Lu *et al.,* 2004). Suggesting that *Gadd45β* has a role in the function of $CD4^+$ T-cells. (Yang *et al.,* 2001; Lu *et al.,* 2004; Lu *et al.,* 2001). Owing to its role in $CD4^+$ T-cells, *Gadd45β* has been shown to be involved in autoimmune diseases.

1.6.1.1Role of *Gadd45β* in experimental autoimmune encephalomyelitis

EAE is a murine model of human multiple sclerosis, primarily caused by the infiltration of autoreactive $CD4^+$ T-cells (Begolka *et al.,* 1998), particularly effector T_H1 and T_H17 cells into the central nervous system (Liu *et al.,* 2005). It has been shown that the apoptotic deletion of autoreactive $CD4^+$ T-cells was related to the downregulation of EAE during recovery (Tabi *et al.,* 1995).

In support to the aforementioned roles of *Gadd45β* in $CD4^+$ effector T cells, recent studies using *Gadd45β*$^{-/-}$ mice have shown that *Gadd45β*$^{-/-}$ T_H1 cells

were more resistant to AICD and also $CD4^+$ T-cells from *Gadd45β*$^{-/-}$ mice underwent more cell division than *Gadd45β*$^{+/+}$ cells after anti-CD3 stimulation (Liu *et al.,* 2005). Similar results were also obtained after IL-12 and IL-18 proinflammatory cytokine stimulation, suggesting that *Gadd45β* might play a key role in $CD4^+$T-cells proliferation. To further understand *Gadd45β's* role in $CD4^+$ T- cells *in vivo, Gadd45β*$^{-/-}$ and *Gadd45β*$^{+/+}$ mice were challenged with myelin oligodendrocyte glycoprotein (MOG) $_{35-55}$. *Gadd45β*$^{-/-}$ mice developed severe EAE and sustained EAE for longer duration compared to *Gadd45β*$^{+/+}$ mice, although the disease onset was same in both the genotypes. This severe disease in *Gadd45β*$^{-/-}$ mice was due to excessive accumulation of AICD resistant effector $CD4^+$ T-cells in CNS, suggesting *Gadd45β's* regulation in $CD4^+$ T-cells was essential for recovery from the EAE (Liu *et al*., 2005).

These studies suggest that *Gadd45β's* expression in effector $CD4^+$ T cells has a physiological role in suppressing EAE, owing to *Gadd45β's* anti-proliferative and pro-apoptotic function. Due to its role in the resolution of EAE, *Gadd45β* could be a potential novel target for MS treatment.

1.6.1.2 Role of *Gadd45β* in rheumatoid arthritis

In addition to its role in EAE by regulating CD4 effector T cells proliferation and survival, *Gadd45β* has also been shown to play a crucial role in rheumatoid arthritis (RA). RA is a chronic inflammatory disease, primarily affecting small diarthrodial joints of the hands and feet and characterised by synovial lining hyperplasia and chronic synovitis. Eventually leading to infiltration of immune cells into the synovium followed by progressive matrix damage that unlimately leads to joint destruction (Firestein G, 2003). In RA, mitogen-activated protein (MAP) kinases, specifically JNK, plays an important role in regulating cytokines and matrix metalloproteinases (MMPs) expression that have been shown to play an important role in extracellular matrix degradation. Indeed, patients with RA, express high levels of MMPs that are involved in joint degradation and remodelling (Han *et al.,* 2001; Han *et al.,* 1998). The upstream kinase in JNK pathway, specifically MKK7, has also been shown to be active in RA patients suggesting its role in extracellular matrix degradation (Yoshizawa *et al.,* 2008).

In a passive K/BxN arthritis model, *Gadd45β* deficiency was shown to be associated with increased JNK levels and increased MMP expression that led to increased disease severity, similar results were also observed in cultured synoviocytes from *Gadd45β*$^{-/-}$ mice (Svensson *et al.,* 2009). However, in the CIA model, *Gadd45β*$^{+/+}$ mice displayed severe arthritis compared to *Gadd45β*$^{-}$

$^{/-}$ mice, and there was no observed difference in JNK levels. This unexpected decrease in susceptibility, in *Gadd45β*$^{-/-}$ mice, was due to increased production of the anti-inflammatory cytokine IL-10 and increased T-regs in *Gadd45β*$^{-/-}$ mice. The source of IL-10 was not exactly known, but this could be related to increase in T-regs (Svensson *et al.,* 2009).

However, in contrast to the RA model, in EAE model *Gadd45β* suppress $CD4^+$ T-cells proliferation and $CD4^+$ T-cells that are lacking *Gadd45β* were more resistant to AICD, leading to severe disease in *Gadd45β*$^{-/-}$ mice compared to *Gadd45β*$^{+/+}$ mice (Liu *et al.,* 2005). The reason for this contrasting result could be attributed to the differences in the model system, the mouse strain used, and also dependent on the stage of the disease.

Thus, the results from the passive K/BxN model suggest that enhancing *Gadd45β* expression in RA patients could be beneficial.

1.6.1.3 Role of *Gadd45β* in type 1 diabetes

Type 1 diabetes is characterised by destruction of pancreatic beta cells (Atkinson and Maclaren, 1994). This damage is mediated by cytokine IL-1β (Eizirik and Mandrup-Poulsen, 2001). A recent study suggested that in addition to its role in type 1 diabetes, IL-1β mediated destruction of beta cells, may also lead to type 2 diabetes (Maedler *et al.,* 2002). Mechanistically, IL-1β mediated induction of iNOS by beta cells acts as a messenger for beta cell destruction (Larsen *et al.,* 1998; Corbett and McDaniel, 1994; Thomas *et al.,* 2002). However, in humans, iNOS independent destruction of beta cells has been reported, suggesting that, there could be additional mechanisms involved in beta cell destruction (Delaney *et al.,* 1997; Kutlu *et al.,* 2003; Loweth *et al.,* 1998). In human beta cells, JNK is readily activated by IL-1β exposure thereby promoting cell death (Ammendrup *et al.,* 2000; Larsen *et al.,* 1998). Moreover, downregulation of JNK activation protects beta cells against T-cell mediated killing (Jaeschke *et al.,* 2005) and increased islet cell survival (Aikin *et al.,* 2004; Noguchi *et al.,* 2005). Thus, suggesting a mechanism to inhibit JNK activation could be crucial for survival and function of beta cells after exposure to stress the response.

Exposure of the beta cells to IL-1β signal induces *Gadd45β* expression. Once induced, *Gadd45β* has been shown to inhibit JNK signalling that has been

associated with decreased apoptosis in beta cells (Ammendrup *et al.,* 2000; Aikin *et al.,* 2004; Eckhoff *et al.,* 2003; Bonny *et al.,* 2001; Bonny *et al.,* 2000). However, despite *Gadd45β's* upregulation, beta cells still undergo apoptosis due to the insufficient inhibition of JNK by *Gadd45β*. This could explain the possible reason for beta cell death in response to cytokine-mediated stress (Larsen *et al.,* 2006). The ability of *Gadd45β* to act as an anti or pro-apoptotic factor is dependent on either activation or inhibition of JNK signalling. *Gadd45β* activation of MAPK kinase kinase (MEKK) 4, lead to MKK4 phosphorylation that promote cell death (Takekawa and Saito, 1998). Whereas inhibition of JNK activity by forming *Gadd45β-MKK7* complex leads to cell death inhibition (Papa *et al.,* 2004).

Modulating *Gadd45β's* expression for effective inhibition of JNK by using small molecule peptide could be a possible future therapy. Thus, *Gadd45β* could act as a potential tool in the prevention of diabetes by protecting beta cells against cytokine-mediated cell death.

1.6.2 Role of *Gadd45β* in liver regeneration

Studies using mouse models have shown that *Gadd45β* plays a crucial role in liver regeneration after partial hepatectomy (PH), by blocking JNK mediated hepatocyte cell death (Papa *et al.,* 2008). These observations suggest that *Gadd45β* protects hepatocyte from damage-induced cell death. Analyses of livers after PH suggest that, there were reduced hepatocyte proliferation and increased PCD in *Gadd45β*$^{-/-}$ livers. In contrast, *Gadd45β*$^{+/+}$ livers showed no signs of injury (Papa *et al.,* 2008). The *in vivo* cytoprotective role of *Gadd45β* was shown to be mediated by inhibition of JNK activity by blocking MKK7. This role of *Gadd45β* was confirmed by increased JNK activity in *Gadd45β*$^{-/-}$ livers (Papa *et al.,* 2008). Mechanistically, transcriptional activity of NF-κB was upregulated after PH, which in turn upregulates *Gadd45β* expression that eventually leads to reduced JNK activity by *Gadd45β* binding to MKK7. Indeed, deletion of JNK in *Gadd45β*$^{-/-}$ reverses the effect of *Gadd45β* loss after PH and exhibited reduced hepatocyte death and increased proliferation (Papa *et al.,* 2008).

Increased compensatory hepatocyte growth was the most important pathogenic element in many liver diseases such as, hepatitis, toxic injuries, and HCC (Taub *et al.,* 2004; Wullaert *et al.,* 2007). Therefore, targeting the

Gadd45β-MKK7 interaction provides a very useful device to develop a new therapy for several liver diseases including cancers.

1.6.3 Role of *Gadd45β* in autophagy

Autophagy is a self-degrading and catabolic process that provides building blocks for macromolecular synthesis and maintenance of homoeostasis during starvation (Rabinowitz and White, 2010). This process is regulated by a set of genes known as autophagy-related (Atg) proteins (He and Klionsky, 2009). The products of autophagy responsive genes atg5, tag/6/beclin, and atg7 form double membrane phagophores during starvation that engulf cellular proteins, lipids, and defective organelles capturing them in autophagosome. These autophagosomes were later degraded in lysosomes (Levine and Kroemer, 2008). Thus, autophagy acts as a recycling mechanism within eukaryotic cells (Mizushima *et al.,* 2011). This process of lysosomal degradation helps to prevent accumulation of toxic proteins, fats, and damaged organelles within the cell. This process of lysosomal degradation is essential for cell survival, development, immunity, and tumour suppression in mammals (Mathew *et al.,* 2007a; Levine & Kroemer, 2008; White *et al.,* 2010; Mizushima *et al.,* 2011; Levine *et al.,* 2011). These findings suggest that autophagy could play a major role in the suppression of chronic tissue damage and cancer (Mathew *et al.,* 2009). The role of autophagy gained further support by an observation suggesting that a defect in the autophagy

mechanism causes accumulation of damaged organelles. In particular, mitochondria, that was associated with the production of reactive oxygen species (ROS) (Hara *et al.,* 2006; Komatus *et al.,* 2006; Mathew *et al.,* 2009).

Atg5 plays a key role in autophagy regulation. Mouse embryonic fibroblasts deficient of Atg5 lack autophagy. Mice deficient of Atg5 die postnatally because they are not able to cope with starvation during the neonatal period (Kuma *et al.,* 2004). Suggesting a crucial role for Atg5 in autophagy and also in cell fate. Consistent with previous findings (Takekawa *et al.,* 1998; Lu *et al.,* 2004), when MEFs were expressed with *Gadd45β-MEKK4* complex, p38 was selectively activated and this activation was specific for the *Gadd45β-MEKK4* complex. Microscopic analyses of these MEFs confirmed the co-localisation of activated p38 with an autophagy marker in cytoplasmic vesicles, suggesting a role of activated p38 in autophagy regulation. Indeed, once activated, p38 directly phosphorylate Atg5, a known autophagy regulatory gene. These observations suggest that *Gadd45β-MEKK4*-p38 regulation of autophagy is mediated by phosphorylation of Atg5. In support of these findings, when MEFs from $Gadd45\beta^{-/-}$ and $Gadd45\beta^{+/+}$ mice were stimulated with LPS, as expected, there was more autophagy is $Gadd45\beta^{-/-}$ MEFs compared to $Gadd45\beta^{+/+}$ MEFs (Keil *et al.,* 2013).

Therefore, *Gadd45β* mediated regulation of autophagy may have a physiological role and suggests induction of *Gadd45β* by inflammatory cytokines or LPS could act as a negative regulator of autophagy.

1.6.4 Role of *Gadd45β* in haematopoietic cells

All the Gadd45 family proteins are rapidly induced by terminal differentiation and genotoxic stresses (Forance, 1989; Papathanasios *et al.,* 1991; Zhan *et al.,* 1994). Although, all Gadd45 proteins share an overall similarity at the level of amino acids, their functions in apoptotic pathways are different. Among all the Gadd45 proteins only *Gadd45β* is induced by TGF-β (Selvakumaran *et al.,* 1994 a, b; Kastan *et al.,* 1992; selvakumaran *et al.,* 1994 a, b; Guillouf *et al.,* 1995 a, b). Knowing the exact mechanism by which *Gadd45β* protein regulates cell survival will be advantageous.

In haematopoietic cells, *Gadd45β* is rapidly induced by exposure to stress responses like UV light or chemotherapy (Gupta *et al.,* 2005) and also by haematopoietic cytokines (Gupta *et al.,* 2006). This induction of *Gadd45β* facilitates the haematopoietic cells to survive in a stress environment. Studies using *Gadd45β*$^{-/-}$ BM cells that *Gadd45β*$^{-/-}$ BM cells undergo rapid apoptosis after the exposure to UV radiation, VP-16 and DNR (a well know anti-cancer

drug) (Gupta *et al.,* 2005). The loss of *Gadd45β* significantly reduced the number of mature macrophage and granulocyte after acute stimulation with cytokines like GM-CSF, IL-3, and M-CSF (Gupta *et al.,* 2006) suggesting its role in myeloid differentiation. This decrease in the number of myeloid cells was due to an increase in apoptosis after acute cytokine stimulation, and this was attenuated by re-introduction of *Gadd45β*. In normal conditions, *Gadd45β* does not influence cell cycle profile unless it is induced by stress. Moreover, mice knockdown for *Gadd45β* were born normal without any abnormalities, suggesting its functional quiescence in steady-state haematopoiesis (Hollander *et al.,* 1999; Hoffmeyer *et al.,* 2001; Lu *et al.,* 2004). Mechanistically, after the induction of *Gadd45β* by genotoxic stress or acute cytokine stimulation or inflammatory signal, *Gadd45β* exerts growth arrest in G2/M phase checkpoint allowing repair of damaged DNA and thus facilitating haematopoietic cell survival. In addition to its role in haematopoietic cells survival, *Gadd45β* also plays a crucial role in myeloid cell differentiation (Adbollahi *et al.,* 1991; Zhang *et al.,* 1999).

In haematopoietic cells, *Gadd45β* acts as an anti-apoptotic protein by inhibiting the activation of JNK signalling (Gupta *et al.,* 2005). This role of *Gadd45β* in inhibiting JNK signalling was further confirmed by studies using

Gadd45β$^{-/-}$ BM cells, after exposure to genotoxic stress, there was sustained and prolonged JNK activity. This sensitivity of *Gadd45β*$^{-/-}$ BM cells to genotoxic stress attenuates by using JNK inhibitor SP600215, suggesting the involvement of JNK pathway (Gupta *et al.,* 2005). In general, JNK is activated *via* phosphorylation of it upstream kinases like MKK4 and/or MKK7, (Roux and Blenis, 2004; Zarubin and Han, 2005) but in haematopoietic cells after exposure to UV radiation, *Gadd45β* acts as an anti-apoptotic protein, by binding to MKK4 instead of MKK7.

These studies confirm the role of *Gadd45β* as an anti-apoptotic protein. This gained further support by few recent studies show that *Gadd45β* mediates the protective effect of CD40 co-simulation against Fas- induced apoptosis in B cells (Zazzeroni *et al.,* 2003). However, the pro-apoptotic role of *Gadd45β* is also well documented in different models, for example, in one study it has been shown that *Gadd45β*$^{-/-}$ hepatocytes were more resistance to TGF-β mediated apoptosis (Yoo *et al.,* 2003). Interestingly, although *Gadd45β* $^{-/-}$ BM cells were more sensitive to stress-induced apoptosis, they were more resistance to TGF-β mediated apoptosis (Gupta *et al.,* 2005). Thus, suggesting the possible role of *Gadd45β* as pro-apoptotic or anti-apoptotic gene was dependent on the encountered stress signal, cell type, and its interaction with

other proteins that eventually play a crucial role in deciding cell fate (Gupta *et al*., 2005).

Thus, suggesting the role of *Gadd45β* on the efficacy of certain cancer drugs and the pathogenesis of leukaemia and response of normal and malignant haematopoietic cells to chemotherapy and radiotherapy.

1.6.5 Role of *Gadd45β* in cardiomyocytes

Apoptosis plays a significant role in many pathologies, it also plays a major role in cardiomyocytes death from ischaemic heart disease and acute myocardial infarction (MI) (Anversa *et al.,* 1998). Every year, millions suffer from ischaemic heart diseases that progressively develop into heart failure (Holly *et al.,* 1999). Animal models of ischaemic heart disease were extensively studied to understand the functional, structural, and molecular association of cardiomyocytes. Results from these models further confirmed that there was a casual association between cardiomyocytes death and ischaemic heart disease (Holly *et al.,* 1999). In the light of *Gadd45β's* role in apoptosis, understanding the mechanism of *Gadd45β's* involvement in cardiomyocytes apoptosis may help to develop new therapeutic strategies.

Studies in animal models of MI (Stanton *et al.,* 2000) and cardiomyocytes models of hypoxia (Mohuczy *et al.,* 2002) has found that several genes that are differentially expressed; however, the role played by these genes have not been thoroughly evaluated. Kim *et al.* used DNA Microarray analysis of hearts obtained from *in vivo* transient ischemia model and cardiomyocytes from *in vitro* hypoxia model to evaluate the unknown role of ischemia/hypoxia-responsive genes. It was shown that *Gadd45β* was upregulated by two fold (Kim *et al.,* 2010). Considering the fact that all phases of ischaemic heart disease were associated with the apoptosis of cardiomyocytes (Abbate *et al.,* 2006; Takemura and Fujiwara, 2004), a possible relation between *Gadd45β* upregulation and increased apoptosis in cardiomyocytes was expected. Indeed, downregulation of *Gadd45β* in cardiomyocytes remarkably reduces ischemia-induced apoptosis in cardiomyocytes (Kim *et al.,* 2010) and blocking *Gadd45β* using their respective siRNAs, dramatically reduced the cardiomyocytes death. Thus confirming the role of *Gadd45β* in ischemia/hypoxia-induced apoptotic cell death. Under normoxic conditions, however, increasing the levels of *Gadd45β* expression had no effect. Only in hypoxia, *Gadd45β* can further worsen the apoptosis of cardiomyocytes. Thus, suggesting *Gadd45β* as an essential component that play a significant role in the apoptosis of cardiomyocytes only in response to hypoxia.

This pro-apoptotic role of *Gadd45β* was further supported in murine hepatocytes where *Gadd45β* plays a pro-apoptotic role that was induced by TGF-β activation (Yoo *et al.,* 2003). In addition to that *Gadd45β* overexpression in ML-1 and HeLa cells induces apoptosis (TakeKawa *et al.,* 1998). However, it is worth noting the cell-type specific role of *Gadd45β*, because overexpression of *Gadd45β* in other cell types like NIH3T3 and INS-1Eβ suppresses apoptosis (Laresn *et al.,* 2006; Engelmann *et al*., 2008). Further studies are necessary to understand the precise mechanism by which *Gadd45β* promotes cardiomyocytes cell death, and these findings may eventually lead to the development of new therapeutic strategies that could target *Gadd45β.*

Collectively, owing to the above-mentioned roles of *Gadd45β* in various pathologies, understanding the mechanisms underlying the roles of *Gadd45β* and NF-κB in tumourigenesis has a fundamental therapeutic significance.

1.7 Macrophage

Macrophages are a plastic cell lineage that acquires functionally distinct phenotypes depending on both the pathological and physiological context (Martinez *et al.,* 2009). Elie Metchinkoff in 1882 was first to describe the large phagocytic cells as macrophages based on phylogenetic studies. He was also the first to describe the presence of macrophages in all vertebrates and invertebrates (Heifets, 1982), for which he received Nobel Prize in 1908. Further studies in 1931 by making an ear chamber in rabbits confirmed that macrophages infiltrate into the inflammatory region. These macrophages were derived from monocytes that were circulating in the blood stream. In normal steady state, macrophages are differentiated from bone marrow-derived monocytes. Recent studies have suggested that tissue resident macrophages develop independent of haematopoietic stem cells (Schulz *et al*., 2012). These macrophages are shown to be derived from the yolk sac, and these cells are genetically distinct from HSC progenitors (Schulz *et al*., 2012). Suggesting that there exist two lineage of macrophage origin; (1) cells that are derived from yolk sac, that give rise to F4/80^{high} cells (2) cells that are derived from bone marrow give rise to F4/80^{low} and CD11b^{high} cells. Macrophages residing in different tissues were given different names; histiocytes in connective tissue, Kupffer cells in liver, alveolar macrophages in lung, free and fixed macrophages in lymph node and spleen, osteoclast in bone, microglia in brain, and Langerhans cells in skin (Davies *et al*., 2013).

The timeline advances in the macrophage polarisation research is shown below (Figure 6) (Liu *et al.,* 2013).

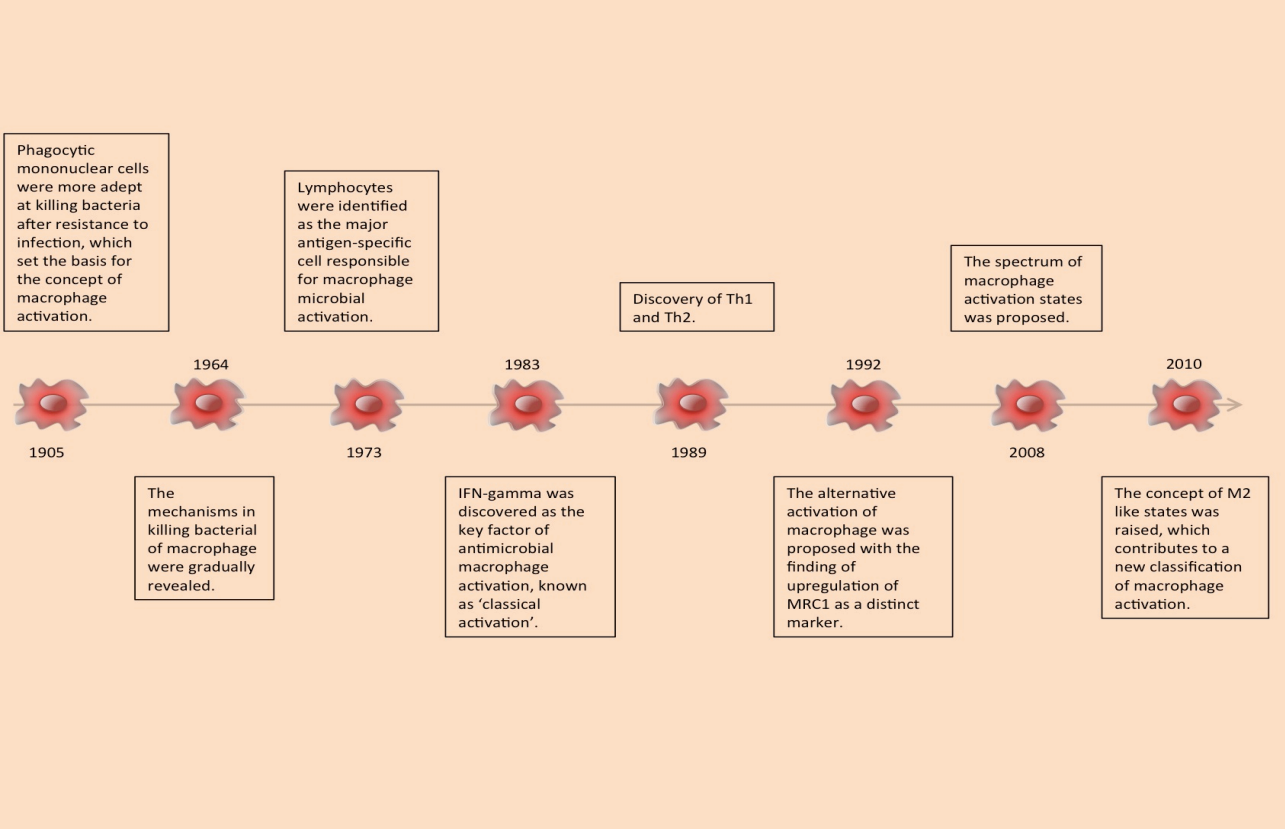


Figure 6. Timeline: Advancement in macrophage polarisation research. (Modified from Liu *et al.,* 2013).

1.7.1 Macrophage activation

Inflammation, infection and trauma results in the disruption of tissue homoeostasis that may eventually lead to macrophage activation, suggesting that these cells are essential for host defence. Macrophage activation was initially thought to destroy microbial pathogens and harmful antigens followed by inactivation. However, recent studies have shown that macrophages once activated may polarise to a distinct phenotype depending on their function and the cytokine milieu (Figure 7). Macrophages were broadly classified into two distinct populations. M1 state of the macrophage is acquired by exposing to high level of T_H1 cytokines such as tumour necrosis factor (TNF), IL-12, LPS, and interferon (IFNs). Macrophage polarised to M1 state release reactive oxygen species, nitrogen intermediates, and proinflammatory cytokines IL-1β, IL-6, IL-12, IL-23, and TNF that exerts a cytotoxic effect on cancer cells. Whereas, macrophages exposed to T_H2 cytokines IL-4, IL-10, IL-13 and transforming growth factor β (TGF- β) polarise macrophage towards M2 phenotype. These cells release several growth factors like VEFG, EGF, FGF, IL-10, and arginase that promote tumour growth and a nutritional advantage to cancer cells. M2 macrophages also express high levels of Ym1, Fizz1, IL-10, and CD206, which are involved in tumour progression. (Lewis and Pollard, 2006; Kosaka *et al.,* 1991; Mantovani *et al.,* 2009; Allavena *et al.,* 2008; Lic *et al.,* 2014). M1 and M2

macrophages express distinct metabolic programmes. M1 macrophage release nitrogen intermediates that inhibit cancer cell division, on the other hand, an increased arginase production by M2 macrophage stimulates cell division (Mills *et al.,* 2000). Unlike T_H1 and T_H2 cells, both M1 and M2 macrophage phenotype can be converted into each other depending on the microenvironment (Wynn *et al.,* 2013). The phenotype of the macrophage in several inflammatory diseases was driven by the interaction of several transcription factors such as STATs, IRFs, NF-κB, AP-1, PPAR-ϒ and CREB (Lawrence and Natoli, 2011; Ohmori *et al.,* 1997; Satoh *et al.,* 2010; Krausgruber *et al.,* 2011; Oeckinghaus *et al.,* 2011; Schonthaler *et al.,* 2011; Odegaard *et al.,* 2007; Bouhlel *et al.,* 2007; Ruffell *et al.,* 2009).

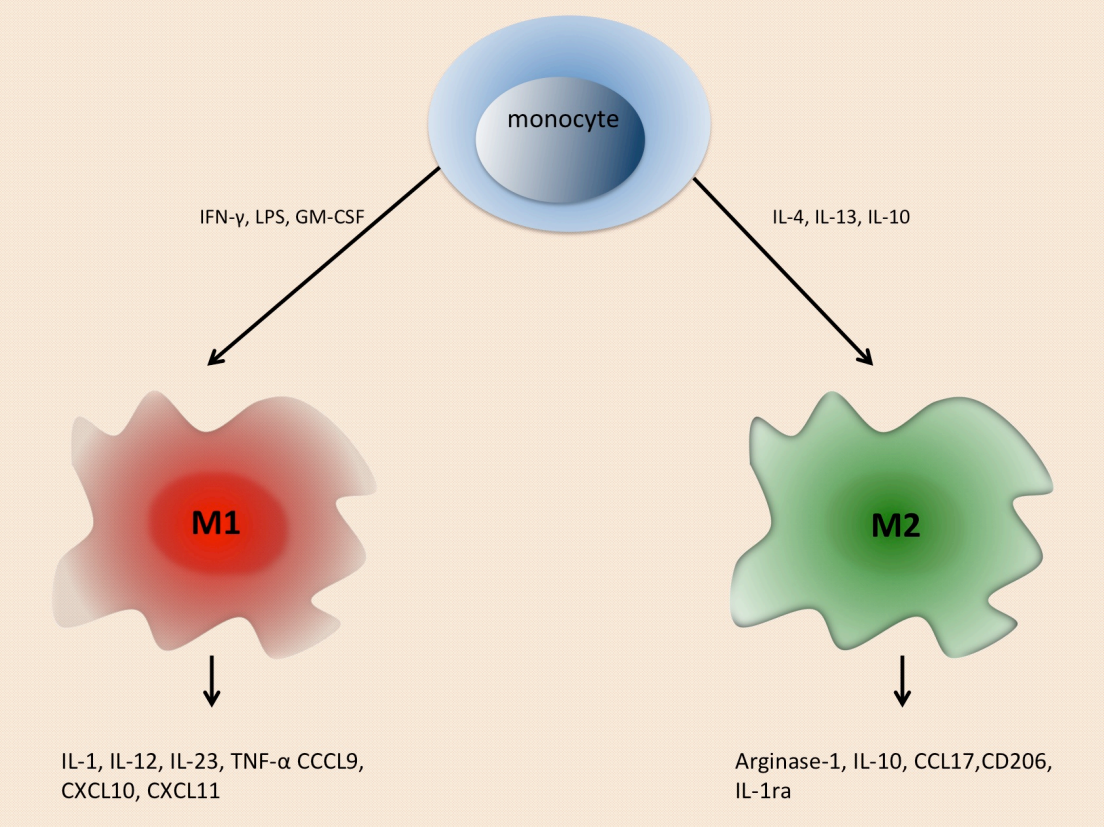


Figure 7. Monocyte differentiation into mature macrophage depends on cytokine milieu. In the presence GM-CSF, LPS and IFN-γ monocyte differentiate into M1 macrophage, M1 cells have anti-microbial activity and cytotoxic to tumour cells. In the presence of IL-4, IL-10 and IL-13 monocytes differentiate into M2 macrophages that favor tumour growth and angiogenesis.

1.7.2 Macrophages in cancer

Macrophages are abundant in all tumours (Sica and Mantovani, 2012). Originally macrophages were thought to be an anti-tumour and proinflammatory cells. However, clinical data has shown that in almost all cancers macrophages are involved in tumour initiation, progression, and metastasis (Wynn *et al.,* 2013). During the chronic inflammation stage, macrophages synthesise copious amount of proinflammatory cytokines that sustain the inflammatory reaction in other immune cell compartments thereby relating chronic inflammation to tumour initiation. The tumour inducing activity by macrophages involves various mechanisms. For examples, skin cancers are induced by IFN-gamma induced by UV exposure. TNF-α causes several other cancers through the production of mutagenic environment, collectively promoting tumour initiation (Zaidi *et al.,* 2011). Once tumours are established, macrophages change their phenotype to pro-tumour phenotype the so-called TAM that has been shown to promote tumour progression, tumour cell migration, and invasion.

1.7.3 Tumour Associated Macrophages (TAM)

TAMs are the monocyte precursors that circulate in the blood (Mantovani *et al.,* 1992) and a major immune cell component that infiltrates into the tumour stroma (Mantovani *et al.,* 2005). Once recruited into the tumour, they

promote tumour growth by releasing cytokines and chemokines that enhance the tumour development (Lewis and Pollard, 2006; Allavena *et al.,* 2008; Sica, 2010). Thus, macrophages act as one of the most important biological markers (Jiang *et al.,* 2012). Suggesting that increased infiltration of macrophages corresponds to poor prognosis and decreased cancer patient survival. (Grugan *et al.,* 2012). The tumour-promoting role of TAM is through lowering its antigen presentation, failing to activate T cell-mediated adaptive immunity (Allavena *et al.,* 2008; Solinas *et al.,* 2009) and also they fail to express proinflammatory cytokines. Many studies in both humans and animal tumour models revealed that majority of the TAMs resemble M2 like macrophages, unlike M1 macrophages that are present in area of acute inflammation (Lewis and Pollard, 2006; Biswas *et al.,* 2006). Recruitment of TAM to the tumour site is influenced by chemotactic factors like CCL2, VEGF, M-CSF, and CXCL12 and also by the secretion of growth factors and tumour derived chemokines (Mantovani *et al.,* 2008; Pollard, 2004).

CCL2/MCP-1 is one of the major chemotactic factors that play a crucial role in the recruitment of TAM (Bottazzi *et al.,* 1983). Accumulation of macrophages at the tumour sites by CCL2/MCP-1 was observed in several types of tumours including breast, melanoma, ovarian, prostate, and pancreatic cancers. (Gazzaniga *et al*., 2007; Monti *et al.,* 2003; Negus *et al.,* 1995; Nesbit *et al.,* 2001; Zhang *et al*., 2010). Recent studies have also shown

that CCL5/RANTES, CXCL1/Gro-alpha, CCL7/MCP-3 and CCL8/MCP-2 also play an important role in the macrophage infiltration. (Balkwill, 2004; Balkwill, 2012; Mantovani *et al.,* 2004). In normal conditions macrophage act as immune regulatory factors, that inhibits tumour initiation and progression. However, when influenced by the tumour signals, macrophages release anti-inflammatory factors, tumour growth and angiogenesis promoting factors and extracellular matrix (ECM) regulatory factors. Altogether, these factors contribute to the inhibition of anti-tumour immune reaction thereby promoting tumour angiogenesis and metastasis (Seki *et al.,* 2000; Espinosa *et al.,* 2010; Soeda *et al.,* 2008; Qian and Pollard, 2010).

TAMs isolated from mouse, and human tumours are broadly characterised as M2 like phenotype (Biswas and Mantovani, 2010). However, it is worth noting that in few mouse and human tumours (Breast, ovarian and renal cell carcinoma, etc.) TAMs express high levels of TNF-α, IL-1β, IL-23p16, IL-6 and CXCL8 that are typical M1 macrophage markers (Langowski *et al.,* 2006; Balkwill, 2004). Suggesting, TAM response may depend on tumour type, stage, and the local signals within the tumour microenvironment. Recent studies have shown that during the inflammatory response bone marrow-derived macrophage replaces tissue resident macrophage and react to a variety of stimuli within the tissue microenvironment. These observations

suggest that TAMs are recruited into the tumours from local circulating blood monocytes, rather than the resident macrophages present within the non-tumour tissue. (Mantovani *et al.,* 2002).

1.7.4 Strategies to target TAMs

TAMs act as a determinant factor to predict the overall survival of various cancer patients (Steidl *et al.,* 2010; Sica *et al.,* 2008) based on the their number, activation, and location of the TAMs within the tumour. Thus, making TAMs as a promising target for tumour therapy and inhibition of TAM tumour promoting role has become a major area of study (Weigert *et al.,* 2009). Following are the anti-tumour approaches that are under development to target TAMs.

(1) Inhibiting macrophage recruitment.

(2) Suppressing TAMs survival.

(3) Enhancing tumouricidal activity of TAMs.

(4) Blocking tumour promoting activity of TAMs.

1.7.4.1 Inhibiting macrophage recruitment

Cytokines and chemokines released by the tumour stroma have been shown to facilitate the recruitment of macrophages and other immune cells, into the tumour. Therefore, targeting macrophage recruitment has become a promising approach for tumour therapy. It has been shown that, overexpression of C-C motif chemokine ligand 2 (CCL2) increases macrophage infiltration that is associated with poor prognosis in human cancers (Roca *et al.,* 2009; Soria and Ben-Baruch, 2008; Zhang *et al.,* 2010). Thus, supporting the role of CCL2 mediated macrophage recruitment into tumours. Similarly, inhibition of CCL2 lead to reduced tumour growth because of the reduced macrophage infiltration (Popivanova *et al.,* 2009; Gazzaniga *et al.,* 2007; Mizutani *et al.,* 2009; Quian *et al*., 2011; Zhu *et al.,* 2010). Pharmacological inhibition of CCL2 by Bindarit has shown some encouraging results by reducing macrophage infiltration, tumour growth, and reduced necrotic tumour mass in melanoma xenograft (Gazzaniga *et al.,* 2007). Trabectedin, a CCL2 targeting agent, which is in clinics for the treatment of human ovarian cancer (Allavena *et al.,* 2005) and myxoid liposarcoma (Germano *et al.,* 2011), contributes to trabectedin induced tumour rejection. This association of CCL2 in macrophage recruitment was further supported by phase II clinical studies, pharmacological inhibitor of CCL2 receptor (CCR2) has been shown to have an effect on macrophage migration (Jin *et al.,* 2010) and humanized monoclonal antibodies (CNT0888

and MLN1202) that specifically target CCL2/CCR2 are under clinical investigation.

M-CSF is another important chemoattractant that facilitates macrophage recruitment. Evidence for M-CSF role in macrophage recruitment was observed in human HCC patients with elevated levels of M-CSF. These patients have high macrophage density in their tumour that is eventually leading to reduce survival (Zhu *et al.,* 2008). This property of M-CSF was further supported by studies from various labs using the mouse model of pancreatic neuroendocrine tumours. In this model, M-CSF deletion reduced macrophage infiltration into the tumour at all the stages of tumour development. (Pyonteck *et al.,* 2011). Further studies, using MCF-7 breast cancer xenograft models has shown that tumour growth was reduced to 40% by using M-CSF blocking antibody (Paulus *et al.,* 2006). Similar results were also obtained by using M-CSF receptor inhibitors (JNJ-28312141 and GW2580) (Manthey *et al.,* 2009; Kubota *et al.,* 2009). These observations place M-CSF as a good target that has the potential to inhibit macrophage recruitment without affecting healthy vascular and lymphatic system outside the tumour site (Kubota *et al.,* 2009). There are several M-CSF/M-CSFR inhibitors that are still under phase I clinical trials.

Several other chemoattractants such as VEGF, CXCL2, and CCL5 also plays an important role in macrophage recruitment. Blocking these chemoattractants has a potential to reduce macrophage density. Furthermore, in orthotropic pancreatic and breast cancer models, blocking VEGFR-2 reduced macrophage density, tumour growth, and angiogenesis (Dineen *et al.,* 2008; Roland *et al.,* 2009). The microenvironment within the tumour is hypoxic, and hypoxia induces hypoxia- inducible factors (HIFs) that activate VEGF and CXCR4 (Fang *et al.,* 2009). In HIF-1α knockout mice, macrophage density and tumour angiogenesis were reduced via blocking matrix metalloproteinase (MMP)-9/VEGF pathway (Du *et al.,* 2008). These observations support HIF's role in macrophage recruitment.

Altogether, inhibiting macrophage infiltration by using CCL-2, M-CSF and HIF inhibitors could act as a potential anti-tumour candidate, not only for its role in the macrophage recruitment but also in reduced angiogenesis. However, recent studies suggest that an interruption to these inhibitor therapies lead to enhanced tumour growth and metastasis, thereby reducing the patient survival (Bonapace *et al.,* 2014). These studies highlight that caution should be taken while considering macrophage infiltration inhibitors as a therapy.

1.7.4.2 Suppressing TAMs survival

Inducing apoptosis specifically in tumour-promoting TAMs is another important approach. Two ways have been reported so far; one is through the induction of apoptosis in the macrophages by chemical reagents, immune conjugated mAbs or attenuated bacteria, and the second strategy is by triggering immune cells to recognise TAM.

Bisphosphonates like clodronate and zoledronic acid that were packed in the liposomes has shown to specifically deplete macrophages (Rogers and Holen, 2011) that lead to a reduced tumour growth, angiogenesis, and metastasis (Zeisberger *et al.,* 2006; Hiraoka *et al.,* 2008; Miselis *et al.,* 2008). Zoledronic acid, a bisphosphonate specifically used in breast cancer patients for cancer therapy has shown to selectively depletes MMP-9 expressing macrophages (Giraudo *et al.,* 2004; Tsagozis *et al.,* 2008). In addition to depleting macrophages, zoledronic acid impairs monocyte differentiation into TAM and also induces tumouricidal activity in macrophages (Tsagozis *et al.,* 2008; Veltman *et al.,* 2010; Coscia *et al.,* 2010; Green and Guenther, 2011) eventually prolonging patient survival (Gnant *et al.,* 2009; Eidtmann *et al.,* 2010; Coleman *et al.,* 2011). In addition to clodronate and zoledronic acid, other bisphosphonates have been studied for their potential to target TAMs (Takahashi *et al.,* 2009). For example, Dasatinib, a pre-clinical drug used in

chronic phase of myeloid leukaemia (Kantarjian *et al.,* 2010) has been reported to induce apoptosis in MMP9 expressing macrophage.

Targeting surface molecules of TAM by using immunotoxin- conjugated agent is an alternative approach used in cancer therapy. Targeting TAM surface proteins such as, scavenger receptor–A by using the immunotoxin-conjugated mAbs is currently studied in patients with ovarian cancer (Pulaski *et al.,* 2009; Bak *et al.,* 2007). Folate receptor beta (FRβ) is another TAMs surface marker associated with high vessel density, high incidence of haematogenous, and poor prognosis in pancreatic patients (Kurahara *et al.,* 2012). Due to its significant tumour promoting ability, targeting FRβ could be a further approach (Nagai *et al.,* 2009). Infecting macrophages with a bacteria *Shigella flexneri* has also been reported to induce apoptosis in macrophages. Single dose of an attenuated strain of *Shigella flexneri* resulted in the reduction of tumour size in few mouse tumour models (Suzuki *et al.,* 2007; Galmbacher *et al.,* 2010). Several other bacterial strains like *S.typhimurium, Listeria monocytogens, Chlamydia pisttaci*, and *Legionella pneumophila* are under investigation. These bacterial strains primarily infect macrophages and could be a potential target for TAM (Weigert *et al.,* 2009).

1.7.4.2.1 T Cell-mediated macrophage depletion

The alternative way to target TAMs is by upregulating macrophage surface markers that enable them to be recognised by cytotoxic T lymphocytes. Lysosomal proteases like legumain is highly expressed in human tumours and overexpressed specifically in TAMs (Luo *et al.,* 2006). When tumour-baring mice were treated with a novel legumain-based DNA vaccine that specifically depletes legumain-expressing TAMs (Luo *et al.,* 2006; Lewen *et al.,* 2008) there was reduced tumour growth, macrophage density, pro-angiogenesis factors, angiogenesis, and metastasis (Luo *et al.,* 2006; Lewen *et al.,* 2008). This vaccine-based approach only target legumain expressing TAMs leaving cytotoxic M1 macrophages unaffected, because M1 macrophages do not express legumain.

1.7.4.3 Enhancing tumouricidal activity of TAMs

Macrophages are highly plastic cells, and their polarisation state is largely dependent on the local cytokine milieu. Although TAMs has an immune suppressive phenotype, they still show plasticity to polarisation (Biswas, 2010). Thus, cancer acts as a paradigm for macrophage diversity and plasticity (Biswas, 2008; Lewis and Polalrd, 2006). The plasticity of macrophage could be a potential target for cancer therapy by re-programming immune suppressive and tumour promoting TAM to a

tumouricidal M1 type macrophage (Biswas, 2010). Macrophages in human and mouse tumours have M2 like phenotype that expresses low levels of proinflammatory cytokine and high levels of anti-inflammatory cytokine (Biswas *et al.,* 2006; Hagemann *et al.,* 2008; Loges *et al.,* 2010). However, macrophages isolated from mammary tumour model are neither M1 nor M2 phenotype although they produce fewer proinflammatory cytokines (Torroella-Kouri *et al.,* 2009). The transcriptional profile of macrophages is dependent on the activation state of the NF-κB transcription factor. In particular STAT1, STAT3, and STAT6 has been shown to transmit the polarising signal to the nucleus and have a distinct role in macrophage polarisation (Sica and Bronte, 2007; Yoshimura, 2006). The expression of proinflammatory cytokines by M1 macrophage is driven by transcription factors STAT1 and NF-κB. On the other hand, tumour promoting and immune suppression status of M2 macrophage is driven by STAT3 and STAT6 transcription factors (Weigert *et al.,* 2009). Hence, by manipulating the inducers and the gene targets of these transcription factors could act as preferential targets for cancer therapy.

Interestingly, STAT1 pathway can reverse the immune suppressive TAMs to tumouricidal M1 macrophages (Sica and Bronte, 2007). IFN a natural agonist to STAT1 was approved by FDA to treat cancer patients, hairy-cell leukaemia and AIDS-related Kaposi's sarcoma (Dunn *et al.,* 2006). Specifically, IFN-α/-β

plays an important role in the inhibition of tumour growth by targeting haematopoietic cells rather than tumour cells (Dunn *et al.,* 2009). However, in human cancers IFN-γ plays an important role in reversing the TAM phenotype (Duluc *et al.,* 2009) by triggering STAT1 as wells as the genes encoding for proinflammatory cytokines such as IL-12, nitric oxide synthase 2 (NOS2) and CXCL-10 in TAMs (Mancino and Lawrence, 2010). From the above observation, it makes favourable conclusion that targeting IFNs may contribute to TAM-re-education. Thus, a better understanding of the regulation of STAT1 is essential before they could be used for cancer therapy.

The crucial role of NF-κB in TAM activation was confirmed by an observation that several tumour promoting gene such as VEGF, IL-6, and TNF-α have been shown to be regulated by NF-κB transcription factor (Hagemann *et al.,* 2009; Karin and Greten, 2005). It has been reported that TAMs are reprogrammed to tumouricidal macrophage M1 phenotype after attenuated NF-κB activity in mouse ovarian cancer (Hagemann *et al.,* 2008). In support of these studies, in mouse model of lewis lung carcinoma TNF-α and other NF-κB activating cytokines promote metastasis and also protect tumour cell and TAMs from apoptosis (Lo *et al.,* 2011; Kim *et al.,* 2009; Perkins, 2012). In some experimental models tumour promoting transcription factor, HIF-1α has shown to be activated by NF-κB (Rius *et al.,* 2008). However, signalling

pathways that promote TAM re-education are yet to be identified to use TAM re-polarisation as cancer therapy.

1.7.4.4 Blocking tumour promoting activity of TAMs

Blocking the essential signals for the differentiation of monocytes to TAM is another strategy that is still under development. Transcription factors STAT3 and STAT6 drives the phenotype of M2 macrophage by targeting the transcription of M2 specific genes like ARG1/2, SOCS3 and SHIP, eventually leads to NF-κB inhibition. Many tumours express high level of STAT3 and have a negative regulatory effect on macrophage activation and inflammatory responses (Lin and Bost, 2004). Blocking STAT3 activation with a dominant negative variant or an antisense oligonucleotide reverse systemic immune responses causing an increased IL-12 and RANTES expression by macrophages (Cheng *et al.,* 2003). WP1066 a small molecular inhibitor of STAT3 was found to reverse immune tolerance in malignant glioma patients (Hussain *et al.,* 2007). Sunitinib and sorafenib a tyrosine kinase inhibitors inhibit STAT3 in macrophages. Sorafenib was shown to restore IL-12 production by suppressing IL-10 expression in prostaglandin E2 macrophages and reversed the immunosuppressive profile of TAMs (Edwards and Emens, 2010). Corosolic acid and oleanolic acid are two new inhibitors of M2 macrophage differentiation by suppressing the activation of STAT3 (Fujiwara *et al.,* 2011a; Fujiwara *et al.,* 2011b). Other STAT3 inhibitors

such as STA-21, IS3 295 and S31-M2001 has been shown to work against tumours. STAT6 is another important transcription factor that maintain M2 macrophage phenotype. Furthermore, in tumour metastasis model, STAT6 deletion led to more than 60% tumour rejection (Sinha *et al.,* 2005). However, the mechanisms of TAM repolarisation by STAT inhibitors are still not known (Mankan and Greten, 2011).

Collectively, by considering the above conflict of findings. Further understanding is essential to apply NF-κB mediators or target genes in re-educating tumour promoting macrophages to tumouricidal macrophages. Furthermore, the NF-κB downstream target genes involved in re-educating tumour enhancing macrophage to anti-tumour macrophage are still unknown. Finally, a further biological understanding of targeting TAMs would help to find a better future therapy.

1.8 Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the third largest cause of cancer death and the fifth most common cancer (El-Serag and Rudolph, 2007). WHO had estimated that in 2008, there were 700,000 deaths worldwide because of liver cancer (Bakiri and Wagner, 2013). The incidence rate, aetiologies, and background of liver disease vary with geographical location, East Asia and Africa with high rates of incidence, Europe and North/South America with

modest rates (Maeda *et al.,* 2005). However, recent evidence has indicated an increased incidence in Europe and North America (Masuzaki *et al.,* 2008; Masuzaki and Omata, 2008). However, hepatitis B virus (HBV) or hepatitis C virus infections are seemingly the major causes for HCC. Cirrhosis associated with chronic inflammation, dietary exposure to fungal contaminant aflatoxin B1, environmental pollutants such as aromatic amines, vinyl chloride, polycyclic aromatic hydrocarbons and nitrosamines (Bosch *et al.,* 2004), alcoholic hepatitis, hemochromatosis and non-alcoholic steatohepatitis (NASH) are considered as the major HCC risk factors. However, the casual relationship between HCC risk factors and the carcinogenic mechanisms has not been clearly elucidated either in human (Thorgeirsson *et al.,* 2002) or animal models (Sell *et al*., 1982).

Accumulated evidence from the genetically manipulated mice has indicated that many genes are associated with HCC and they all fall into two different categories: 1) those that augment spontaneous HCC 2) those that increase susceptibility to chemically induced HCC. In few mouse models, HCC develops spontaneously with overexpression of HBV surface antigen, hepatomitogen, transforming growth factor-α (TGF-α) and the c-myc proto-oncogene (Fausto, 1999). In few other models augmented HCC in mice deficient in type II TGFβ receptor (Im *et al.,* 2001) and Mdr2 P-glycoprotein transporter (Mauad *et al.,* 1994). The majority of these mouse strains have displayed an

enhanced susceptibility to the chemically induced carcinogenesis, as evidenced in IKKβ and JNK1 ablation in hepatocytes, but the underlying molecular and cellular mechanisms were poorly understood. However, recent studies have indicated that a sustained inflammatory reaction in the liver is a major contributing factor for the development of HCC. For example, during chronic hepatitis C infection, the host immune response to HCV is not strong enough to completely clear the infection, resulting in the chronic stimulation of an antigen-specific immune response (Budhu *et al.,* 2006; Budhu and Wang, 2006). These continued expression of cytokines and subsequent recruitment of activated inflammatory cells into the liver results in liver damage, which eventually leads to compensatory hepatocyte proliferation. The persistent cycles of hepatocyte damage, inflammation and hepatocyte proliferation provides a mitogenic and mutagenic environment that could result in the development of HCC (Elsharkawy and Mann, 2007).

The current treatment options for HCC include surgical resection, percutaneous ablation, and liver transplantation. Liver transplantation has shown to be a better alternative; however, this has a major drawback because of the limited organ donors (Mazzaferro *et al.,* 1996). The alternative treatment option would be surgical removal of the tumour. However, the long-term prognosis has not been satisfactory because of the frequent recurrence and also because of lack of effective treatment for advanced HCC.

Recent advances in antiviral treatment and molecular targeted therapy are also not satisfactory.

Thus, gaining further understanding of the molecular mechanisms underlying the process of carcinogenesis is important to discover new molecular targets that prevent occurrence and recurrence of HCC. As well as treatment options for the patients with even an advanced stage of HCC.

2: Aims and Objectives

Aims and Objectives

NF-κB is a master coordinator of immunity, inflammation and differentiation. It also controls cell survival (Hayden and Ghosh, 2004; Claudio *et al*., 2006; Papa *et al.,* 2006). The activation of NF-κB antagonises PCD induced by TNF-α and other cytokines. The inappropriate protective activity of NF-κB against PCD is a key pathogenic element prevalent in human diseases such as inflammation and cancer (Edinger and Thompson, 2004; Karin, 2006; Papa *et al*., 2006). Inhibition of apoptosis is perhaps the most obvious way through which NF-κB signalling promotes the development of cancer (Van Antwerp *et al*., 1996). Numerous NF-κB target genes such as *Gadd45β* (De Samele *et al*., 2001), ferritin heavy chain (Pham *et al.,* 2004), Bcl-xL, IAPs (inhibitors of apoptosis), and cFLIP, prevent apoptosis – a normal mechanism by which immune and genomic surveillance mechanisms can remove premalignant or malignant cells (Karin, 2006). Downstream of TNF-Rs, the protective activity of NF-κB involves suppression of c-Jun N-terminal kinase (JNK) cascade. Prior work carried out in our laboratory utilising an unbiased screen identified *Gadd45β* as a pivotal mediator for the suppression of JNK cascade. *Gadd45β* has been shown to be required for liver regeneration post partial hepatectomy (Papa *et al.,* 2008). The precise mechanism by which *Gadd45β* regulates hepatic function during this process and the relevance of this regulation to NF-κB dependent tumourigenesis, however are not known. In order to elucidate these mechanisms we propose to examine:

(1) The role of *Gadd45β* in the NF-κB mediated link between inflammation and cancer.

(2) The role of *Gadd45β* in tumour initiation and tumour progression.

(3) The cell type specific role of *Gadd45β* within the tumours.

Understanding the basis of NF-κB mediated link between inflammation and cancer will not only provide valuable insight into the mechanisms of tumourigenesis but also helps to identify new targets for anti-cancer therapy.

3: Materials and Methods

3.1 Solutions and Buffers

Blocking buffer	1x TBS 5% BSA (w/v)
FACS buffer	1x PBS 5% FBS (v/v)
Freezing medium	90% FBS (v/v) 10% DMSO (v/v)
TAE buffer (10x)	400 mM Tris/HCl 200 mM Acetic Acid 10 mM EDTA
Transfer buffer	192 mM Glycine 25 mM Trizma Base

	0.01 % EDTA (w/v)
	20 % Methanol (v/v)
Wash Buffer	1x TBS
	0.05 % Tween-20 (v/v)
PBST	1x PBS
	0.05 % Tween-20 (v/v)

Working Solution: - Lithium Carbonate 1.36%

Lithium Carbonate, 47g

dH_2O, 3500 ml

Working Solution: - 0.25% Acid Alcohol

95% Ethanol, 2578 ml

dH_2O, 950ml

HCL, 9ml

Primary antibody	Catalogue number	Application	Source
anti- p38	8690	WB	Cell Signalling
anti- p-p38	9215	WB	Cell Signalling
anti- JNK	9258	WB	Cell Signalling
anti- p-JNK	9251	WB	Cell Signalling
anti- ERK	4695	WB	Cell Signalling
anti- p-ERK	4377	WB	Cell Signalling
anti- F4/80	Ab6640	IF, IHC	Abcam
CD45.1 FITC	11-0453	IF, FACS	e-Bioscience
CD45.2 FITC	11-0454	IF	e-Bioscience
Cd45.2 APC	17-0454	FACS	e-Bioscience

Kit	Source
DAB reagent kit	DAKO
Vector ABC kit	Vector lab
BrdU kit	BD Pharmigen
TUNEL assay kit	Abdserotec

Secondary antibody	Catalogue number	Application	Source
HRP-labelled anti-Rabbit	NA9340V	WB	GE health-care
Biotinulated anti-Rat	E 0468	IHC	DAKO
Texas red Goat anti-Rat	T-6392	IF	Invitrogen Life technologies

Instruments	Source
Flow Cytometer	Dako CyAn
Microscope Axiovert	Zeiss
Digital Caliper	VWR
Irrdiator	IBL 637
Microscope	Zeiss
Nano Drop Spectrophotometer ND-1000	NanoDrop Technologies
Incubator	Fisher scientific
Electrophoresis chamber	Biorad
Electrophoresis power pack	Fisher scientific
Hyper processor X-Ray film developer	Amersham Bioscience
SpectraMax-MII	Molecular Devices
Heat blocks	VWR
Centrifuge	Sorvall
Balance	Fisher scientific
Freezers	Eppendorf
Humidified chamber	Sigma-Aldrich
70u Cell strainers	Becton Dickinson
19 gauge needle	Becton Dickinson

25 gauge needle	Becton Dickinson
27 gauge needle	Becton Dickinson
Nito-cellulose membrane	GE healthcare
X-Ray films	Sigma-Aldrich
Sterile pestle	Sigma-Aldrich

Other important reagents	Source
Diethylenitrosoamine (DEN)	Sigma-Aldrich
Phenobarbital	Sigma-Aldrich
PFA	Sigma-Aldrich
OCT	VWR
Acrylamide	Biorad
Bradford reagent	Biorad
Protein ladder	Biorad
Clodronate liposome	Clodronateliposme foundation
Rabbit serum	Monosan
FBS	Sigma-Aldrich
Haematoxylin	Sigma-Aldrich
Eosin	Sigma-Aldrich
PBS	Invitrogen

3.2 Methods

3.2.1 Mice

All the mice that were used for bone marrow adoptive transfer experiments, tumour allograft model and *in vitro* bone marrow-derived macrophage studies were maintained under pathogen-free facility located at Hammersmith campus, Imperial College London. *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ were on C57BL/6 (CD45.2^{+}) background and were bred in-house. For adoptive transfer experiment, all the bone marrow donor mice are CD45.1 positive. Mice procedures were performed in accordance with the Home Office regulations. At the end of the experiment, all the mice were sacrificed by asphyxiation in $C0_2$ chamber.

3.2.2 Tumour induction

Two weeks old male *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ (C57BL/6) mice were challenged with a single dose of DEN (Sigma-Aldrich, UK) 25 mg/kg by intraperitoneal injection (*i.p*) using 25 G needles (BD Falcon). Mouse serum and livers were analysed ten months after the DEN injection.

3.2.3 Tumour analysis

DEN injected mice were sacrificed, and mice livers and lungs were removed and separated into individual lobes. Externally visible tumours (≥0.5 mm) were counted, tumours size was measured by using a digital caliper and tumour-occupied area was calculated. Remaining lobes were microdissected into tumour and non-tumour tissue and stored at −80°C for further analysis or fixed in 4% PFA (Sigma-Aldrich; UK) for histological examination. Liver images were captured using a canon digital camera.

3.2.4 Hematoxylin and eosin stain

Livers from HCC bearing *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ (C57BL/6) mice were removed, washed with PBS, fixed in 4% PFA at 4°C overnight and then paraffin-embedded. Five-micron thick sections were cut through the complete block and stained with hematoxylin and eosin stain (H&E) by using a standard protocol. Briefly, Paraffin sections were de-waxed in xylene (Sigma-Aldrich, UK) three times for ten minutes each. Sections were rehydrated three times for ten

minutes by using different concentrations of ethanol (100%, 95% and 75%) (Thermo Fisher Scientific, Inc). Slides were rinsed in dH_20 for three times. Sections were then stained with hematoxylin (Sigma-Aldrich, UK) for about five minutes and rinse them with tap water for about 15 minutes. De-colourise the sections in acid alcohol for about five seconds and rinsed with tap water for five minutes. Sections were then immersed in lithium carbonate for five seconds and rinsed with tap water for five minutes. Counterstain with eosin (Sigma-Aldrich, UK) for 20 seconds and dehydrate the slides in increasing ethanol concentrations (75%, 95% and 100%). Clear the slides in xylene and mount the slides with a hard set mounting media (Merck Millipore) and slides were stored in the dark until images were acquired.

3.2.5 Short –term DEN injection

Two weeks old male *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ (C57BL/6) mice were challenged with a single dose of DEN 25mg/kg or 100mg/kg intra-peritoneal injection using 25 G needle. Mice livers were analysed 2, 4, 6, 24 and 48 hours after the DEN exposure. Individual liver lobes were dissected and stored at −80°C for further analysis or fixed in 4% PFA for histological examination.

3.2.6 TUNEL assay

Paraffin-embedded liver blocks were cut into five-micron thick sections. TUNEL assay was performed following manufacturer's instructions (Abdserotec).

3.2.7 BrdU assay

Two weeks old male *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ (C57BL/6) mice that were challenged with 100mg/kg DEN *i.p* were injected with 100mg/kg BrdU (Sigma-Aldrich, UK) 3 hour prior to the sacrifice. Paraffin-embedded sections were stained using BrdU detection kit (BD Pharmigen).

3.2.8 RNA isolation

RNA was isolated from DEN treated liver tissues by using RNA extraction kit (Life Technologies, UK) according to the manufacture's guidelines. The concentration of RNA was evaluated by using Nano Drop, samples were then stored in -80^{o}C for later use.

3.2.9 RT-PCR conversion of mRNA

To 11µl of DNase-treated RNA add 1µl of random primers (Life Technologies, UK) and 1µl of 10mM dNTPs (Life Technologies, UK). Heat the mixture to 65^{o}C for about five minutes and incubate the samples on ice for at least one minute followed by a brief centrifuge. To this reaction mixture add 4µl of 5X first strand buffer, 1µl of 0.1M DTT, 1ml RNaseOUT and 1µl of superscript III reverse transcriptase (Life Technologies, UK). Mix the samples by pipetting gently up and down and incubate samples at: 25˚C for 5 minutes with primers (Life Technologies, UK), 50˚C for 50 minutes with reverse transcription (Life Technologies, UK) and 70˚C for 15 minutes of enzyme inactivation. The cDNA samples were then stored at -20˚C for future use.

3.2.10 qRT-PCR

Prepare a master mix by adding 1µl of Taqman assay, 1µl of 18s assay (Life Technologies, UK), 10µl of PCR master mix and 7µl of H_20. Vortex the master mix and to this add 1µl of cDNA making the total volume to 20µl followed by a

brief vortex and centrifuge. Add 9.6µl of this sample to the appropriate designated plate wells (Life Technologies, UK). Cover the plate and centrifuge for 1 minute at 1000rpm.

3.2.11 Western blot

3.2.11.1 Protein isolation

Liver tissues or macrophage cell pellets that were stored in Eppendorf tubes in −80°C were taken out and thawed on ice for 10 minutes, 200µl of NP-40 lysis buffer with protease and phosphatase inhibitors were added into the Eppendorf containing tissue or cell pellet. Samples were then minced with a sterile pestle (VWR, International) and homogenised with the help of 19G needle and syringe. Samples were centrifuged at 38,000g for one hour at 4°C, supernatants with homogenates were isolated into a fresh sterile Eppendorf tube. Samples were stored at -80°C for later use.

3.2.11.2 Protein quantification

To determine the protein concentration of liver tissue lysates, Bradford assay (Bio-Rad, UK) was performed. One µl of tissue lysates was added to 200µl of Bradford reagent and incubated at room temperature for about five to ten minutes, light absorption was measured by spectra max plate reader at 595nm range (Molecular Devices, USA).

3.2.11.3 Sodium dodecyl sulfate- polyacrylamide gel electrophoresis

Samples were mixed with sample buffer and heated at 95°C for about 10 minutes, samples were cooled and centrifuged before loading onto the gel. Protein samples were separated using 12% acrylamide gel. A pre-stained protein ladder (Bio-Rad, UK) was used as molecular weight marker. Once samples were load into the wells, electrophoresis was performed at 50 V for 10-20 minutes and subsequently to 120 V for another 1 hour in running buffer.

3.2.11.4 Western blotting

After protein separation by SDS-PAGE method, proteins were transferred onto pre-activated nitrocellulose membrane (GE Healthcare, USA) by applying a current of 300mA for 1.5 hour in the cold room; transfer buffer was used for this technique. After successful transfer nitrocellulose member was incubated for one hour using blocking buffer at room temperature to prevent non-specific protein binding. Afterwards, nitrocellulose membranes were incubated with desired primary antibody overnight in the cold room, following day membranes were washed in wash buffer and incubated with HRP labeled secondary antibodies for about one hour at room temperature. Membranes were washed with wash buffer and TAE buffer, proteins were visualized by using ECL reagent (GE Healthcare, USA) membrane were then developed on to x-ray films (Sigma-Aldrich, UK) by using a developer.

3.2.12 Adoptive transfer and carcinogenesis

3.2.12.1 Bone Marrow chimeras

Mice were lethally irradiated to 950 rads (IBL 637 Irradiator), after irradiation total bone marrow from donor mice was isolated from tibiae and femur of *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ (CD45.1^{+}) mice flushed into DEME complete medium. BM cells were washed twice in PBS and re-suspended with PBS at a density of 50x10^{6} cells/ml. All the mice that were irradiated to 950-rad were *i.v* injected with 5x10^{6} BM cells from either *Gadd45β*$^{+/+}$ or *Gadd45β*$^{-/-}$ (CD45.1^{+}) mice. Leading to four possible chimeric combinations. Bone marrow reconstitution was verified by staining for CD45.1 and CD45.2 surface markers (e-Bioscience, UK).

3.2.12.2 Protocol: A

28 days old male *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ (C57BL/6) mice were lethally irradiated with 950 rads. Within 24 hours of irradiation, five million bone

marrow cells were injected *i.v* into all irradiated mice using 27 G needle, leading to four possible bone marrow chimeric combinations. Four weeks after the adoptive transfer clodronate-encapsulated liposomes (ClodronateLiposomes.org) were injected *i.v* into each mouse using 27 G needle to deplete all the endogenous tissue resident macrophages within liver and spleen. Fourteen weeks after the irradiation, mice were challenged with a single dose of DEN 100mg/kg *i.p* using 25 G needles. Four weeks after DEN injection, mice received 0.07% of phenobarbital in the drinking water until the end of the study. Mice livers and lungs were analysed 12 months after the DEN injection.

3.2.12.3 Flow-Cytometry (FACS)

Spleens and bone marrows from both Protocol A and B were harvested for FACS analysis. Single cell suspension was prepared by mashing tissues in RMPI compete media (Life Technologies, UK) and passing through 70µm cell strainers (BD Falcon). Adjust cell concentration of 1-5 x 10^6 cells/ml in ice-cold FACS buffer. Cells were usually stained in polystyrene round bottom 12 x 75 mm^2 falcon tubes (BD falcon). Cells should be centrifuged sufficiently so that the supernatant fluid can be removed with little loss of cells. Cells were then

incubated with anti-CD16/CD32 (Fc Block) (eBioscience, UK) to minimise the non-specific binding of the antibody to Fc receptor. Add 0.1-10μg/ml of labeled antibody in FACS buffer. Ten minutes after the Fc block incubation, cells were washed with ice-cold FACS buffer and then incubated with relevant FACS antibody/ isotype control (eBioscience, UK) for at least 30 minutes on ice. After antibody incubation cells were washed two to three times and re-suspend cells in 500ul of ice-cold PBS. Place the cells in dark until analysed by the Dako Cyan flow cytometer instrument. In all the experiments, single stained and unstained cells were used as controls. All the data acquired by Cyan flow cytometer instrument were compensated and analysed by suing Flowjo software (Tree star Inc, USA).

3.2.12.4 Macrophage depletion and F4/80 staining

Mice were injected with 200μl of liposome-encapsulated clodronate and PBS liposome (ClodronateLiposomes.org) as a control. Liver and spleens were harvested at 24 hour and 72 hour after the injection, tissues were washed in ice-cold PBS, fixed in 4% PFA for at least 24 hour and then processed for histological examination. Tissues were paraffin embedded, and four-micron

thickness sections were prepared to stain for macrophage marker F4/80. Slides were de-waxed in xylene at room temperature for 10-15 minutes. To rehydrated the sections incubate slides in two washes of 100% ethanol for 10 minutes each and repeat the same with 95% ethanol. Wash the sections twice with dH_20 for five minutes. Endogenous peroxidase was blocked by incubating sections in 1% H_20_2 (Sigma-Aldrich, UK) at room temperature. After endogenous peroxidase blocking wash the slides in PBS. To prevent no specific binding of the antibody, blocking step was carried by adding 125 µl of normal rabbit serum (Sigma-Aldrich, UK) to each slide and leave for 30 minutes at RT in a humidified chamber. After blocking slides were rinsed in PBS. Dilute primary antibody in PBST/5% normal rabbit serum. Make up 1:300 dilution of primary antibody F4/80 (Abcam, UK) and add 120ul to each slide, incubated with primary antibody for one hour at RT. After incubation wash the slides in PBST buffer for 10 minutes. Secondary antibody was diluted according to manufacturers instruction (Life Technologies, UK) and incubated at room temperature for 30 minutes. Staining was visualized by using DAB kit (Dako) following manufacturers instruction.

3.2.12.5 Immunofluorescence

Liver tissues that were embedded in OCT media (VWR, International) were stored in -80^{o}C for future use. Day before preparing the sections, cryo-blocks was stored in -20^{o}C freezer to prevent any crack formation on the blocks. Using a cryostat, very thin sections (8µm) were made and mounted on to Poly-L-Lysine coated slides (Thermo Fishers). Sections were then air dried for at least one hour and fixed in ice-cold methanol- acetone (Sigma-Aldrich, UK) at 1:1 ratio for about ten minutes. Air-dry the sections for about 15 minutes, once the sections were dry, using a wax pen (Vector Laboratories, UK) draw boundaries for the tissues sample, this will reduce the quantity of antibody used, sections were then rinsed in PBS-T. Block the sections with appropriate blocking serum for about 30 minutes at the room temperature to prevent any non-specific binding of the antibody. Sections were then stained for the macrophage marker F4/80 at a 1:100 dilution (Abcam, UK) at 4 ^{o}C overnight in a humidified chamber (Agar Scientific, USA). After over-night incubation sections were rinsed in PBS-T and labelled with appropriate secondary antibody (Texas-red, Life Technologies, UK) at a dilution of 1:200 for about 30 minutes at room

temperature followed by a quick rinse in PBS-T and PBS. Sections were then stained with FITC-conjugated CD45.1 antibody at a dilution of 1:25 or FITC-conjugated CD45.2 antibody at a dilution of 1:50 (BD Bioscience PharMingen) for about 30 minutes at 4 ^{o}C in a humidified chamber (Agar Scientific, USA). Slides were then washed in PBS for about five minutes and mounted using DAPI mounting media (Vector Laboratories, UK) and stored in light protective container until images were acquired using Zeiss deconvolution microscope, images were later analysed using MetaMorph software.

3.2.12.6 Tumour analysis

DEN injected mice were sacrificed, and mice livers were removed and separated into individual lobes. Externally visible tumours (≥0.5 mm) were counted, tumours were measured by using a digital caliper (VWR, International) and tumour-occupied areas were also calculated. Remaining lobes were microdissected into tumour and non-tumour tissue and stored at −80°C for further analysis. Lungs were verified for metastasis. Both liver and lung tumours were fixed in 4% PFA (Sigma-Aldrich, UK) or cryo-blocks were

prepared in OCT medium (VWR International) for histological examination.

3.2.12.7 Protocol: B

28 days old male *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ (C57BL/6) mice were challenged with a single dose of DEN 75mg/kg (*i.p*). Four weeks after the DEN injection mice have received phenobarbital (0.07%) in drinking water. Eight weeks later mice were lethally irradiated to 950 rads. Within 24 hour after irradiation five million bone marrow cells were injected *i.v* into each mouse, four weeks after adoptive transfer clodronate encapsulated liposomes were injected into each mouse to deplete all endogenous tissue resident macrophages within liver and spleen. Mice livers and lungs were analysed eight months after clodronate liposome injection.

3.2.12.8 Tumour analysis

DEN injected mice were sacrificed, and mice livers were removed and separated into individual lobes. Externally visible tumours (≥0.5 mm) were counted, tumour size was measured using a digital caliper and tumour-occupied areas

were also calculated. Remaining lobes were microdissected into tumour and non-tumour tissue and stored at −80°C for further analysis. Lungs were verified for metastasis. Both liver and lung tumours were fixed in 4% PFA (Sigma-Aldrich, UK) or cryo-blocks were prepared in OCT medium (VWR international) for histological examination.

3.2.13 Freezing and thawing of cells

For long-term storage cells were suspended in freezing medium. The cell numbers were adjusted to 1-2x10^6 cells/ml and stored in cryo-vials (Star-labs, UK) in liquid nitrogen tank. For thawing cells, cryo-vials were placed in 37^0C water bath, and cells were immediately transferred to warm complete medium.

3.2.14 Cell culture medium

All the cell culture work was carried out in DMEM medium (with glutamines, without sodium pyruvate), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, UK), 100µgml^{-1} penicillin, 100µgml^{-1} streptomycin and 1mM glutamine (Gibco, UK). Complete DMEM medium was

passed through 0.22μm pore size filter (Thermo fishers, UK)) before use.

3.2.15 Allograft model

MCA-203 tumour cells were grown to 80% confluent, and cells were trypsinised using 1x Trypsin/EDTA solution (Gibco, UK); cells were then washed twice with DMEM medium and washed twice with PBS. Cells were centrifuged for five minutes at 1500 RPM at 4 ^{0}C. Tumour cells were re-suspended in PBS at a concentration of 1x 10^{6} cells/ml. Tumour cells were inoculated subcutaneously into the right flank of *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ (C57BL/6) mice, each mouse receives 1x10^{5} cells. Cell number was determined with a Neubauer chamber slide. Tumour growth was monitored alternative day starting from day 9, and tumour volumes were estimated using the formula length x width^{2}/2. All the tumour-bearing mice were analysed on day 18 or 21 after tumour cell inoculation. Excised tumours were incubated in a falcon tube with 5mg/ml of Collagenase-D (Roche, UK) in a complete DMEM media for about 35 minutes in tissue culture incubator with an occasional shaking. Tissue samples were minced and passed through 40μm cell strainer, single cell suspensions were washed twice with a complete media and proceed to CD11b positive selection

using CD11b microbeads (Miltenyi Biotec, UK) following manufacturer's instructions. In parallel, spleens from tumour bearing mice were meshed and pass through 40μm cell strainer. Red blood cells were lysed by incubating cell for about 10 minutes in RBC lysis buffer (Sigma-Aldrich, UK). Cells from tumour and spleen were then washed, and cell number was determined with a Neubauer chamber slide.

3.2.16 Generation of BM-derived macrophages

Total bone marrow was obtained from mice by flushing femurs and tibiae in DMEM by using a 12cc syringe filled with DMEM complete medium. Push out the bone marrow from both the ends with medium into a 50ml screw top Falcon tube. BM cells were made single cell suspension by passing through a 70μm cell strainer (BD falcon) and centrifuged at 1500RPM for about five minutes. Red blood cells were depleted by adding 2ml of RBC lysis buffer (Sigma-Aldrich, UK) and incubated for about ten minutes and stop this reaction by adding 10ml of complete media. Re-suspend BM cells in 10ml Medium and repeat the centrifugation, re-suspend in 5ml of medium. Total BM cells were counted in a haemocytometer. To generate BM-derived macrophages, $2x10^6$ BM cells were

plated into 10cm Petri dish (BD Falcon) with 10ml of complete medium supplemented with MCSF-1 (Peprotech, UK). Cells were incubated in 37^0C incubator in 5% $C0_2$. A week after, non- adherent cells were washed with complete medium, adherent cells were harvested by incubating with cell dissociation buffer (Gibco, UK) for five minutes. The purity of harvested macrophages was typically more than 90%.

3.2.17 Macrophage tumour cell co-culture

Bone marrow-derived macrophages from *Gadd45β*$^{-/-}$ and *Gadd45β*$^{+/+}$ mice were seeded at a density of 4 x 10^5 cells/insert (VWR International). Tumour cells were seeded at a density of 4 x 10^5 or 8 x 10^5 cells/well in a 6 well plate (BD Falcon). Incubate the cells overnight at 37°C in DEME complete media, the next day check the cell attachment and confluence. Transfer the cell culture inserts (VWR International) with *Gadd45β*$^{-/-}$ and *Gadd45β*$^{+/+}$ bone marrow-derived macrophages into the 6-well plates that contained seeded tumour cells. Macrophage cells attached to the inserts but without tumour cells will act as a control. RNA was extracted from macrophages 12 hours after as done before

(Hagemann *et al.,* 2008). Gene expression levels were analysed by qRT-PCR for both M1 and M2 macrophages markers.

3.2.18 Macrophage polarisation

Bone marrow-derived macrophages from *Gadd45β*$^{-/-}$ and *Gadd45β*$^{+/+}$ mice were seeded at a density of 0.5 x 10^6 cells/well in a six-well plate (BD Falcon). For M1 macrophage polarisation, BM-derived macrophages were treated with 100ng/ml of LPS (Sigma-Aldrich, UK) and 20ng/ml of IFN-gamma (Sigma-Aldrich, UK) for 24 hours, samples were harvested for RNA extraction at different time points. For M2 macrophage polarisation, BM-derived macrophages were treated with 20ng/ml of IL-4 and IL-13 (Peprotech, USA) for 24 hours, samples were harvest for RNA extraction using Trizol reagent (Ambion, UK). Bone marrow-derived macrophages from *Gadd45β*$^{-/-}$ and *Gadd45β*$^{+/+}$ mice that were not treated with any cytokines acted as a control.

3.2.19 Statistical analysis

P-values less than 0.05 were considered as statistically significant. Student's t-test was used to determine statistical significance.

4: Results

4.1 *Gadd45β* is required for DEN-induced hepatocellular carcinogenesis

NF-κB activation within the tumour cell promotes their survival (Dutta *et al.,* 2006); the basis for this protective activity of NF-κB is best understood in the context of TNF-R1 stimulation. This control of TNF-R1 included suppression of programmed cell death (PCD) by NF-κB is crucial for tumourigenesis and chemoresistance in cancers. Although signalling through TNF-R1 receptor can trigger PCD in tumour cells. However, tumour cells survive from this signalling due to the activation of NF-κB. Indeed, selectively active NF-κB is most commonly found in many solid tumours, including lymphomas and certain other solid tumours (Gilmore, 2006).

Previous studies have shown that the protective activity of NF-κB is linked to the suppression of sustained activation of the JNK cascade (Papa *et al.,* 2004), a major MAPK cascade in all the eukaryotes (Bardwell and Shah, 2006). To better understand the precise mechanism that control TNF-R1 inflicted PCD and tumourigenesis, *Gadd45β* has been found as a pro-survival gene that is under the direct transcriptional control of NF-κB (De Smaele *et al.,* 2001). *Gadd45β* is rapidly induced by TNF-*α* through a transcriptional mechanism that requires NF-κB; *Gadd45β* expression abrogates TNF-*α* induced JNK activation thereby protecting cells from PCD (De Smaele *et al.,* 2001). This protective activity of *Gadd45β* against TNF-*α* induced PCD involves suppression of JNK cascade by means of MKK7 inactivation (Papa *et al.,* 2004;

Tornatore *et al.,* 2014). These findings suggest that *Gadd45β* afforded suppression of JNK signalling acts as a crucial factor for the pro-survival activity of NF-κB (Papa *et al.,* 2004). This effect of *Gadd45β* was reported in a recent study, in which it has been shown that *Gadd45β* is required for liver regeneration after PH (Papa *et al.,* 2008), a process that is controlled by TNF-R1 through a negative crosstalk between NF-κB and JNK signalling (Papa *et al.,* 2008). The role of NF-κB in linking inflammation and cancer has been widely documented in several animal models. Definitive examples of these models are the model of CAC and hepatocellular carcinogenesis model by the administration of DEN (Greten *et al.,* 2004; Maeda *et al.,* 2004).

We proposed that using the established DEN model of inflammation driven hepatocellular carcinogenesis (Maeda *et al.,* 2005) would allow to assess the precise role of *Gadd45β* in the NF-κB mediated link between inflammation and cancer. It also helps to determine whether *Gadd45β* contributes to these activities of NF-κB during hepatocellular carcinogenesis. This understanding may help to develop a preferable therapeutic approach that would block the critical NF-κB downstream target genes that promote cancer cell survival, rather than blocking NF-κB itself. Current NF-κB targeting drugs used in the treatment of human malignancies have serious side effects, especially upon prolonged use (Orlowski and Baldwin Jr, 2002; Yamamoto and Gaynor, 2001).

To this end, we used a DEN model in which the relevance of crosstalk between TNF-R1 induced NF-κB and JNK activity was previously established (Sakurai *et al.,* 2006). In the DEN model of hepatocellular carcinoma (HCC), NF-κB orchestrates crosstalk between hepatocytes and the resident macrophages (so-called Kupffer cells), the two cell types that are critical for the formation of HCC (Maeda *et al.,* 2005). The NF-κB regulated genes that promote cell survival in HCC and linking this with inflammation, however, are unknown. As NF-κB upregulates *Gadd45β* within the pre-cancerous liver (Pikarsky *et al.,* 2004; Papa *et al.,* 2008), the role of *Gadd45β* in DEN induced HCC was examined.

Previously, it has been found that a single DEN injection to two-week-old male mice resulted in an efficient HCC induction (Sarma *et al.,* 1986), whose course of development was similar to human HCC (Verna *et al.,* 1996). To determine the role of *Gadd45β* in DEN induced hepatocellular carcinogenesis, we used *Gadd45β* knockout (*Gadd45β*$^{-/-}$) mice that are generated earlier by using standard gene targeting strategies (Papa *et al.,* 2008). In agreement with previous reports (Lu *et al.,* 2004; Gupta *et al.,* 2005), *Gadd45β*$^{-/-}$ mice are born with expected frequency, developed normally, showed no obvious abnormalities and died of old age. In this tumour initiation model, two-week-old *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ male mice were injected *i.p* with the chemical carcinogen DEN (25mg/kg) (Figure 1). Both the *Gadd45β*$^{+/+}$ and *Gadd45β* $^{-/-}$ mice survived 25mg/kg dose of DEN exposure.

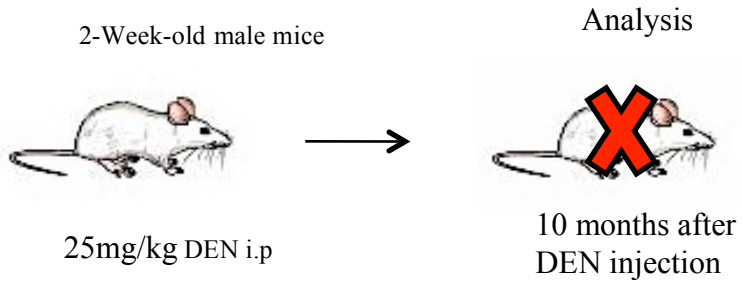


Figure 1: The schematic representation of DEN induced HCC experimental protocol. Two-week-old male C57BL/6 *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ mice were treated with a single *i.p* injection of DEN (25mg/kg). Mice were analysed ten months after the DEN injection.

Previous studies have shown that male mice are more sensitive than female mice to DEN induced carcinogenesis due to hormonal factors (Sarma *et al.,* 1986). Only 10% of the females treated with DEN developed HCC (Maeda *et al.,* 2005). In the light of this, only male mice were used throughout the experiments described in this thesis.

Mice were analysed ten months after DEN injection. Almost all the *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ mice given DEN developed typical HCC within ten months (Figure 2 A and B). Representative photographs of *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ liver were shown (Figure 2 A and B). Histological examination of H and E

staining revealed that most of the tumour nodules seen in the livers of DEN treated mice were indeed HCC (Figure 2 C and D). Representative microscopic HCC images of the liver were shown in (Figure 2 C and D). Strikingly, the numbers of detectable HCC were four-fold lower in *Gadd45β*$^{-/-}$ mice compared to *Gadd45β*$^{+/+}$ mice (Figure 3 A), and so was the tumour occupied area (Figure 3 B). The maximum tumour area was also two-fold lower in the *Gadd45β*$^{-/-}$ mice compared to *Gadd45β*$^{+/+}$ mice (Figure 3 C). However, there was no significant difference in the HCC incidence between the two genotypes (Figure 3 D). Notably, many tumours in *Gadd45β*$^{+/+}$ mice, but none of the *Gadd45β*$^{-/-}$ mice exhibited signs of neovascularization. Furthermore, H and E staining on liver sections revealed that there were more necrotic regions in *Gadd45β*$^{+/+}$ tumours (Figure 2 E). Some *Gadd45β*$^{+/+}$ mice had HCC metastasis in their lungs, whereas *Gadd45β* $^{-/-}$ mice did not. Furthermore, there was no detectable difference in the mortality rates between the genotypes, because of HCC development.

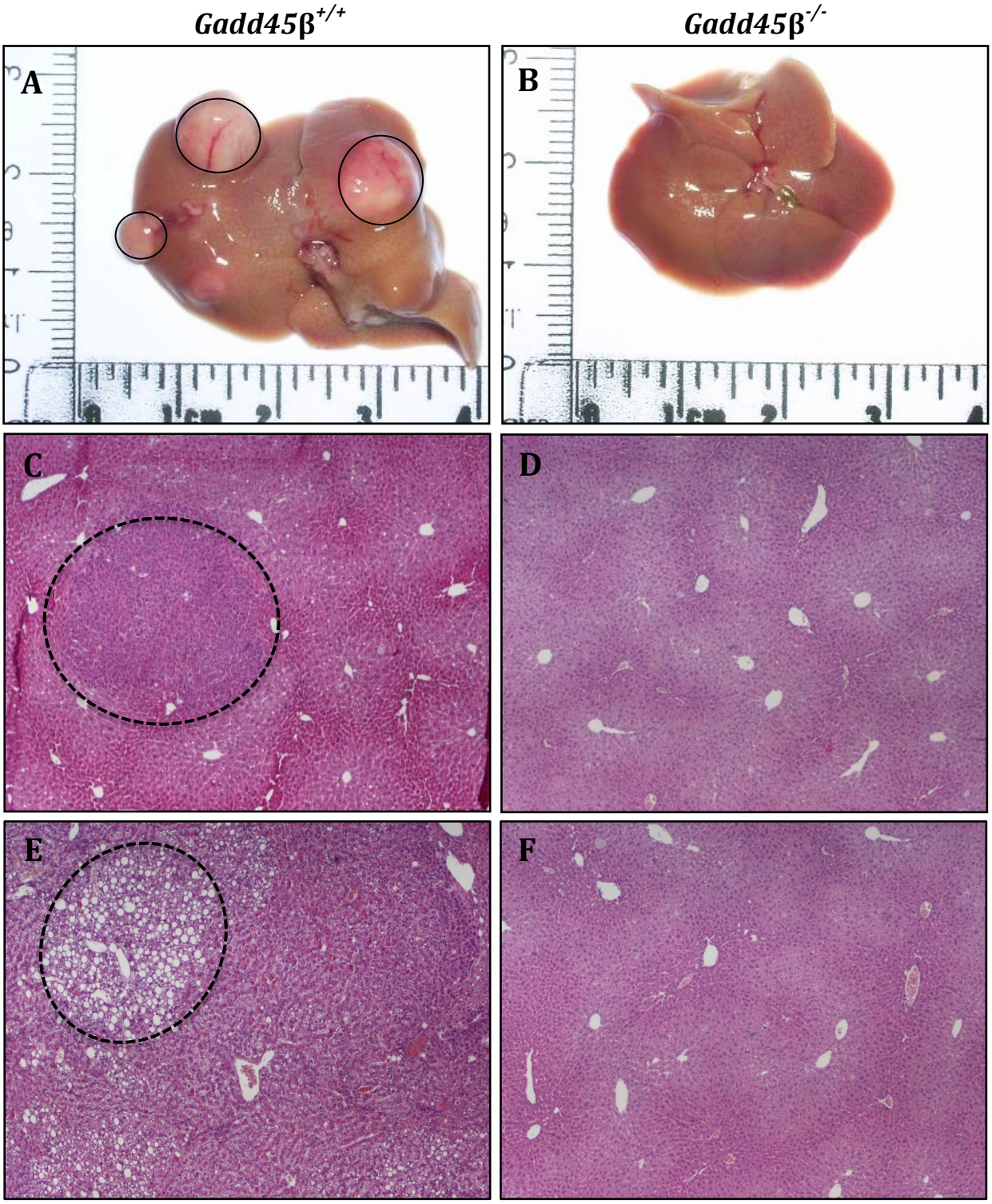


Figure 2: Reduced DEN induced tumour development in *Gadd45β*$^{-/-}$ mice liver. (A) Represent photographs of the liver of *Gadd45β*$^{+/+}$ (n=20) and (B) *Gadd45β*$^{-/-}$ (n=26),

mice that were given with high dose of DEN (25mg/kg) at 15 days of age, circle represents tumour area. Typical liver histology of DEN treated *Gadd45β*$^{+/+}$ (C,) and *Gadd45β*$^{-/-}$ (D,) broken circles represent the tumour location. Histological examination of *Gadd45β*$^{+/+}$ (E) and *Gadd45β*$^{-/-}$ (F) liver tumours, broken circle represents necrotic region.

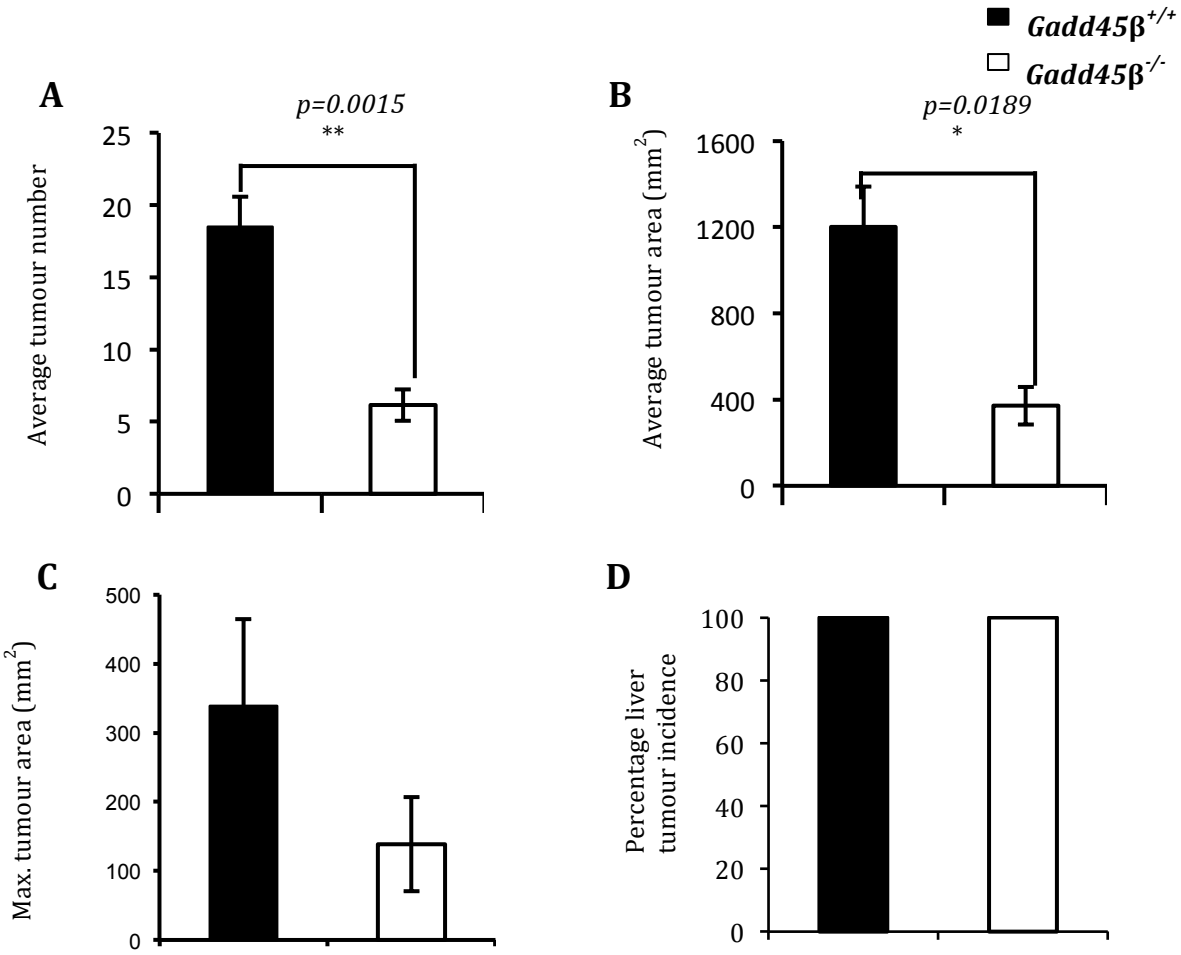


Figure 3: Reduced HCC growth in *Gadd45β*$^{-/-}$ mice. (A) Average tumour number (>0.5mm), average tumour area (B), maximum tumour area (C) and percentage of liver tumour incidence (D) in livers of male *Gadd45β*$^{+/+}$ *and Gadd45β*$^{-/-}$ mice ten months after DEN treatment. Values denote mean ±SEM (*Gadd45β*$^{+/+}$, n=20 and *Gadd45β*$^{-/-}$, n=26). *, p<0.05; **, p< 0.01.

Collectively, these results suggest that *Gadd45β* is required for efficient HCC induction and loss of *Gadd45β* reduces susceptibility to develop DEN induced hepatocellular carcinogenesis. Thus, *Gadd45β* has a tumour-promoting role at least in this model of inflammation-elicited carcinogenesis, and therefore *Gadd45β* may have acted as an important component of the NF-κB mediated link between inflammation and cancer. These results, however, stand in marked contrast to *Gadd45β* downregulation, which has been associated with differentiation and nuclear grade of HCC in humans. (Qiu *et al.,* 2003). The reason for this contrasting result could be because of the differences in the experimental model system used.

4.2 Effects of loss of *Gadd45β* in acute DEN injected mice

To understand the basis for the decreased susceptibility of *Gadd45β* $^{-/-}$ mice to the DEN induced hepatocellular carcinogenesis, it is important to understand the mechanisms involved in early stages of tumour development and also it is important to investigate the short-term effects of DEN exposure. To this end, two week old male *Gadd45β* $^{+/+}$ and *Gadd45β* $^{-/-}$ mice were injected with either 25 or 100 mg/kg of DEN *i.p* and livers were harvested at different time points, as described previously (Maeda *et al.,* 2005). Both the *Gadd45β* $^{+/+}$ and *Gadd45β* $^{-/-}$ mice survived acute DEN exposure. Previous studies have suggested that the initial hepatic injury and compensatory hepatocyte proliferation that immediately followed after DEN injection are the important factors that allows HCC formation in this model of DEN induced carcinogenesis (Naugler *et al.,* 2007; Karin, 2009). To this end analyses were performed to investigate the effects of loss of *Gadd45β* on hepatocyte survival, proliferation and signal transduction that are crucial for DEN induced carcinogenesis (Sakurai *et al.,* 2006).

4.2.1 Loss of *Gadd45β* has no effect on hepatocyte survival shortly after DEN injection

It has been previously reported that *Gadd45β* participate in liver regeneration after partial hepatectomy by promoting hepatocyte survival and

proliferation (Papa *et al.,* 2008). To this end, acute DEN injected *Gadd45β* $^{+/+}$ and *Gadd45β* $^{-/-}$ mice were analysed for the circulating liver enzyme, ALT, a marker for hepatic cytolysis (Sakurai *et al.,* 2006; Papa *et al.,* 2008). In accordance with the previous findings, at 48 hours after DEN injection *Gadd45β* $^{-/-}$ mice displayed a slight increase in the circulating levels of ALT (Figure 4 B) that indicates the extent of liver damage. However, this effect was not significant (Figure 4 B). TUNEL assay was performed to detect hepatocyte cell death via apoptosis. TUNEL assay results revealed a slight increase in hepatocyte cell death only in the liver of *Gadd45β* $^{-/-}$ mice 48 hours after DEN injection (Figure 4 A). These findings suggest that the DEN induced injury was mild, as evidenced by having comparable levels of TUNEL positive cells in the livers of *Gadd45β* $^{+/+}$ and *Gadd45β* $^{-/-}$ mice 72 hours after DEN injection (Figure 4 A).

The above results suggest that unlike what we see in PH model, *Gadd45β* upregulation may not be required for hepatocyte survival at least in this model of DEN induced hepatocellular carcinogenesis.

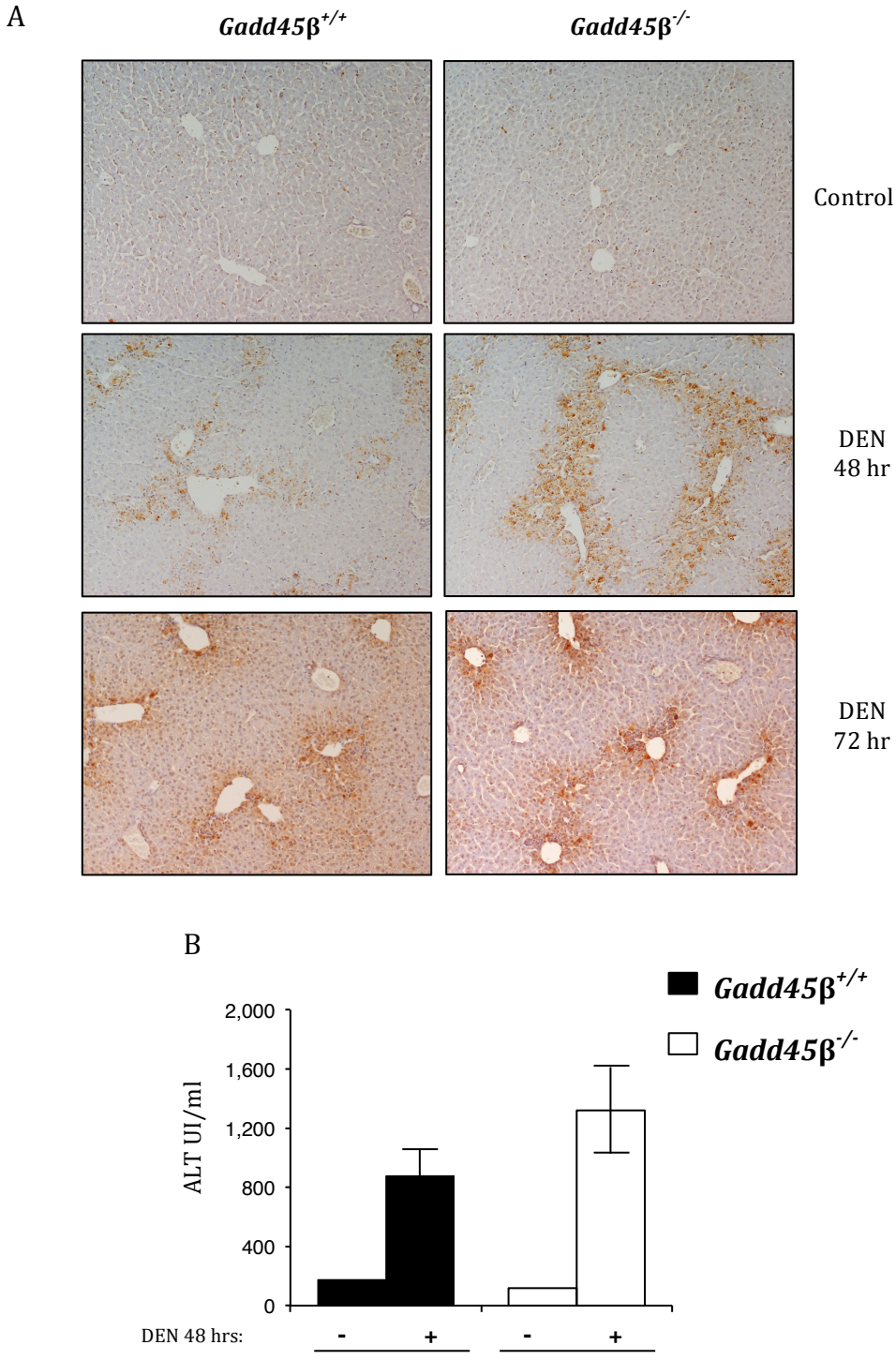


Figure 4: Increased hepatocyte cell death after DEN injection. A) Images of *Gadd45β* $^{+/+}$ and *Gadd45β* $^{-/-}$ liver sections showing representative clusters of TUNEL positive cells from DEN treated mice. Original magnification (x10). (B) ALT levels were determined in

serum from two week old male *Gadd45β* $^{+/+}$ (n=5) and *Gadd45β* $^{-/-}$ (n=6) mice treated i.*p.* with 25 mg/kg of DEN.

4.2.2 Loss of *Gadd45β* attenuates DEN induced hepatocyte proliferation

To further investigate the DEN induced compensatory hepatocyte proliferation (Sakurai *et al.,* 2006), BrdU labelling was performed on the liver of DEN treated mice at different time points. The results of this analysis revealed the presence of BrdU positive cells in the livers of all DEN treated *Gadd45β*$^{+/+}$ mice, as reported previously (Maeda *et al.,* 2005). Earlier studies using the PH model demonstrated that the liver of *Gadd45β*$^{-/-}$ mice displayed impaired hepatocyte proliferation (Papa *et al.,* 2008). As expected, and in accordance with the previous studies, the proliferative response to DEN injection was modest, but significantly reduced in *Gadd45β*$^{-/-}$ livers (Figure 5 C). Indeed, more BrdU positive cells were detected in *Gadd45β*$^{+/+}$ livers 48 hours after DEN injection compared to the livers of similarly treated *Gadd45β* $^{-/-}$ mice, which contained relatively fewer BrdU positive cells at the same time point (Figure 5 A and B). These proliferating cells, which stained positive for BrdU, were most common around the cluster of apoptotic cells (Sakurai *et al.,* 2006). Suggesting that the DEN induced hepatocyte cell death promotes compensatory hepatocyte proliferation (Sakurai *et al.,* 2006), however, this proliferative response after DEN injection was significantly reduced in *Gadd45β* $^{-/-}$ mice (Figure 5 C). It should be noted that, because of the high

regenerative capacity of the liver, even a small fraction of hepatocyte cell death in response to DEN exposure could trigger compensatory proliferation of the surviving hepatocytes that may eventually lead to HCC development (Maeda *et al.,* 2005; Sakurai *et al*.,2006).

The defective proliferative response observed in *Gadd45β*$^{-/-}$ liver after the DEN injection may not be a direct consequence of *Gadd45β* ablation. Because, there were a similar number of BrdU positive cells (Figure 5 C) in untreated *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ livers (Figure 5 C).

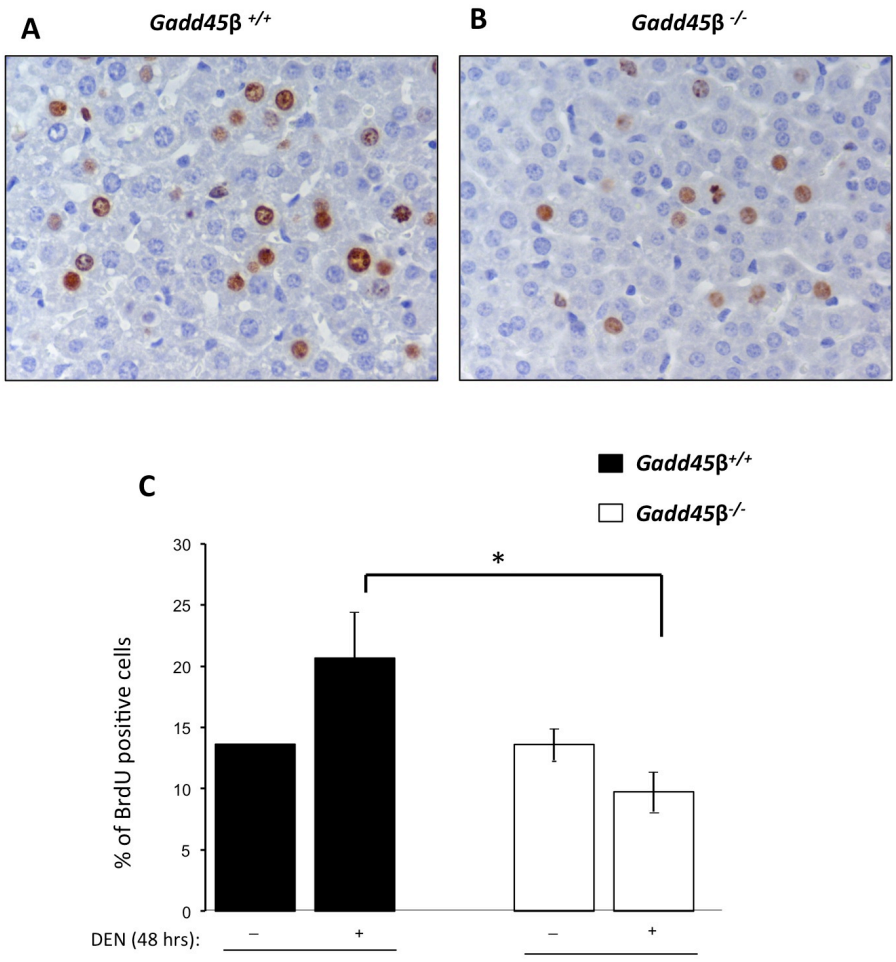


Figure 5: BrdU incorporation assay. Histological examination of paraffin-embedded liver sections 48 hours after DEN treatment, (A) and (B) represent *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ livers labelled for BrdU. Percentage of BrdU positive cells per focal area 48

hours after DEN injection *i.p* 100mg/kg (c). Values denote mean ±SEM (*Gadd45β*$^{+/+}$, n=6; *Gadd45β*$^{-/-}$, n=9). *, p<0.05; **, p< 0.01.

Results obtained from BrdU assay suggest that *Gadd45β* upregulation promotes hepatocyte proliferation after acute DEN injection. However, the mechanism by which *Gadd45β* promotes hepatocyte proliferation in this DEN induced carcinogenic mode, still need to be elucidated.

4.2.3 Loss of *Gadd45β* attenuates DEN induced mitogen factor production

Next we examined whether the impaired hepatocyte proliferative response observed in *Gadd45β*$^{-/-}$ mice after acute DEN injection was due to impaired production of factors that are known to drive compensatory hepatocyte proliferation (Maeda *et al.,* 2005; Sakurai *et al.,* 2006). Hepatic mitogen factor production by *Gadd45β*$^{+/+}$ and *Gadd45β* $^{-/-}$ livers shortly after DEN injection was assessed by qRT-PCR. Because, it has been shown in various experimental models that the cytokines such as TNF-α, IL-1β and IL-6 produced by Kupffer cells promotes compensatory hepatocyte proliferation (Sakurai *et al.,* 2006; Fausto *et al.,* 2000). Quantitative real-time PCR results from DEN treated livers revealed that the mRNA levels of IL-1β in *Gadd45β* $^{-/-}$ mice were sharply and significantly reduced four hours after DEN administration compared to those of similarly treated *Gadd45β*$^{+/+}$ mice

(Figure 6A). This sharp and immediate decrease of IL-1β is most likely to limit the extent of liver injury and compensatory hepatocyte proliferation (Figure 6 A) (Sakurai *et al.,* 2006). The proinflammatory cytokine IL-1β involves in tumour progression and also induces IL-6 and MCP-1, a chemokine that has an important role in angiogenesis and tumour growth (Sica *et al.,* 2000). Furthermore, four hours after DEN treatment, there was a decrease in IL-6 mRNA levels in *Gadd45β* $^{-/-}$ mice livers (Figure 6 C). TNF-α is a potent inducer of cell death but it also involves in liver regeneration. This effect of TNF-α prompted us to determine the intrahepatic levels of TNF-α. Four hour after DEN injection TNF-α mRNA was rapidly induces in both *Gadd45β* $^{-/-}$ and *Gadd45β*$^{+/+}$ livers (Figure 6 B). Surprisingly, there was no obvious difference in the TNF-α mRNA levels between the two genotypes (Figure 6 B).

To investigate the reason for elevated levels of cytokine production by *Gadd45β*$^{+/+}$ Kupffer cells, mRNA levels of *Gadd45β* were assessed by qRT-PCR in DEN treated mice. Results revealed that the mRNA level of *Gadd45β* has increased by four-folds in DEN treated *Gadd45β*$^{+/+}$ livers compared to those of untreated *Gadd45β*$^{+/+}$ mice (Figure 6D). Suggesting that *Gadd45β* may have a critical role in promoting hepatocyte proliferation shortly after DEN injection.

Histological examination for the macrophage marker F4/80 was performed to understand the reason for the decreased cytokine production in *Gadd45β*$^{-/-}$ liver shortly after DEN injection. Histology analysis revealed that Kupffer cells virtually disappeared in all the *Gadd45β*$^{-/-}$ livers compared to *Gadd45β*$^{+/+}$ livers 48 hours after DEN injection (Figure 7). Suggesting that *Gadd45β*$^{-/-}$ Kupffer cells harbour additional defects.

Together, these results suggest that the DEN exposure enhances the liver damage in both *Gadd45β*$^{+/+}$ and *Gadd45β* $^{-/-}$ mice. However, there was a significant reduction in hepatocyte proliferation and cytokine production in *Gadd45β* $^{-/-}$ liver shortly after DEN injection. These results precisely explain the basic mechanism by which *Gadd45β* promotes compensatory hepatocyte proliferation in response to DEN exposure. Thus, establishing a co-relation between hepatocyte proliferation and cytokine production by Kupffer cells. Suggesting that the upregulation of *Gadd45β* in non-parenchymal cell compartment is important for their survival and the production of factors that are known to drive hepatocyte proliferation.

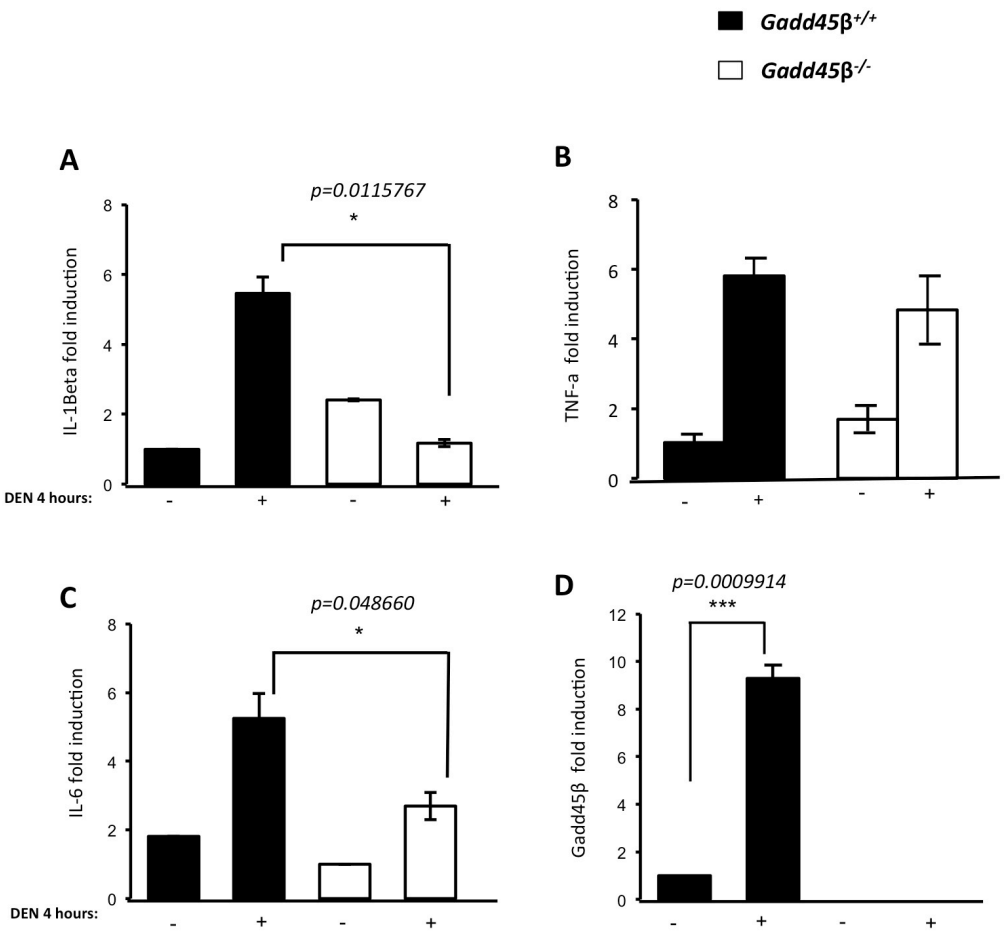


Figure 6: Decreased expression of proinflammatory factors shortly after DEN injection. *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ mice treated with DEN (25mg/kg) *i.p,* total liver RNA was extracted at indicated time point. Expression of proinflammatory gene (A, B,

and C) and the mRNA of *Gadd45β* after DEN injection (D). Values denote mean ±SEM (*Gadd45β*$^{+/+}$, n=5; *Gadd45β*$^{-/-}$, n=5). *, p<0.05; **, p< 0.01; ***, p<0.001.

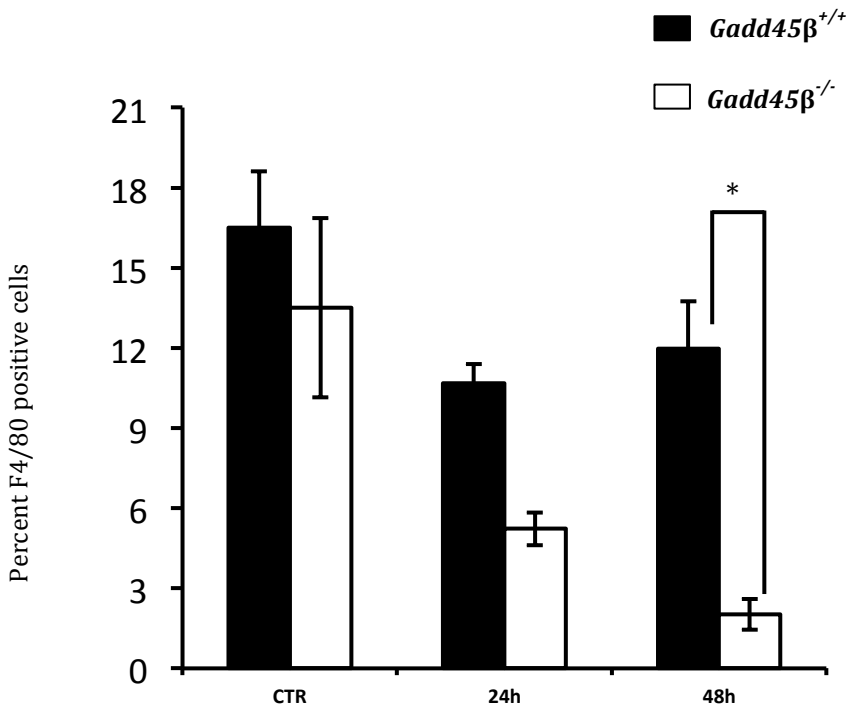


Figure: 7 Loss of *Gadd45β* augment Kupffer cell death after DEN treatment. *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ mice treated with DEN (25mg/kg) *i.p.* Livers were harvested 24 and 48 hours after treatment and stained with the macrophage marker F4/80 antibody. The cells stained with F4/80 were counted per focal area. Values denote mean ± SEM (*Gadd45β*$^{+/+}$, n=5; *Gadd45β*$^{-/-}$, n=5). *, p<0.05.

4.2.4 Loss of *Gadd45β* has no effect on MAP kinase signalling in the livers of DEN treated mice

Previous studies have shown that DEN administration activates JNK signalling (Sakurai *et al.,* 2006). Prolonged and robust JNK activation has been shown to promote hepatocyte cell death (Sakurai *et al.,* 2006). Additionally, recent studies using the PH model suggest the mechanism by which *Gadd45β* upregulation promotes hepatocyte survival is through the inhibition of the prolonged activation of JNK signalling (Papa *et al*., 2008; Papa *et al.,* 2004). It has also been shown that the protective activity of *Gadd45β in vitro* was linked to a control of JNK signalling (De Smaele *et al.,* 2001; Papa *et al.,* 2004; Tornatore *et al*., 2014).

To investigate changes in the major MAP Kinase signalling immediately after DEN exposure and to determine whether the defective hepatocyte proliferation observed in *Gadd45β*$^{-/-}$ liver associated with elicited JNK activity. The livers of acute DEN injected *Gadd45β*$^{+/+}$ and *Gadd45β* $^{-/-}$ were, therefore, analysed for JNK activation by using the western blot technique. In accordance with the previous findings (Papa *et al.,* 2008; Sakurai *et al.,* 2006), JNK activation was rapidly induced in both *Gadd45β*$^{+/+}$ and *Gadd45β* $^{-/-}$ livers after DEN exposure (Figure 8). This activation of JNK in both *Gadd45β*$^{+/+}$ and

Gadd45β $^{-/-}$ livers was observed early on and was relatively strong at most of the time points that were examined (Figure 8). These findings were confirmed by the use of phospho-specific anti-JNK antibodies (Papa *et al.,* 2008) establishing the activation of both JNK1 and JNK2 (predominantly present as p46 and p54 isoforms) (Figure 8). The high JNK expression in both *Gadd45β*$^{+/+}$ and *Gadd45β* $^{-/-}$ livers suggested that JNK pathway may have contributed to the enhanced hepatocyte death after DEN injection. However, in contrast to what was seen in the PH model (Papa *et al*., 2008) there was no significant difference in the expression of JNK activation between both the genotypes. This data suggest that *Gadd45β* ablation had no effect on JNK signalling and either in the p38 or in the ERK pathways (Figure 8). Very few *Gadd45β* $^{-/-}$ mice expressed elevated levels of phosphorylated-p38 early after DEN exposure, and this was not consistent. However, in those mice enhanced p38 expression might have played a tumour suppressing role. Recent studies show that p38α prevents ROS accumulation, liver fibrogenesis, and subsequent hepatocellular carcinogenesis in mice that were exposed to chemicals that normally induce liver cirrhosis and cancer (Sakurai *et al.,* 2013). Hence, *Gadd45β* activation does not influence MAPK activation in the livers of DEN treated mice.

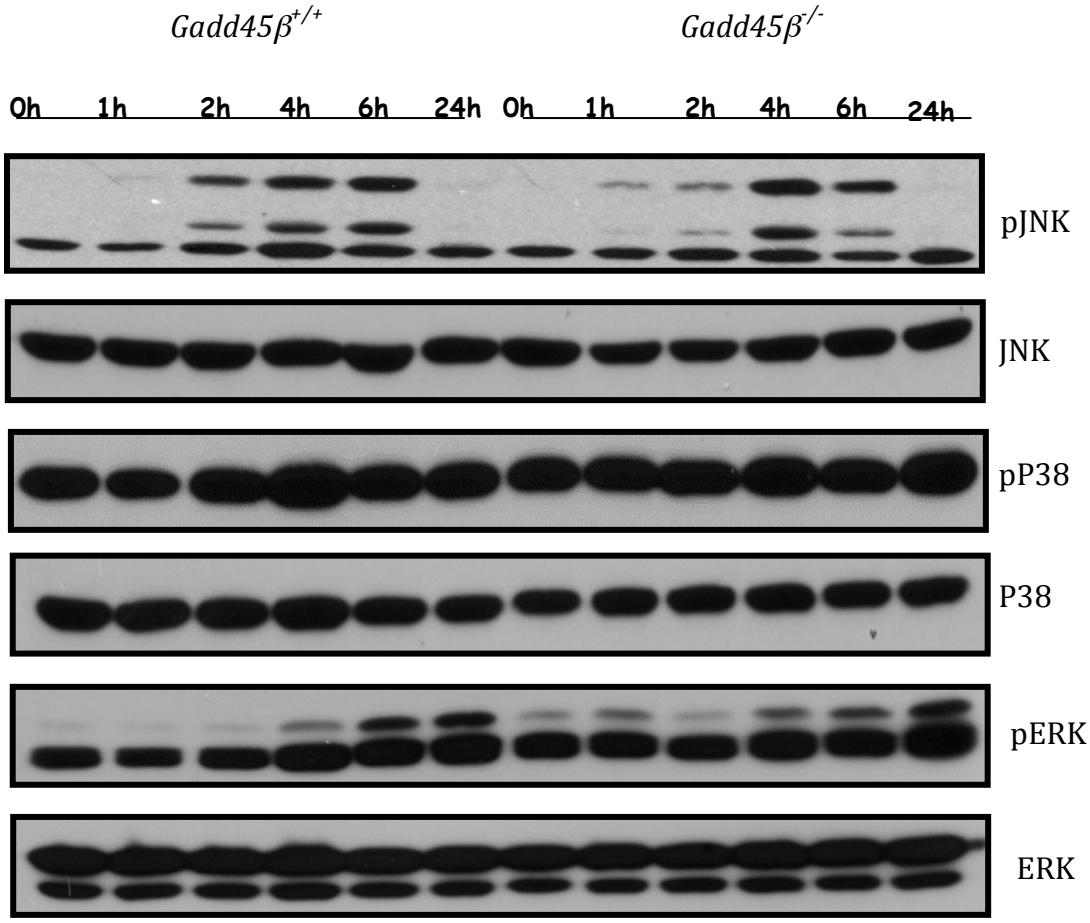


Figure 8: Biochemical characterisation of MAPK signalling in the liver after DEN injection. Expression of major MAP kinase proteins in the liver tissue at indicated time points in *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ livers after the DEN treatment. Liver tissues were dissected, and samples were lysed, gel separated and analysed by immunoblotting with antibodies to the indicated proteins at different time points.

Thus, the early effects of DEN exposure experimental data suggests, in *Gadd45β*$^{-/-}$ mice there was no significant difference in the hepatocyte cell death and the activation of major MAP Kinases. However, there was moderate and significantly reduced hepatocyte proliferation in *Gadd45β* $^{-/-}$ liver, this is most likely because of the impaired cytokine production by the

Kupffer cells. Based on the above results, it is plausible that *Gadd45β* upregulation promotes hepatocyte proliferation and the production of factors that are known to drive compensatory hepatocyte proliferation. These results may provide a mechanistic basis for the decreased susceptibility of *Gadd45β*$^{-/-}$ mice to DEN induced hepatocellular carcinogenesis. However, the cell-type specific roles of *Gadd45β* and its precise role either in tumour initiation or in tumour progression remain to be investigated.

4.3 Loss of *Gadd45β* in haematopoietic cells reduces the DEN induced hepatocellular carcinogenesis

The next important step is to investigate in which cell type *Gadd45β* primarily exercises its pro-tumourigenic action, whether in hepatocytes or the proinflammatory cells, such as Kupffer cells. To address this question a bone marrow chimeric mouse model was established, harbouring all possible combinations of *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ parenchymal and haematopoietic cells (Figure 9). This model will allow us to examine the DEN induced carcinogenesis in mice harbouring selective *Gadd45β* deficiency in either hepatocyte or Kupffer cells. Evidence suggests that Kupffer cells are the major cell types that are responsible for the production of cytokines that stimulate hepatocyte proliferation during compensatory hepatocyte proliferation and HCC development (Fausto *et al.,* 2000).

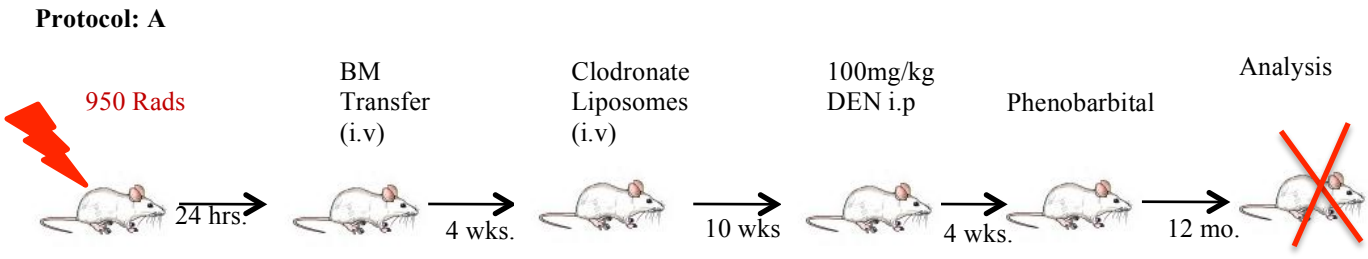


Figure 9: Schematic diagram of the bone marrow chimeric experimental "Protocol A" .

Briefly, 28-day-old male mice were lethally irradiated with 950 rads. After irradiation *Gadd45β*$^{+/+}$ recipients (CD45.2) were injected *i.v* with five million *Gadd45β*$^{+/+}$ (CD45.1) bone marrow cells, *Gadd45β*$^{+/+}$ recipients (CD45.2) were injected with *Gadd45β*$^{-/-}$ bone marrow cells (CD45.1), *Gadd45β*$^{-/-}$ recipients (CD45.2) were injected with *Gadd45β*$^{+/+}$ (CD45.1) bone marrow and *Gadd45β*$^{-/-}$ recipients (CD45.2) were injected with *Gadd45β*$^{-/-}$ bone marrow (CD45.1). Thus, leading to four possible bone marrow chimeric combinations of *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ parenchymal and haematopoietic cells, from here onwards referred as WT→WT, KO→WT, WT→KO and KO→KO respectively. Spleen and bone marrow were harvested four weeks after adoptive transfer. The percentage of reconstitution was verified using flow cytometry. The results from FACS analysis revealed more than 90% reconstitution by donor's bone marrow-derived cells in all the bone marrow chimeric mice, suggesting a successful bone marrow reconstitution (Figure 10). Four weeks after the adoptive transfer, liposome-encapsulated clodronate was injected *i.v* to deplete the endogenous tissue resident macrophages within the liver and spleen, thus allowing the recruitment of donor's bone marrow-derived monocytes into the liver. To confirm the complete depletion of macrophages in the liver, a small group of mice injected with clodronate liposome and another group of mice injected with PBS liposome as a control. Liver and spleen from

both the groups were harvested 24 and 72 hours after injection. The histological examination was performed to detect macrophage marker, F4/80. Results have revealed a complete depletion of macrophages both in liver and spleen at 24 and 72 hours after clodronate liposome injection (Figure 11). No analyses were performed at the later time points.

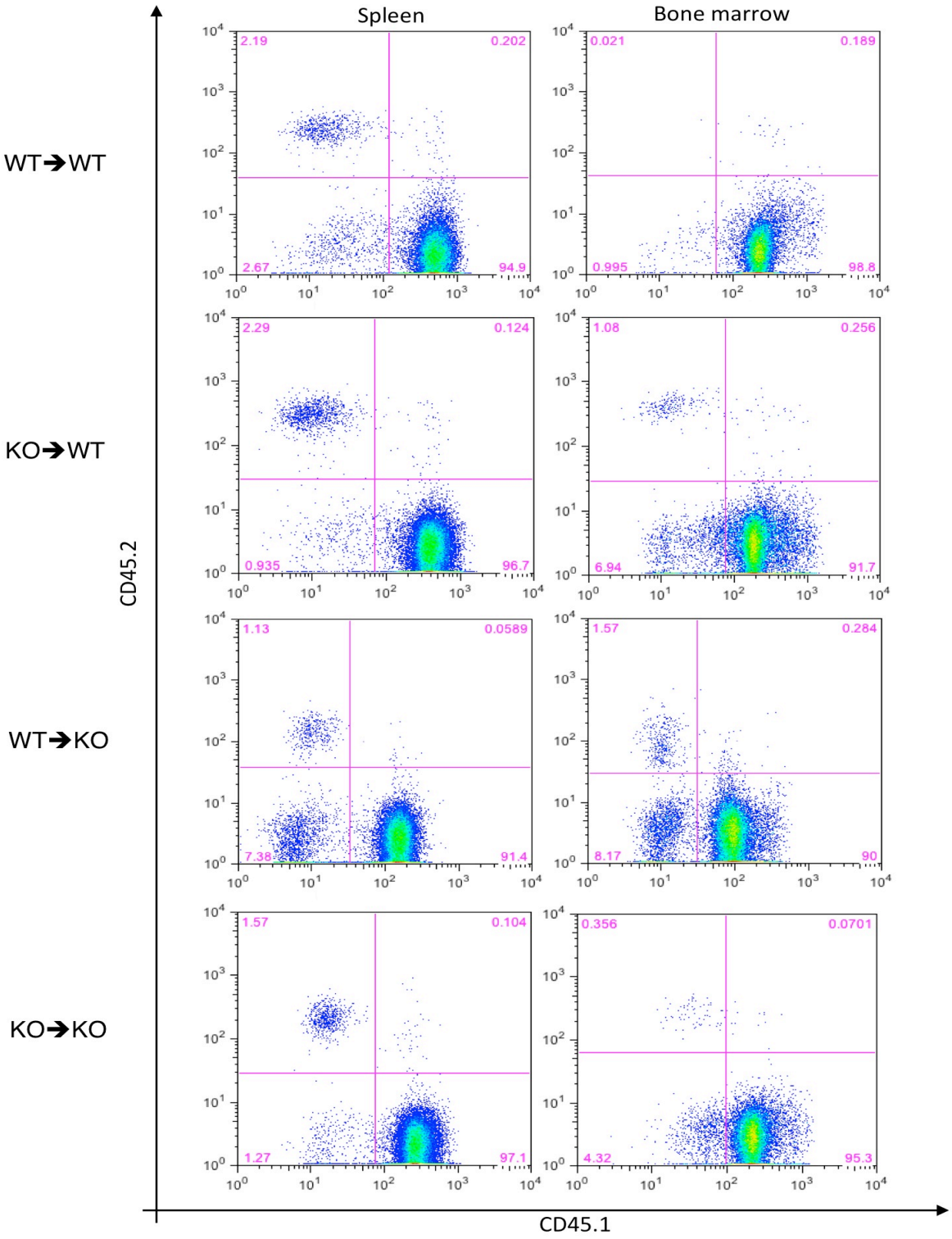

Spleen
Bone marrow
WT➔WT
KO➔WT
WT➔KO
KO➔KO
CD45.2
CD45.1
2.19
0.202
2.67
94.9
0.021
0.189
0.995
98.8
2.29
0.124
0.935
96.7
1.08
0.256
6.94
91.7
1.13
0.0589
7.38
91.4
1.57
0.284
8.17
90
1.57
0.104
1.27
97.1
0.356
0.0701
4.32
95.3

Figure 10: Bone marrow reconstitution. FACS analysis performed for leukocyte marker CD45.1 (donor) and CD45.2 (recipient). Spleen and bone marrow were harvested and stained with anti-CD45.1 and anti-CD45.2 antibodies, results showing percentage of reconstitution by donor bone marrow-derived cells (CD45.1).

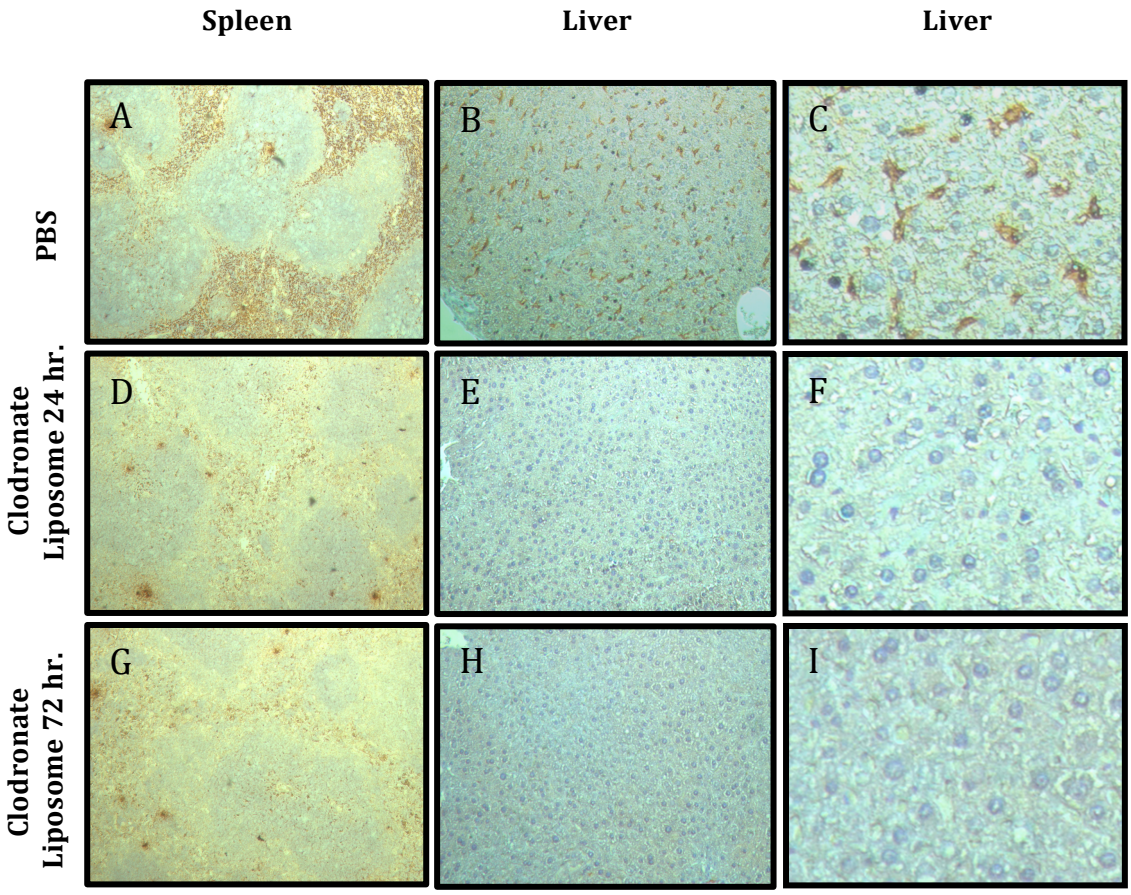


Figure 11: Macrophage depletion by clodronate liposome injection. Mice injected with clodronate liposome *i.v* (D-I) and clodronate PBS as a control (A-C). Spleen and livers were dissected 24 and 72 hours after injection and stained for the macrophage marker, F4/80. (C, F, I 100 x magnification).

In order to rule out any possible role played by endogenous liver resident macrophages in this tumour initiation and promotion model and to verify the successful replacement of endogenous tissue resident macrophages with macrophages of donor bone marrow origin. Frozen liver sections from the bone marrow chimeric combinations stained with anti-CD45.1+anti-F4/80 or anti-CD45.2+anti-F4/80. Immunofluorescence results revealed anti-CD45.1+anti-F4/80 staining in the livers of chimeric mice (Figure 12). Immunofluorescence results suggest that only the donor bone marrow-derived (CD45.1) macrophages recruited into the liver might have played a key role in this model of DEN induced HCC.

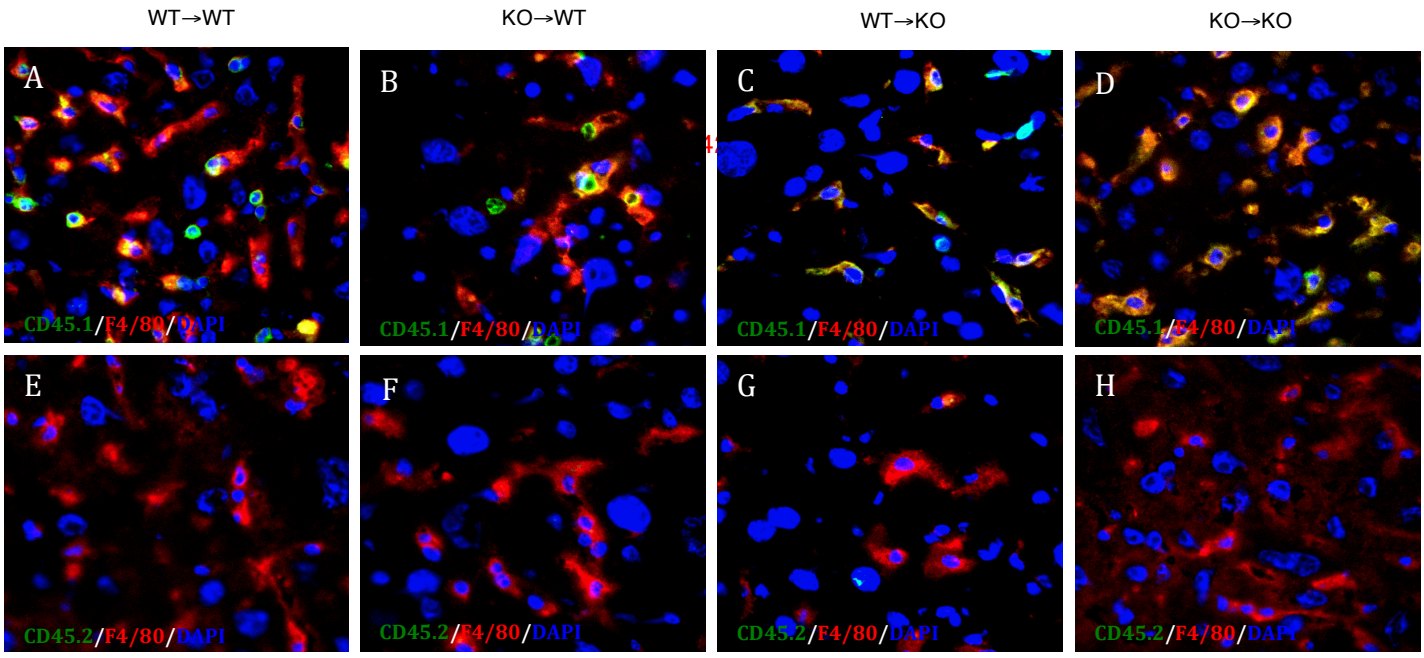


Figure 12: Successful macrophage replacement in the liver by donor bone marrow-derived cells. Histological examination of frozen liver sections stained for the leukocytes marker CD45.1 (green) (Donor) or CD45.2 (green) (recipient) and the macrophage marker F4/80 (red). (A, B, C, D) represents a complete replacement of macrophages derived from donor bone marrow and (E, F, G, H), represents complete deletion of endogenous (CD45.2) macrophages.

After the successful bone marrow transfer (Figure 10) and depletion of tissue resident macrophages (Figure 11). Bone marrow chimeric mice challenged with the chemical carcinogen DEN (*i.p)* (100mg/kg). All the mice that received DEN survived. Four weeks after the DEN injection, mice were given 0.07% phenobarbital in their drinking water until the end of the experiment.

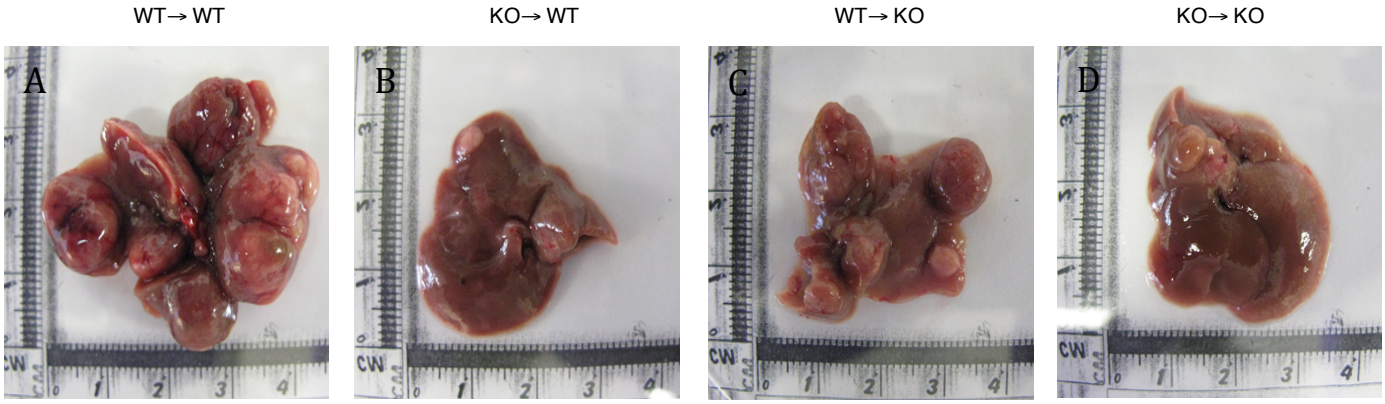


Figure 13: The loss of *Gadd45β* in haematopoietic compartment reduces DEN induced HCC. Liver images of adoptively transferred mice from protocol A, 13 months after the DEN (100mg/kg) treatment.

Mice were sacrificed 13 months after DEN injection, and their livers and lungs were analysed. The results of this analysis revealed that almost all the WT→WT and WT→KO mice developed HCC with large and visible tumours, which were present all over the liver (Figure 13 A and C). Strikingly, in KO→WT and KO→KO chimeric mice HCC development was poor (Figure 13 B and D). Tumours that are greater than 0.5mm in size were counted, and the tumour occupying area was measured using a digital calliper. In KO→KO mice, there was a three to four-fold reduced tumour number and tumour surface area as compared to WT→WT mice (Figure 14 A and B). These results are in line with those obtained in the initial DEN injection experiment (Figures 2 and 3). Correspondingly, the maximum tumour size in KO→KO mice was two-fold lower than in WT→WT mice (Figure 14 C), and so was the maximum surface area (Figure 14 D). HCC incidence was also dramatically reduced in KO→KO livers as compared to the WT→WT livers (Figure 16 A). The average liver weight were also significantly reduced in KO→KO mice compared to WT→WT mice; this is most likely due to the reduced HCC burden in KO→KO mice (Figure 16 B). Interestingly, in WT→KO mice, there was a significant three-fold increase in tumour numbers and average tumour surface area as compared to KO→KO mice (Figure 14 A). The maximum tumour size,

maximum surface area and percentage of liver tumour incidence were also dramatically increased (Figure 14 C, D and Figure 16 A). Similarly, the average liver weights were slightly, but not significantly increased in WT→KO mice compared to KO→KO mice (Figure 16 B). This increase in the liver weight could be because of infiltration of *Gadd45β*$^{+/+}$ bone marrow-derived immune cells into *Gadd45β*$^{-/-}$ liver that may have favoured the tumour growth.

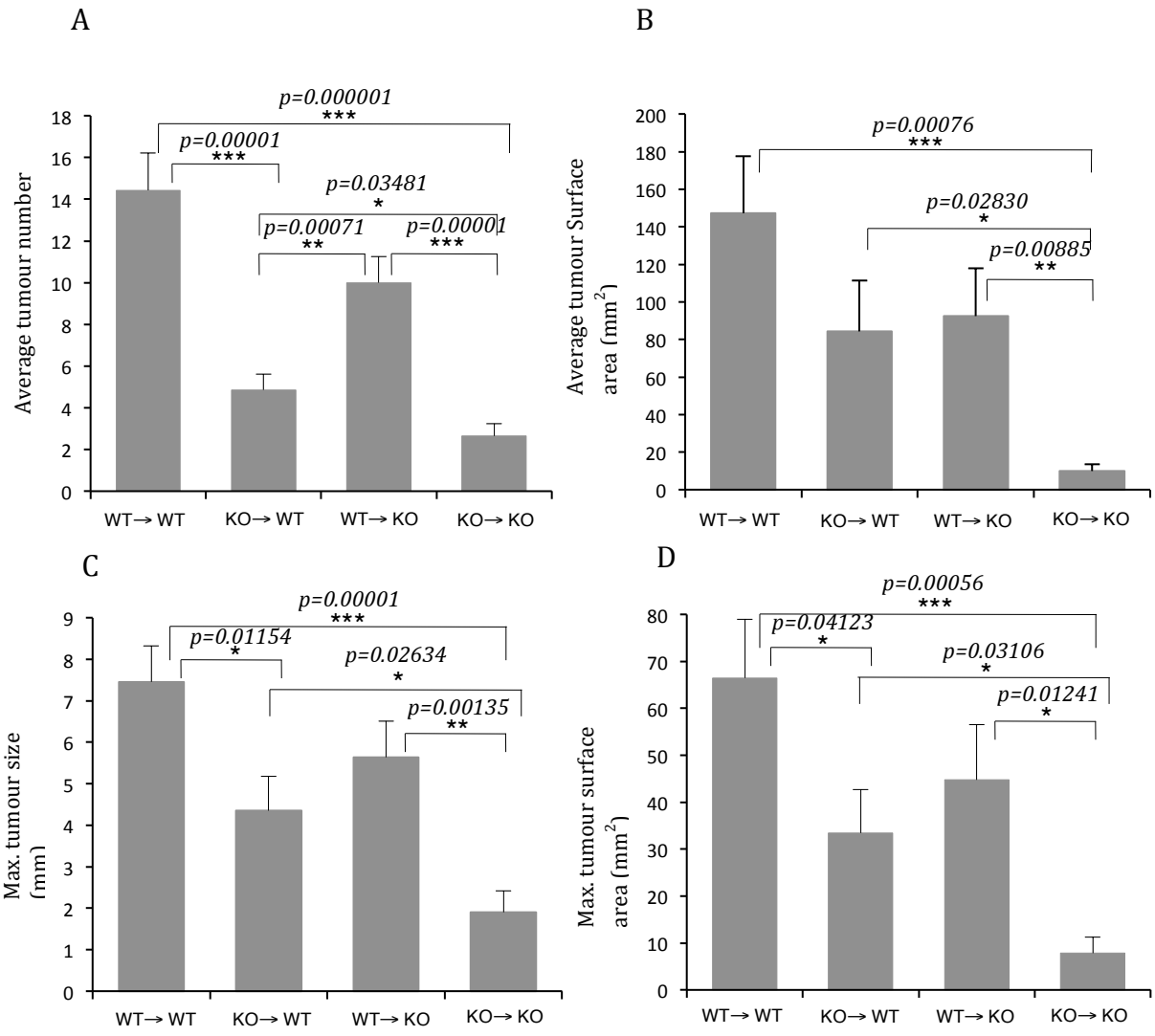


Figure 14: The loss of *Gadd45β* in haematopoietic cells reduces DEN induced HCC formation. Average tumour number (A), average tumour surface area (B), maximum tumour size (C) and maximum surface area of the tumours (D). Values denote mean ± SEM (wt→wt, n=40; ko→wt, n=36; wt→ko, n=35; ko→ko, n=25). *, $p<0.05$; **, $p< 0.01$; ***, $p<0.001$.

On the other hand, in the KO→WT mice there was a significant reduction in the tumour number by about three-folds compared to WT→WT and WT→KO mice (Figure 14 A). The maximum tumour size and maximum tumour surface area were also reduced significantly in KO→WT mice compared to WT→WT mice (Figure 14 C and D). This significant reduction in the HCC burden in KO→WT livers was evident by a slightly lower liver weight and also the percentage of liver tumour incidence (Figure 16 A and B). Finally, there was a significant increase in tumour number, tumour surface area, maximum tumour size and maximum tumour surface area in KO→WT mice compared to KO→KO chimeric mice.

The significantly lower HCC burden in KO→WT mice compared to WT→WT and WT→KO mice could be because of reduced proliferation of *Gadd45β*$^{+/+}$ hepatocytes because of reduced hepatic cytokine produced by *Gadd45β*$^{-/-}$ Kupffer cells. Thus, the presence of *Gadd45β*$^{-/-}$ bone marrow-derived cells in the *Gadd45β*$^{+/+}$ liver restricted the HCC development.

The increased tumour burden observed in WT→KO mice compared to KO→KO and KO→WT chimeric combination could be attributed to the role of *Gadd45β* in Kupffer cells to induce hepatic cytokine production in response to hepatocyte death. Based on these results, it is plausible that in WT→KO mice, after DEN exposure, there was an abundant hepatic cytokine production by *Gadd45β*$^{+/+}$ Kupffer cells collectively contributed to the increased HCC burden in WT→KO mice.

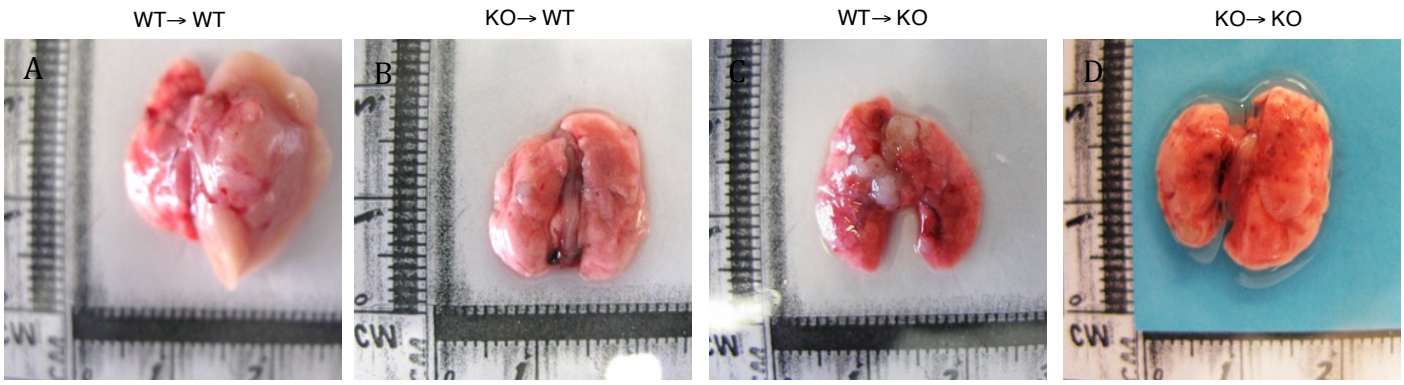


Figure 15: lung metastasis in the chimeric mice from experimental "protocol A" . Representative images showing the reduced lung metastasis in KO→WT and KO→KO bone marrow chimeric mice (C and D).

In line with the liver tumour incidence and HCC burden, the metastatic ability of HCC was also assessed in all the DEN treated bone marrow chimeric mice. The results revealed that the metastatic ability in KO→KO mice was two-fold

lower compared to WT→WT mice (Figures 15 and 16 C) and also the presence of *Gadd45β*$^{-/-}$ bone marrow-derived cells reduced the lung metastasis by two-fold in *Gadd45β*$^{+/+}$ mice (Figures 15 and 16 C). One the other hand, the presence of *Gadd45β*$^{+/+}$ bone marrow-derived cells in *Gadd45β*$^{-/-}$ mice increased lung metastasis by three-fold (Figures 15 and 16 C). In most of the cases, lung tumour mass was single rather than multiple, and the size of the tumour varied from 0.1 to 1 cm in diameter (Figure 15). Thus, the lung metastasis ability could be attributed to the severity of the HCC burden in the respective bone marrow chimeric combinations.

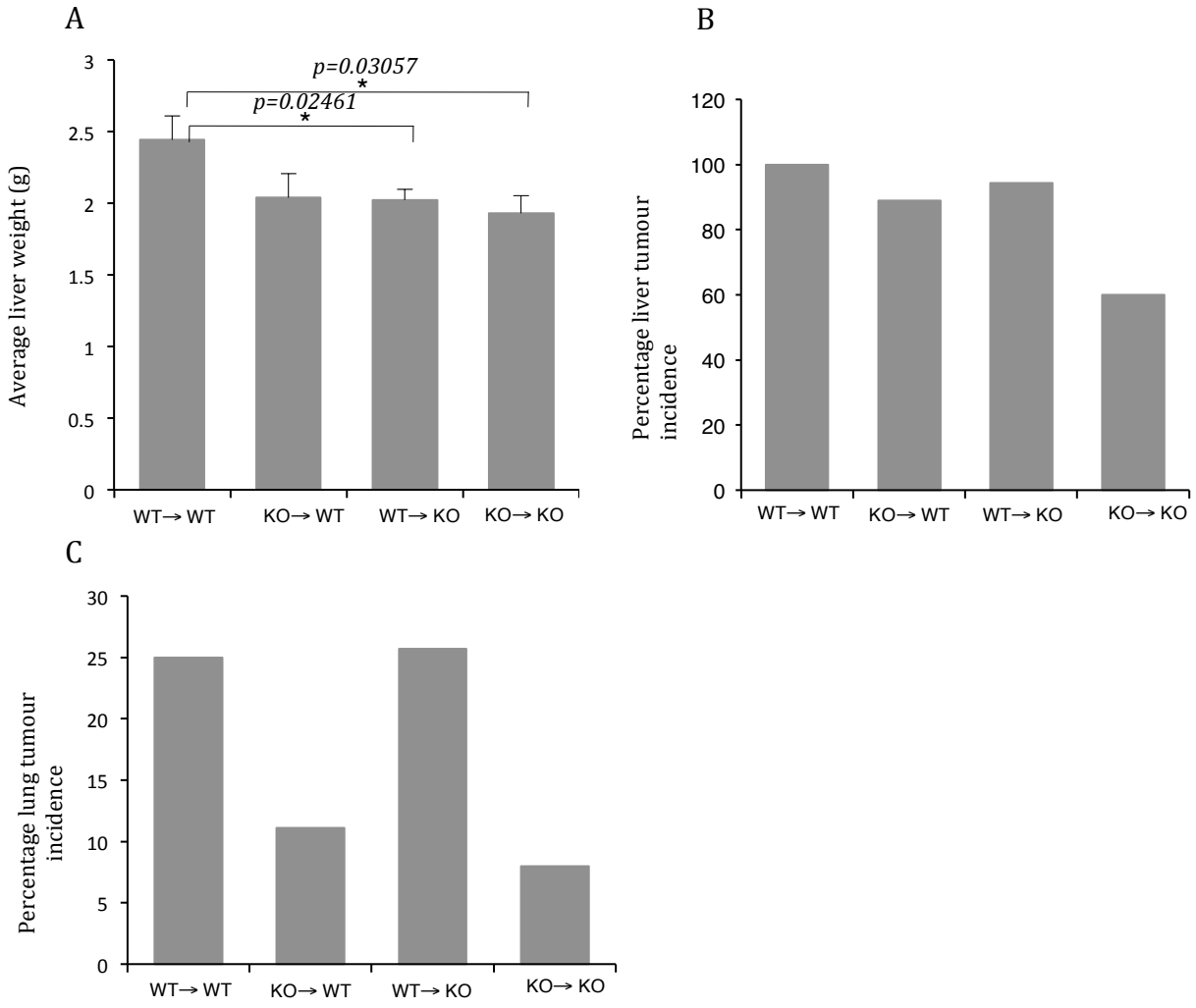


Figure 16: The loss of *Gadd45β* in haematopoietic cells reduces DEN induced HCC formation. (A) Average liver weight (B) Liver tumour incidence in adoptively transferred mice 13 months after DEN injection in the four possible BM chimeric combinations. Number of mice that has liver tumour in each BM chimeric combinations (WT→WT=40; KO→WT=32; WT→KO=33; KO→KO=15). (C) lung tumour incidence. Number of mice that has lung tumour in each BM chimeric combinations (WT→WT=10; KO→WT=4; WT→KO=9; KO→KO=2)Values denote mean ±SEM (wt→wt, n=40; ko→wt, n=36; wt→ko, n=35; ko→ko, n=25). *, $p<0.05$.

The results from "protocol A" suggested that *Gadd45β* exerts its tumourigenic action primarily within the inflammatory cells derived from the haematopoietic system. Loss of *Gadd45β* within these inflammatory compartments resulted in a reduced HCC burden and lung metastasis. However, the precise role of *Gadd45β* during tumour progression and whether the absence of *Gadd45β* in inflammatory cells can limit the growth of transforming neoplastic tissue to a fully-grown tumour, remain to be elucidated.

Since, "protocol A" could not distinguish between the role of *Gadd45β* in tumour initiation and tumour progression, "protocol B" was adopted to clarify this point (Figure 17).

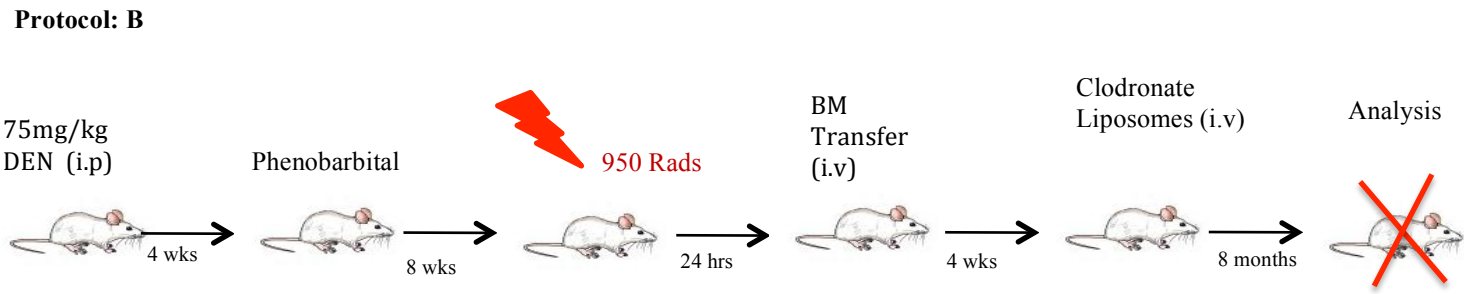


Figure 17: Schematic diagram of the bone marrow chimeric experimental "protocol B".

Four-week-old *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ male mice were injected with 75mg/kg DEN *i.p* and then administered 0.07% phenobarbital in their drinking water starting one month after the DEN injection. Eight weeks after, mice were lethally irradiated with 950 rads. Mice were then injected with five million bone marrow cells *i.v*, leading to four possible bone marrow chimeric combinations as described in "protocol A". From here onwards chimeric mice are represented as WT→WT, KO→WT, WT→KO and KO→KO respectively. Four weeks after the bone marrow adoptive transfer, liposome-encapsulated clodronate was injected *i.v* to deplete macrophages within the liver and spleen as shown in (Figure 11).

Bone marrow reconstitution was verified by FACS analysis. The results of this analysis revealed more than 90% reconstitution by donor bone marrow-derived cells. The percentage of reconstitution with donor bone marrow was comparable to that of "protocol A" (Figure 18). In order to verify the successful replacement of endogenous tissue resident macrophages with macrophages of donor bone marrow origin, frozen liver sections from the chimeric mice were stained with anti-CD45.1+anti-F4/80 and anti-CD45.2+anti-F4/80. Immunofluorescence revealed only anti-CD45.1+anti-F4/80 staining (Figure

19), suggesting that only donor bone marrow-derived (CD45.1) macrophages recruited into the liver might have played a key role in this model of DEN induced HCC.

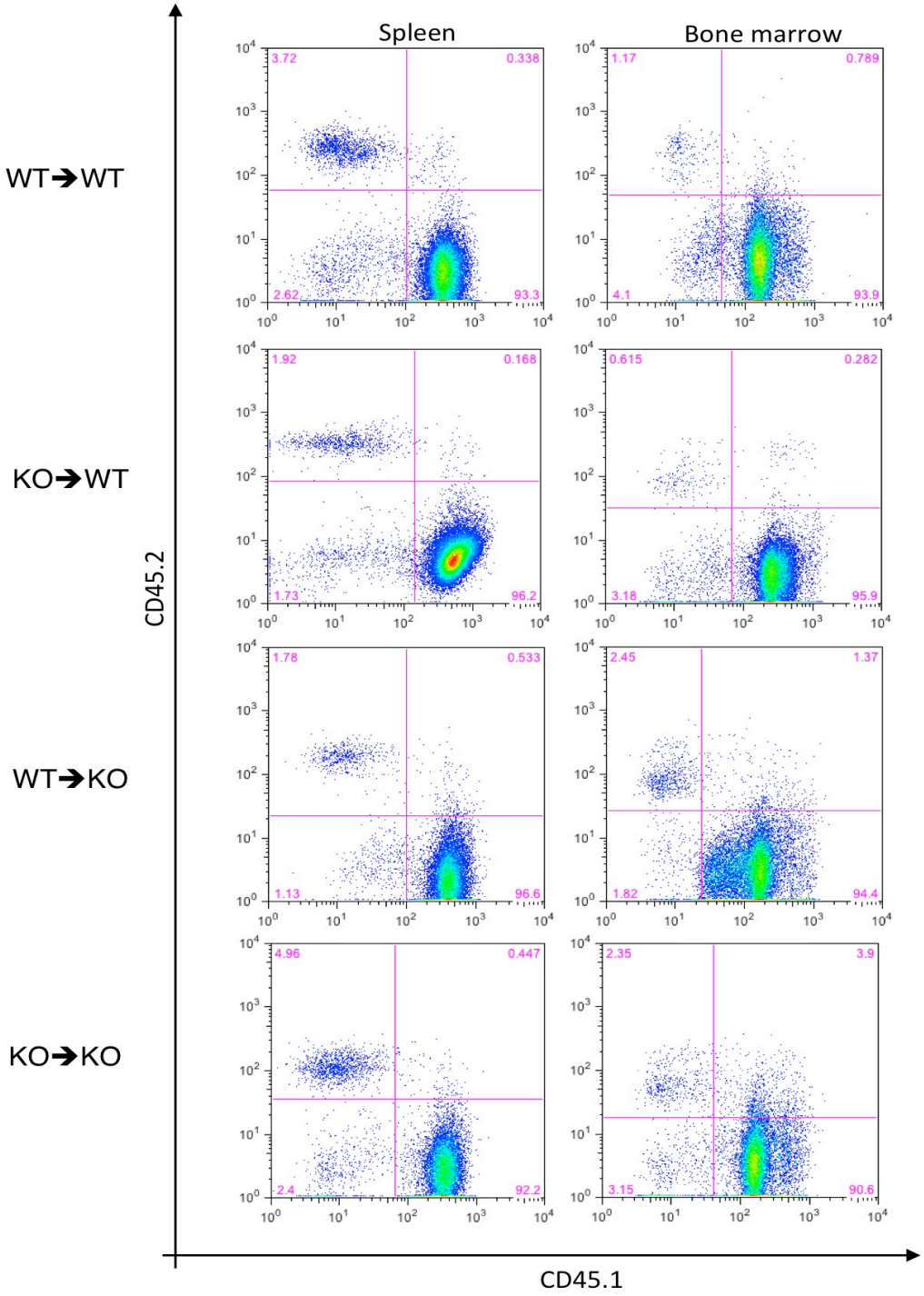

Spleen
Bone marrow
WT➔WT
KO➔WT
WT➔KO
KO➔KO
CD45.2
CD45.1
3.72
0.338
2.62
93.3
1.17
0.789
4.1
93.9
1.92
0.168
1.73
96.2
0.615
0.282
3.18
95.9
1.78
0.533
1.13
96.6
2.45
1.37
1.82
94.4
4.96
0.447
2.4
92.2
2.35
3.9
3.15
90.6

Figure 18: Bone marrow reconstitution. FACS analysis performed for the leukocyte markers CD45.1 (donor) and CD45.2 (recipient). The spleen and bone marrow were harvested and stained with anti-CD45.1 and anti-CD45.2 antibodies.

Twelve months after the DEN injection, most of the WT→WT and WT→KO mice had developed HCC with large and visible tumours (Figure 20). Tumours that were greater than 0.5mm were considered. The results revealed that in KO→KO mice, the average tumour number, total tumour surface area, maximum tumour size and maximum tumour surface area were approximately two-fold lower compared to WT→WT mice livers (Figure 21 A, B, C and D). On the other hand, in WT→KO mice livers, there was a significant increase in tumour number by two-fold and the tumour surface area had also increased significantly compared to KO→KO mice (Figure 21 A and B). The maximum tumour size, maximum surface area and percentage of liver tumour incidence were also dramatically increased in WT→KO mice compared to KO→KO (Figure 21 C, D and F).

Surprisingly, in KO→WT mice there was a significant reduction in tumour number and maximum tumour size by two-fold as compared to WT→WT mice (Figure 21 A and B). The maximum tumour size and maximum surface area of the

tumours of KO→WT mice were also significantly reduced compared to WT→WT mice. By contrast, in the WT→KO mice there were no significant reduction in maximum tumour size and maximum surface area (Figure 21 C and D). The percentage of liver tumour incidence was also reduced in KO→KO and KO→WT mice compared to WT→WT and WT→KO mice (Figure 21 F).

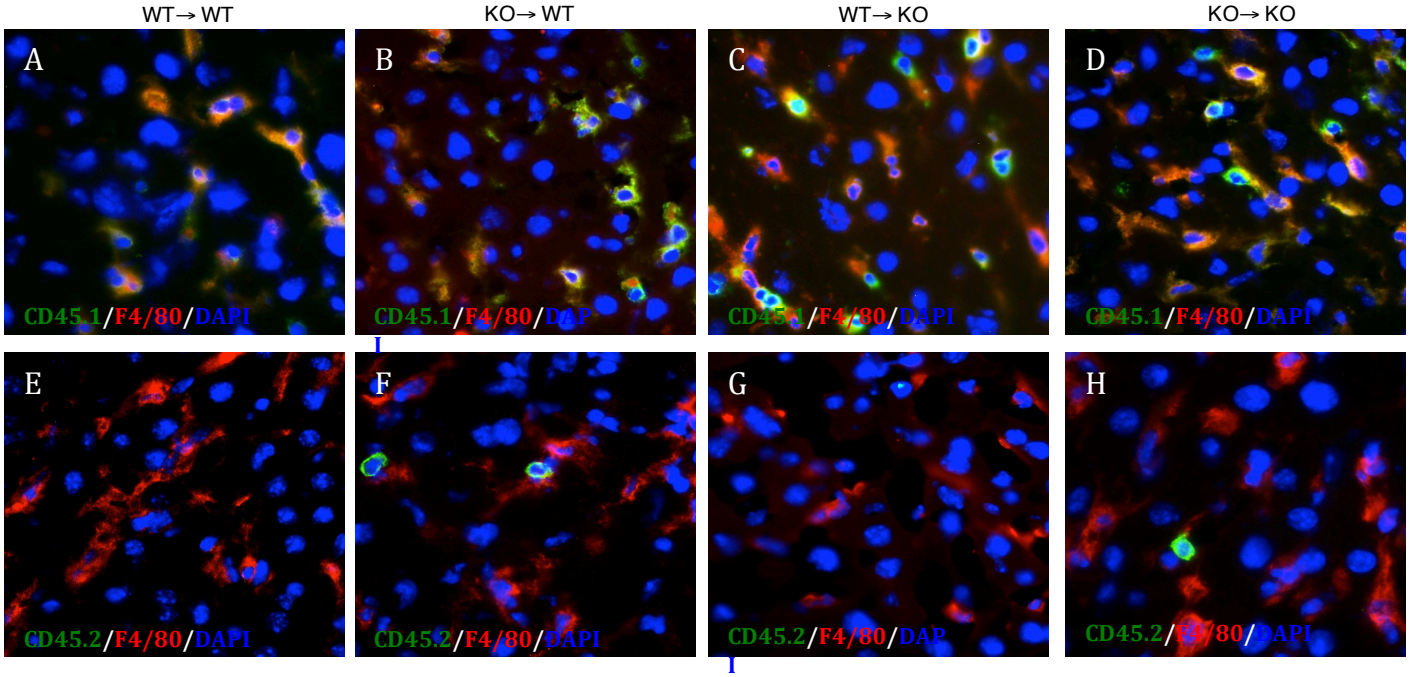


Figure 19: Macrophage replacement in the liver by donor bone marrow-derived cells. Histological examination of frozen liver sections stained for leukocytes marker CD45.1 green (Donor) or CD45.2 green (recipient), and macrophage marker, F4/80 (red). (A, B, C, D) Representative figures showing a complete replacement of liver the macrophage and (E, F, G, H), represents the complete deletion of endogenous (CD45.2) macrophages.

In line with the liver tumour incidence and HCC burden, the metastatic ability of HCC was also analysed in all the bone marrow chimeric mice. Results revealed that the metastatic ability of KO→KO mice was higher among all the combination of bone marrow chimeric mice (Figure 21 E). However, the presence of *Gadd45β*$^{-/-}$ bone marrow- derived cells reduced the lung metastasis in *Gadd45β*$^{+/+}$ recipient mice (Figure 21 E).

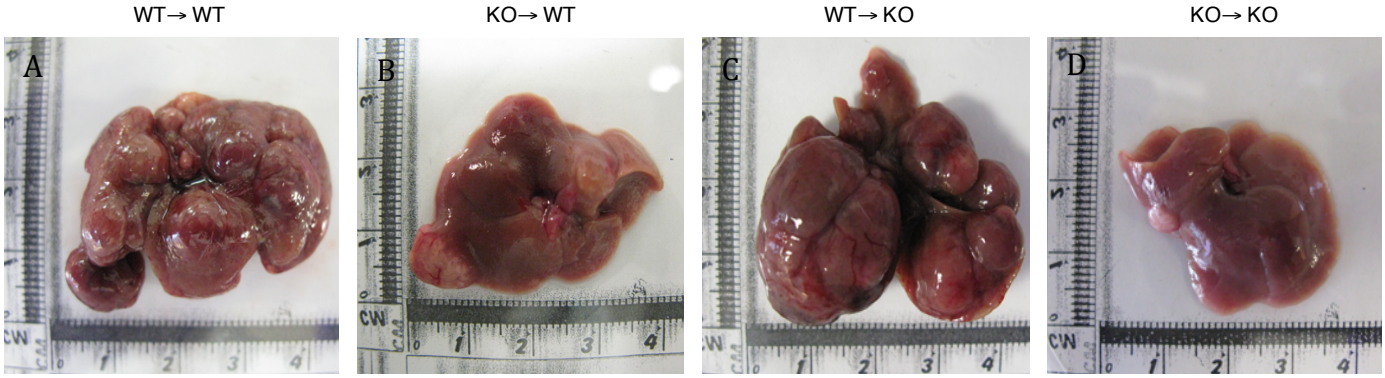


Figure 20: The loss of *Gadd45β* in haematopoietic cells during tumour progression reduces the DEN induced HCC development. Images of livers from "protocol B".

Interestingly, most of these results mirrored those obtained with “protocol A” . Results obtained using “ protocol B” suggest that *Gadd45β* exerts its tumourigenic role primarily within proinflammatory cells during tumour progression than in tumour initiation. Accordingly, *Gadd45β* deficiency diminished tumourigenesis in KO→WT bone marrow chimaeras, which contained *Gadd45β*$^{+/+}$ tissue only at the time of DEN injection, and *Gadd45β*$^{-/-}$ bone marrow-derived cell during tumour progression. By contrast, the inflammatory cells derived from *Gadd45β*$^{+/+}$ bone marrow supports the development of HCC even in the absence of *Gadd45β* within hepatocytes.

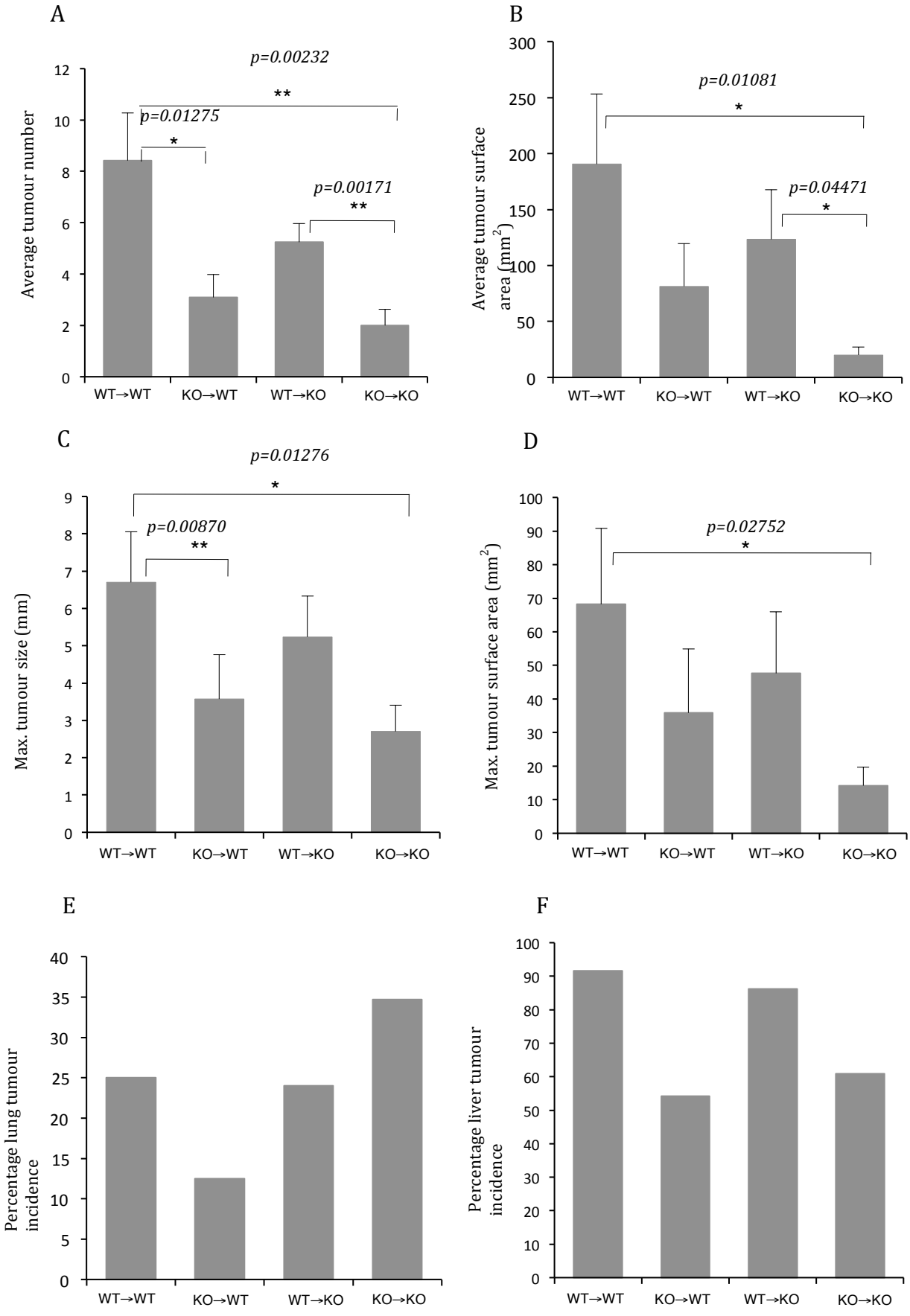

A
p=0.00232
**
p=0.01275
*
p=0.00171
**
Average tumour number
0
2
4
6
8
10
12
WT→WT
KO→WT
WT→KO
KO→KO
B
p=0.01081
*
p=0.04471
*
Average tumour surface area (mm2)
0
50
100
150
200
250
300
WT→WT
KO→WT
WT→KO
KO→KO
C
p=0.01276
*
p=0.00870
**
Max. tumour size (mm)
0
1
2
3
4
5
6
7
8
9
WT→WT
KO→WT
WT→KO
KO→KO
D
p=0.02752
*
Max. tumour surface area (mm2)
0
10
20
30
40
50
60
70
80
90
100
WT→WT
KO→WT
WT→KO
KO→KO
E
Percentage lung tumour incidence
0
5
10
15
20
25
30
35
40
WT→WT
KO→WT
WT→KO
KO→KO
F
Percentage liver tumour incidence
0
10
20
30
40
50
60
70
80
90
100
WT→WT
KO→WT
WT→KO
KO→KO

Figure 21: The loss of *Gadd45β* in haematopoietic cells during tumour progression reduces DEN induced HCC formation. Average tumour number (A), average tumour surface area (B), maximum tumour size (C), maximum tumour surface area (D), percent lung tumour incidence, number of mice that has lung tumour in each BM chimeric combinations (WT→WT=6; KO→WT=3; WT→KO=7; KO→KO=8) (E) and percent liver tumour incidence, number of mice that has liver tumour in each BM chimeric combinations (WT→WT=22; KO→WT=13; WT→KO=25; KO→KO=14) (F) of bone marrow chimeric mice from "protocol B". Values denote mean $\pm$ SEM (wt→wt, n=24; ko→wt, n=24; wt→ko, n=29; ko→ko, n=23;). *, $p<0.05$; **, $p< 0.01$.

4.4 The reduced MCA-203 tumour growth in *Gadd45β* knockout mice

To better understand the role of NF-κB-*Gadd45β* pathway that has promoted DEN induced HCC progression, and to further investigate the precise mechanism by which *Gadd45β* promoted tumour progression. We used an allograft tumour model, in which the role of tumour infiltrating inflammatory cells was very well established during tumour progression (Elinav *et al.,* 2013). In this model, any difference in the tumour growth observed between *Gadd45β*$^{-/-}$ and *Gadd45β*$^{+/+}$ mice would depend exclusively on the role that *Gadd45β* plays in the tumour infiltrating inflammatory cells.

The allograft experiment was performed by a single subcutaneous injection of MCA-203 murine fibrosarcoma tumour cells into *Gadd45β*$^{+/+}$ (WT) and *Gadd45β*$^{-/-}$ (KO) mice. The injection of MCA-203 cells resulted in a tumour growth in all the injected mice (Marigo *et al.,* 2010). In *Gadd45β*$^{+/+}$ mice, tumours grew rapidly compared to *Gadd45β*$^{-/-}$ mice as evidenced by measuring the tumour volume every other day using a digital calliper (Figure 22 A). Throughout the experimental period, the tumour growth in *Gadd45β*$^{-/-}$ mice was reduced as compared to *Gadd45β*$^{+/+}$ mice (Figure 22 A). Nearly three weeks after the tumour cell injection, spleens and tumours were excised from tumour bearing mice. The reduced tumour growth observed in *Gadd45β*$^{-/-}$ mice was confirmed by counting the total number of cells isolated from tumours after enzymatic digestion (Figure 22 C). In all tumour bearing *Gadd45β*$^{+/+}$ mice, spleens were enlarged, and the total number of splenocytes

isolated were significantly higher compared to the number of cell isolated from tumour bearing *Gadd45β*$^{-/-}$ mice (Figure 22 B). Previous studies have shown that, the spleen contributes to the tumour growth in this model by acting as a reservoir for the tumour infiltrating inflammatory cells (Cortez-Retamozo *et al.,* 2012). It was also shown that there was a significant reduction in tumour growth in the splenectomised mice (Cortez-Retamozo *et al.,* 2012). The impaired tumour progression observed in *Gadd45β*$^{-/-}$ mice was associated with a smaller spleen size and lower spleen cell number consistent with the slow growth of MCA-203 tumour in these mice.

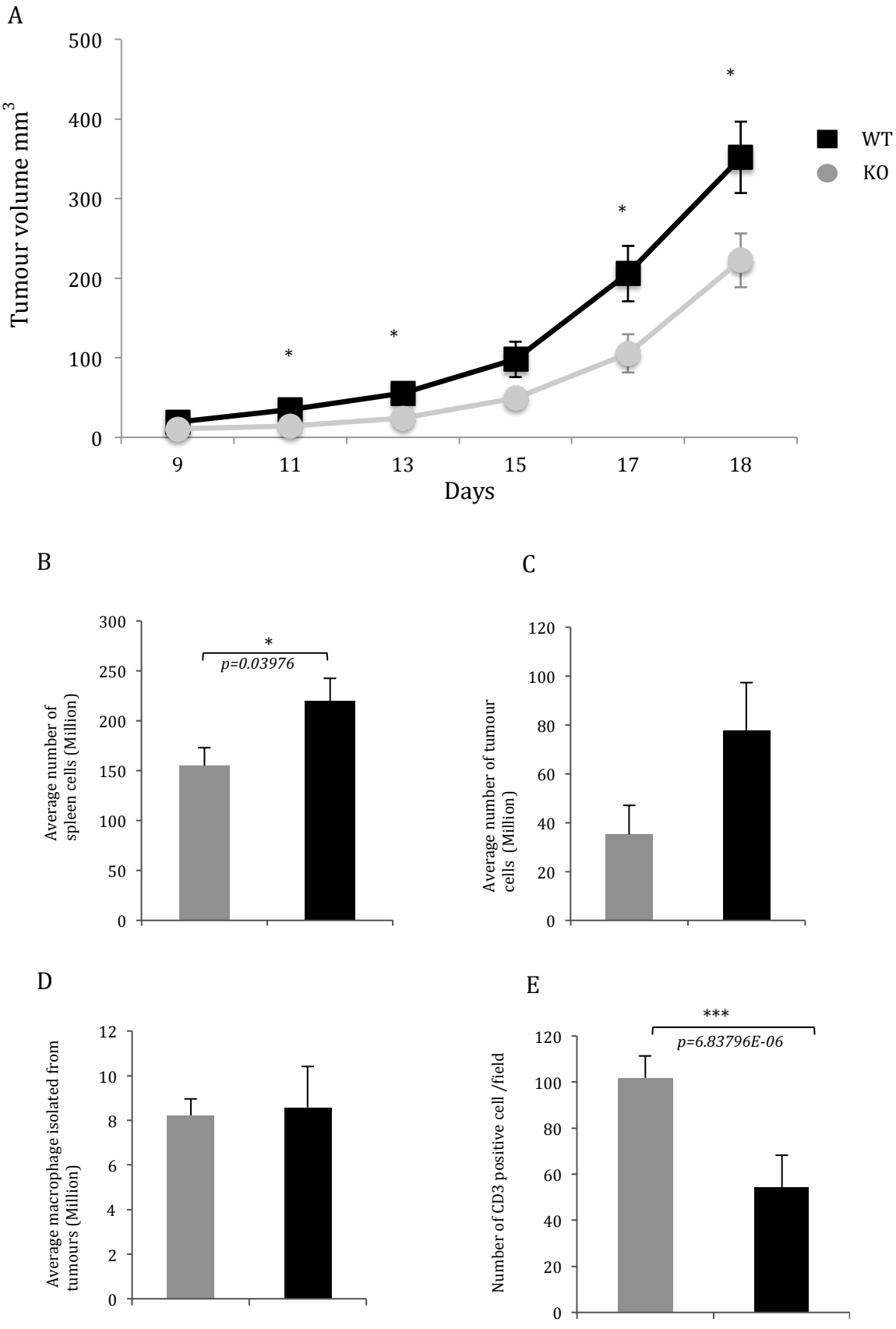

A
Tumour volume mm3
500
400
300
200
100
0
9
11
13
15
17
18
Days
*
*
*
*
WT
KO
B
Average number of spleen cells (Million)
300
250
200
150
100
50
0
*
p=0.03976
C
Average number of tumour cells (Million)
120
100
80
60
40
20
0
D
Average macrophage isolated from tumours (Million)
12
10
8
6
4
2
0
E
Number of CD3 positive cell /field
120
100
80
60
40
20
0
***
p=6.83796E-06

Figure 22: The reduced tumour growth in *Gadd45β*$^{-/-}$ mice. *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ mice were injected *s.c* with MCA-203 tumour cells, and tumour volumes were measured alternative days until day 18. (A) Graph showing the tumour volumes in *Gadd45β*$^{+/+}$ (n=10) and *Gadd45β*$^{-/-}$ (n=9. (B) Average number of spleen cells isolated from *Gadd45β*$^{+/+}$ (n=7) and *Gadd45β*$^{-/-}$ (n=8) tumour bearing mice. (C) Average number of cells isolated from *Gadd45β*$^{+/+}$ (n=7) and *Gadd45β*$^{-/-}$ (n=7) tumours after enzymatic digestion. (D) Average number of macrophages isolated from *Gadd45β*$^{+/+}$ (n=8) and *Gadd45β*$^{-/-}$ (n=6) tumours using CD11b magnetic beads. (E) Number of cells stained positive for CD3 marker within the *Gadd45β*$^{+/+}$ (n=3) and *Gadd45β*$^{-/-}$ (n=3) tumours, examined by immunohistochemical analysis. Values denote mean $\pm$ SEM.*, $p<0.05$; ***, $p<0.001$.

Next, to determine the composition of the tumour inflammatory infiltrates in *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ tumours. We performed immunohistochemical analysis of serial frozen sections of excised MCA-203 fibrosarcoma tumours. The analysis revealed that there were significantly high in numbers of CD3^{+} T-cells in *Gadd45β*$^{-/-}$ tumours compared to *Gadd45β*$^{+/+}$ tumours (Figure 22 E). These analyses suggest that there could be possible differences in the levels of recruitment of other tumour infiltrating inflammatory mediators (Saccani *et al.,* 2006). In addition to CD3 T-cell, tumour-infiltrating neutrophils (Gr-1), macrophages (F4/80) and B-cell (B220) (Figure 23 A, B, C) that are generally associated with inflammation within the tumours were also investigated (Galon *et al.,* 2006; Mantovani *et al.,* 2008; Fridlender *et al.,* 2009; Ammirante *et al.,* 2010). The results of these analyses revealed no significant difference in the number of tumour infiltrating B-cells or neutrophils in the centre and the periphery of *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ tumours (Figure 23 A and B).

By contrast, in *Gadd45β*$^{-/-}$ mice there was significantly high number of F4/80^{+} cells at the periphery but not in the centre of the tumour as compared to *Gadd45β*$^{+/+}$ mice (Figure 23 C). However, there were no obvious differences in the total number of macrophages that were infiltrating into the tumour (Figure 22 D). This was evident by counting the number of macrophages that were isolated from tumours using CD11b micro-beads (Figure 22 D). The recruitment of macrophages into the tumour and its role during tumour progression has been documented in several studies (Qian *et al.,* 2010; Doedens *et al.,* 2010; Qian *et al.,* 2011; Bingle, 2002). But the precise role of the macrophage subtypes in tumour development has only recently been appreciated. M1 macrophages are considered as anti-tumour and proinflammatory that are characterised by the expression of high levels of inflammatory gene such as iNOS, IL-12, COX-2, CXCL-9, CXCL-10 and CXCL-11 (Martinez *et al.,* 2006; Biswas *et al.,* 2006; Porta *et al.,* 2009; Movahedi *et al.,* 2010). Conversely, pro-tumour and anti-inflammatory M2 macrophages express high levels of anti-inflammatory genes such as ARG-1, CD206, HO-1 and IL-10 (Martinez *et al.,* 2006). The anti-tumour properties of M1 macrophages are linked to the upregulation of proinflammatory gene iNOS that generates nitric oxide that acts as a tumouricidal agent (MacMicking *et al.,* 1997).

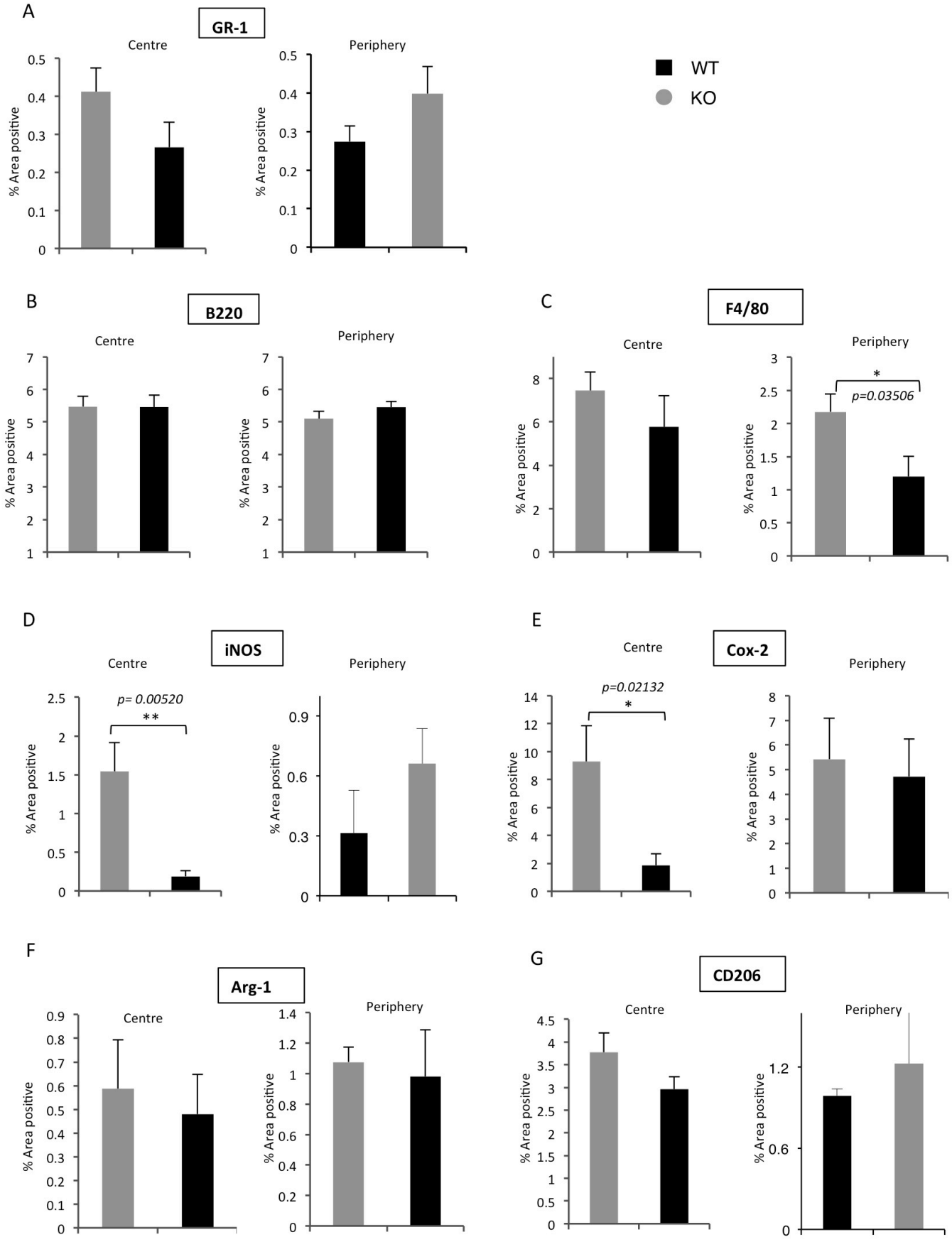

A
GR-1
Centre
Periphery
% Area positive
WT
KO
B
B220
C
F4/80
*
p=0.03506
D
iNOS
p= 0.00520
**
E
Cox-2
p=0.02132
*
F
Arg-1
G
CD206

Figure 23: Histological examination of the inflammatory infiltrates of MCA-203 tumours from *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ mice. Serial frozen sections from *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ tumours were stained with antibodies for tumour infiltrating inflammatory mediators. The percentages of positive areas were quantified: Gr-1, Neutrophils; B220, B-cells; F4/80, macrophages (A, B, C). M1 macrophage markers iNOS and Cox-2 (D, E). M2 macrophage markers Arg-1 and CD206 (F, G). Values denote mean SEM± (*Gadd45β*$^{+/+}$, n =7 and *Gadd45β*$^{-/-}$, n=8). *, p<0.05; **, p< 0.01.

Based on the above results, we hypothesised that the reduced tumour growth observed in *Gadd45β*$^{-/-}$ mice could be associated with the increased number of tumouricidal macrophages. This, prompted us to quantify the different subsets of the macrophage both M1 and M2 that are infiltrating into the *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ tumours. Macrophage markers were evaluated on the frozen tumour sections by immunohistochemistry, iNOS and COX-2 were chosen as M1 markers, and Arg-1 and CD206 were chosen as M2 marker. Immunohistochemical analysis for M1 and M2 macrophage markers have revealed that, in *Gadd45β*$^{-/-}$ tumours there were significantly high number of areas that were stained positive for macrophage M1 marker iNOS and COX-2 only at the centre of the tumour as compared to *Gadd45β*$^{+/+}$ tumours (Figure 23 D and E). From this observation, it is plausible to hypothesise that the high number of accumulation of M1 macrophages at the centre of *Gadd45β*$^{-/-}$ tumours may have played a detrimental role in reducing tumour growth in the *Gadd45β*$^{-/-}$ mice. By contrast, there was no significant difference in the number of areas within the tumour that were stained for macrophage M2

associated marker Arg-1 and CD206 in *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ tumours (Figure 23 F and G).

Collectively, these results suggest that the loss of *Gadd45β* promotes M1 macrophage polarisation and also facilitate the accumulation of M1 macrophages within the tumour. From the above results, it is evident that *Gadd45β* promoted tumour growth during tumour progression by acting primarily during the tumour infiltrating macrophages and by polarising macrophages towards M1 like phenotype without affecting M2 phenotype. This data established a critical role for *Gadd45β* in tumour progression within the proinflammatory compartment.

4.5 The loss of *Gadd45β* promotes M1 macrophage differentiation *in vitro*

Previous studies have shown that macrophages are one of the major cell types that are important for HCC development as well as development of other cancers (Kuang *et al.,* 2007; Cheng *et al.,* 2007; Kuang *et al.,* 2009; Lin *et al.,* 2006). Examining these cells has become important because of the recent discovery suggesting that IKKβ a subunit of IKK complex is important for the differentiation and function of so-called tumour associated macrophages (TAMs) which resemble M2 macrophages (Hagemann *et al.,* 2008). TAMs have been shown to promote tumour growth, angiogenesis and metastasis in various types of cancers. (Biswas and Mantovani, 2010). Results obtained from MCA-203 allograft tumour model suggest that *Gadd45β* promotes tumour development possibly by regulating macrophage phenotype.

Further, to investigate the role of *Gadd45β* in macrophage differentiation *in vitro* macrophage co-culture experimental system was used. Experiments were set up using bone marrow- derived macrophages from both $Gadd45\beta^{-/-}$ and $Gadd45\beta^{+/+}$ mice. These experiments would enable us help to understand the role of *Gadd45β* in macrophages.

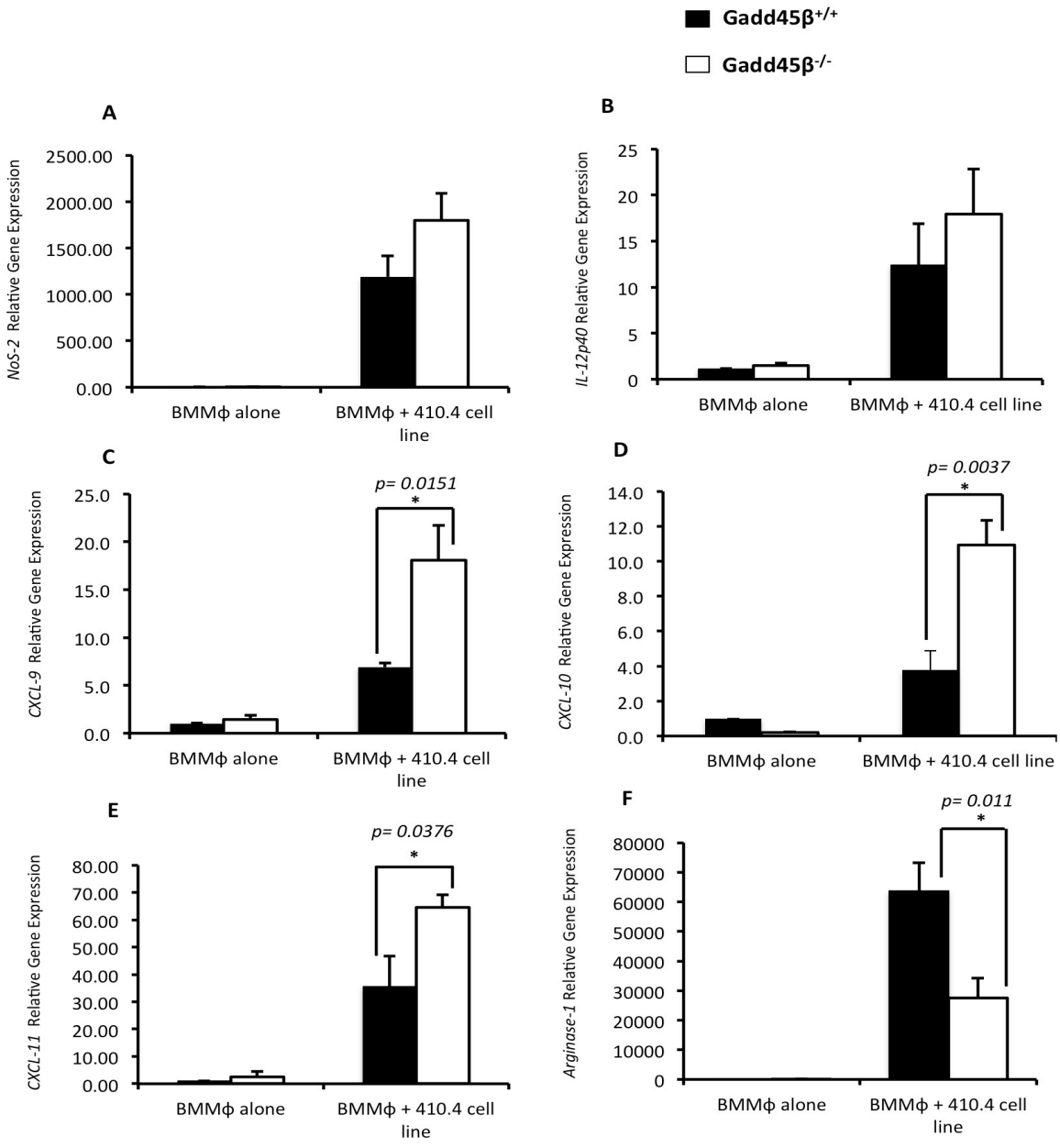


Figure 24: Quantitative real-time PCR analysis of gene expression in a macrophage co-culture system. Increased levels of M1 related proinflammatory genes in *Gadd45β*$^{-/-}$ macrophages upon co-culture with murine mammary adenocarcinoma 410.4. mRNA levels of M1 macrophage associated genes Nos-2, Il-12p40, CXCL-9, CXCL-10

and CXCL-11 (A, B, C, D, and E). mRNA levels of M2 macrophage associated gene Arg-1 (F). Values denote mean ± SEM (*Gadd45β*$^{+/+}$, n=5 and *Gadd45β*$^{-/-}$, n=5). *, p<0.05.

BM-derived macrophages upon co-culture with murine tumour cells influenced the cytokine profile of macrophages. Suggesting a direct cell-to-cell contact was not necessary for the observed effect (Figure 24). Co-culture experiment was set up using BD-derived macrophages from *Gadd45β*$^{-/-}$ and *Gadd45β*$^{+/+}$ mice with murine mammary tumour cells 410.4. Quantitative real-time PCR analysis was performed to examine the expression of both M1 and M2 macrophage associated genes. The analysis revealed that *Gadd45β*$^{-/-}$ macrophages displayed an increased expression of classically activated M1 macrophage associated proinflammatory genes iNOS, IL-12B, CXCL9, CXCL10 and CXCL11 (Figure 24 A-E). Consequently, the high expression of M1 macrophages related genes in *Gadd45β*$^{-/-}$ BMDM were associated with the decreased expression of Arg-1, a typical M2 associated gene (Figure 24 F). This data suggests that the loss of *Gadd45β* in BM-derived macrophages switched macrophages from a tumour promoting M2 phenotype to tumouricidal M1 macrophage phenotype upon co-culture with 410.4 tumour cells (Figure 24). Similar results were also obtained upon macrophage co-culture with MN/MCA-1 murine fibrosarcoma tumour cells (Figure 25). Compared to *Gadd45β*$^{+/+}$ BM-derived macrophage, *Gadd45β*$^{-/-}$ macrophages exhibited an increased polarisation towards “classically” activated M1 macrophages and a reduced polarisation towards “alternatively” activated

M2 macrophages resembling TAMs that promote tumourigenesis. These results are consistent with previous results obtained with IKKβ$^{-/-}$ macrophage co-culture experimental system and suggest that *Gadd45β* is an essential downstream target of the IKKβ/NF-κB pathway in controlling macrophage polarisation (Hagemann *et al.,* 2008).

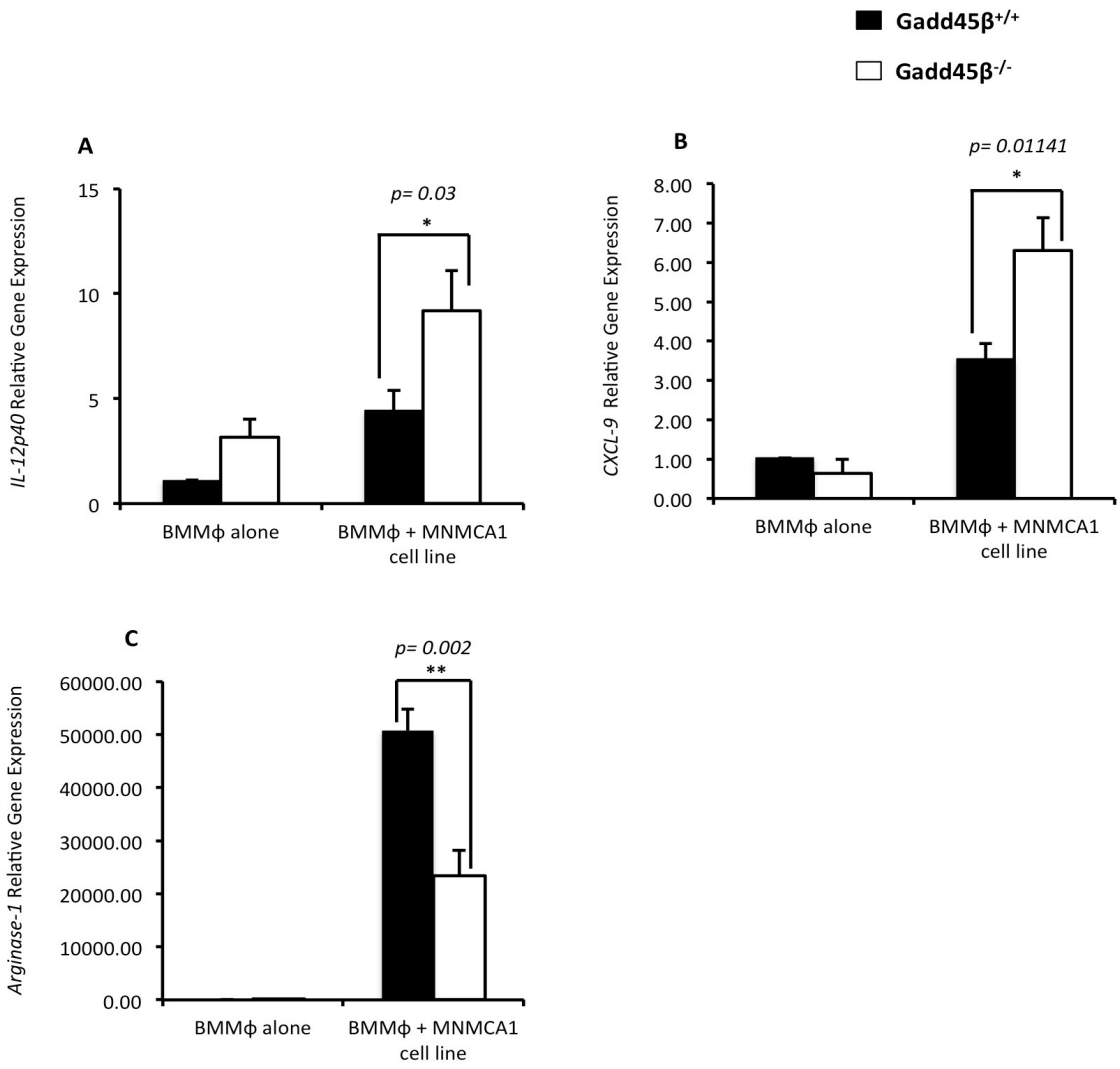


Figure 25: Quantitative real-time PCR analysis of gene expression in a macrophage co-cultures system. Upregulation of macrophage M1 markers in *Gadd45β*$^{-/-}$ macrophage upon co-culture with the murine fibrosarcoma cell line MN/MCA-1, levels of M1 macrophage associated inflammatory genes Il-12p40 and CXCL-9, (A and B).

Levels of M2 macrophage associated gene Arg-1 (C). Values denote mean ± SEM (*Gadd45β*$^{+/+}$, n=5 and *Gadd45β*$^{-/-}$, n=5). *, p<0.05; **, p< 0.01.

The results obtained from co-culture system suggest that *Gadd45β*$^{-/-}$ BM-derived macrophages upon co-culture with tumour cells elicited a hyper-production of proinflammatory cytokine that promotes tumouricidal M1 macrophage phenotype. However, the mechanism by which *Gadd45β* acts primarily in the macrophages remains to be investigated.

4.6 The loss of *Gadd45β* promotes M1 macrophage polarisation upon stimulation with cytokines *in vitro*

To further investigate the role of *Gadd45β* in macrophages polarisation, we used an *in vitro* experimental model system of macrophage polarisation by cytokines stimulation. Experiments were set up using an established macrophage polarisation protocols (Hagemann *et al.,* 2008). Stimulating macrophages with LPS will lead to the greatest induction of inflammatory cytokines than the macrophages in any other culture conditions (Holt *et al.,* 2010).

In the first step, to establish an *in vitro* macrophage polarisation protocols, *Gadd45β*$^{+/+}$ BM-derived macrophages were stimulated with either LPS/IFN-gamma or IL-4/IL-3. Results from the real-time PCR analysis revealed that there was an increased M1 associated cytokines eight hours after LPS/IFN-gamma stimulation compared to unstimulated *Gadd45β*$^{+/+}$ BM-derived macrophages (Figure 26 A-E). On the other hand, after IL-4/IL-13 stimulation there was an increased M2 associated cytokine levels in *Gadd45β*$^{+/+}$ BM-derived macrophage (Figure 26 F and G). This data suggests that, the culture conditions that were used results in a complete polarisation of BM-derived macrophages to either M1 or M2 phenotype depending on the cytokines used to stimulate.

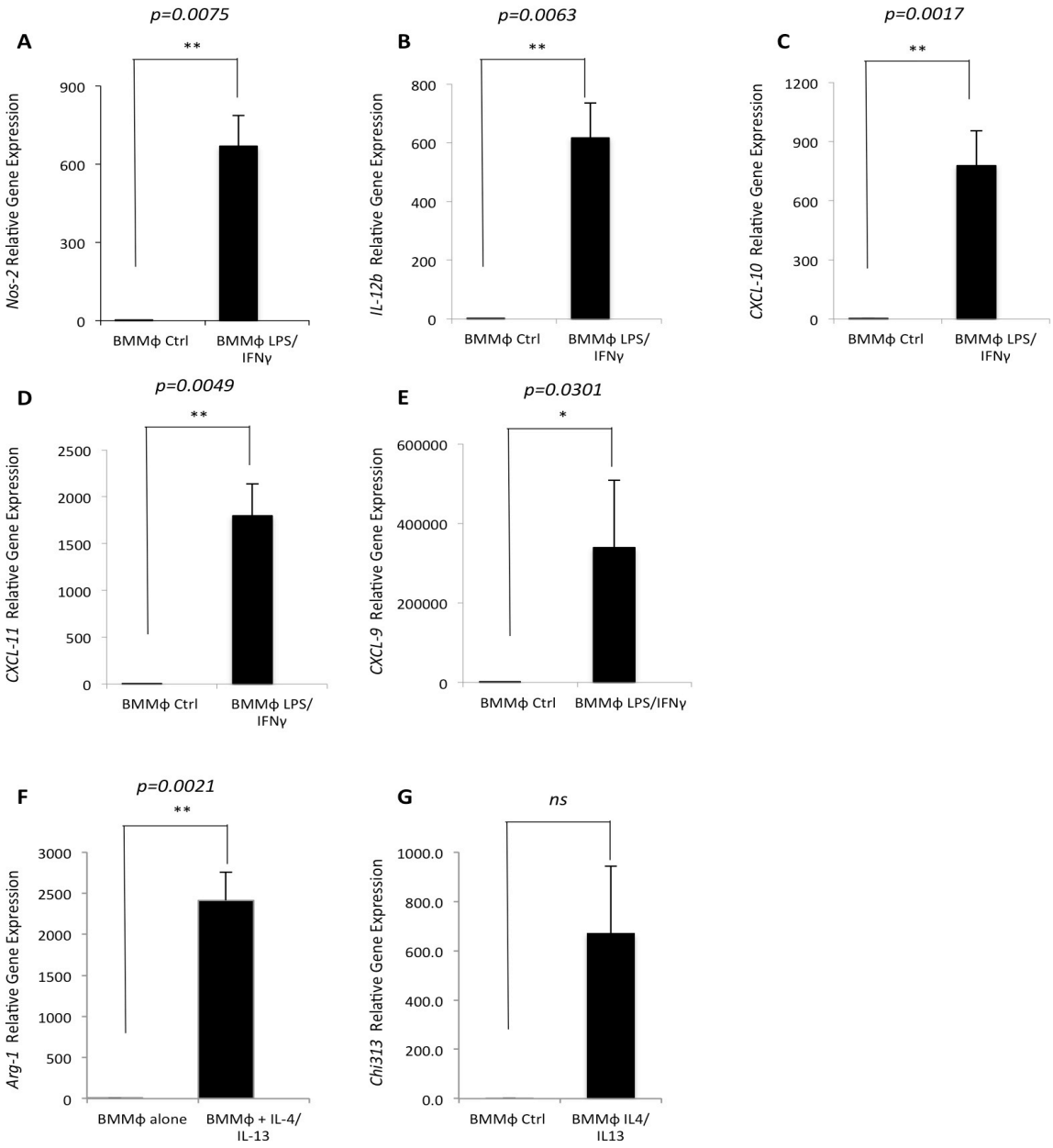


Figure 26: Cytokine stimulation promotes the expression of macrophage lineage-specific cytokines. Bone marrow-derived macrophages from *Gadd45β*$^{+/+}$ mice stimulated with either LPS/IFN-gamma or IL-4/IL-3. Eight hours after the stimulation, total RNA was isolated and qRT-PCR analysis was performed for macrophage

lineage-specific cytokines. (A, B, C, D and E) Real-time PCR analysis showing the relative gene expression of BM-derived macrophages stimulated LPS/IFN-gamma. (F and G) Real-time PCR analysis showing the relative gene expression BM-derived macrophages stimulated IL-4 and IL-13.

To evaluate *Gadd45β's* role in macrophage polarisation under cytokine-stimulated conditions, BM-derived macrophages from *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ mice were polarised *in vitro* to either the M1 or M2 macrophage phenotypes. M1 macrophages express high levels of proinflammatory cytokine genes iNOS, IL-12B, CXCL9, CXCL10, and CXCL11. On the other hand, M2 macrophage phenotype that was induced by IL-4/IL-3 stimulation is characterised by the expression of Arg-1, CD206 and Chi3l3. Macrophages from *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ mice exhibited similar morphology in each of the culture conditions and time points. Total RNA was extracted eight hours after cytokine stimulation, and the expression of respective M1 and M2 genes was quantified by qRT-PCR. The results revealed that there was a much strong induction of iNOS, IL-12b, CXCL10 and CXCL11 in *Gadd45β*$^{-/-}$ macrophages compared to *Gadd45β*$^{+/+}$ macrophages at the eight hour stimulation with LPS/IFN-gamma (Figure 27 A-D), this induction of M1 related genes were very strong and reproducible in *Gadd45β*$^{-/-}$ macrophages. By contrast, there was no significant difference in the expression of CXCL-9 chemokine (Figure 27 E). Upon stimulation with LPS/IFN-gamma macrophages exhibited high *Gadd45β* expression (Figure 27 F), suggesting that the upregulation of *Gadd45β* expression in macrophage plays a critical

role in the inhibition of M1 macrophage polarisation. Most of the results obtained by cytokine-driven macrophage polarisation were consistent with those obtained in macrophage-tumour cell co-culture experiments and suggested an essential role for *Gadd45β* in restraining the development of M1 macrophage (Figure 24 and 25). It is, therefore, plausible to expect similar results by using cytokine-driven polarisation. Surprisingly, the stimulation of BM- derived macrophage with IL-4/IL-13 revealed that the macrophage M2 associated genes Arg-1, CD206 and Chi3l3 expression displayed no obvious difference between both the genotypes (Figure 28). The reason for this observed differences in the results obtained from co-culture system to the results obtained using cytokine-driven polarisation results could be because of the difference in the model system used.

The above data shows that loss of *Gadd45β* in macrophage facilitates the polarisation effectively towards M1 phenotype, whereas loss of *Gadd45β* in macrophage had no effect on M2 phenotype.

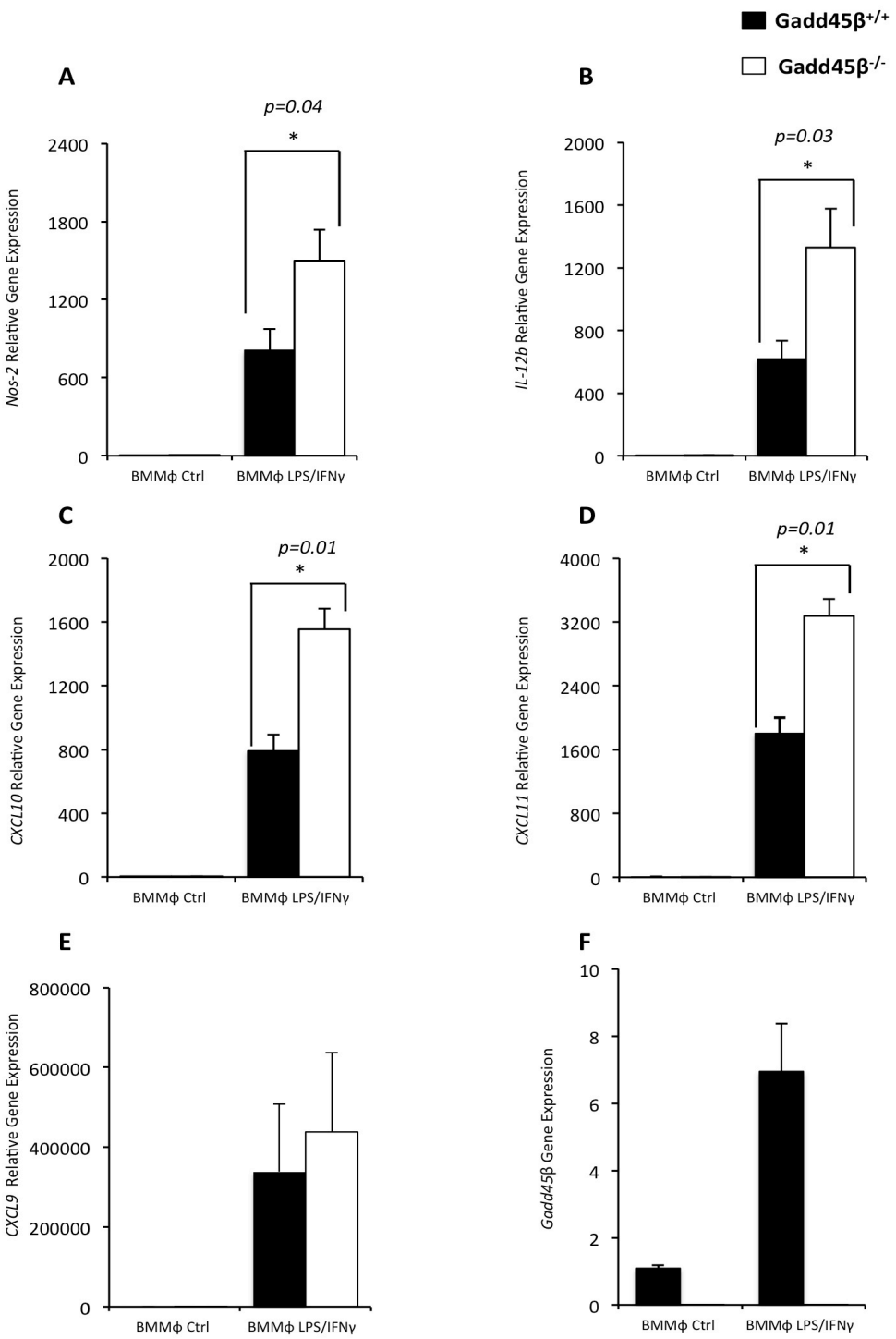


Figure 27: The *In vitro* polarisation of bone marrow- derived macrophage towards the M1 phenotype. Bone marrow-derived macrophages from *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ mice stimulated with LPS/IFN-gamma. (A, B, C and D) Quantitative real-time PCR analysis showing the increased expression of M1 macrophage

associated genes in *Gadd45β*$^{-/-}$ macrophages upon cytokine stimulation. (F) *Gadd45β* upregulation eight hours after stimulation with *LPS/IFN-gamma.* Values denote mean ± SEM (*Gadd45β*$^{+/+}$, n=3 and *Gadd45β*$^{-/-}$, n=3) *, p<0.05.

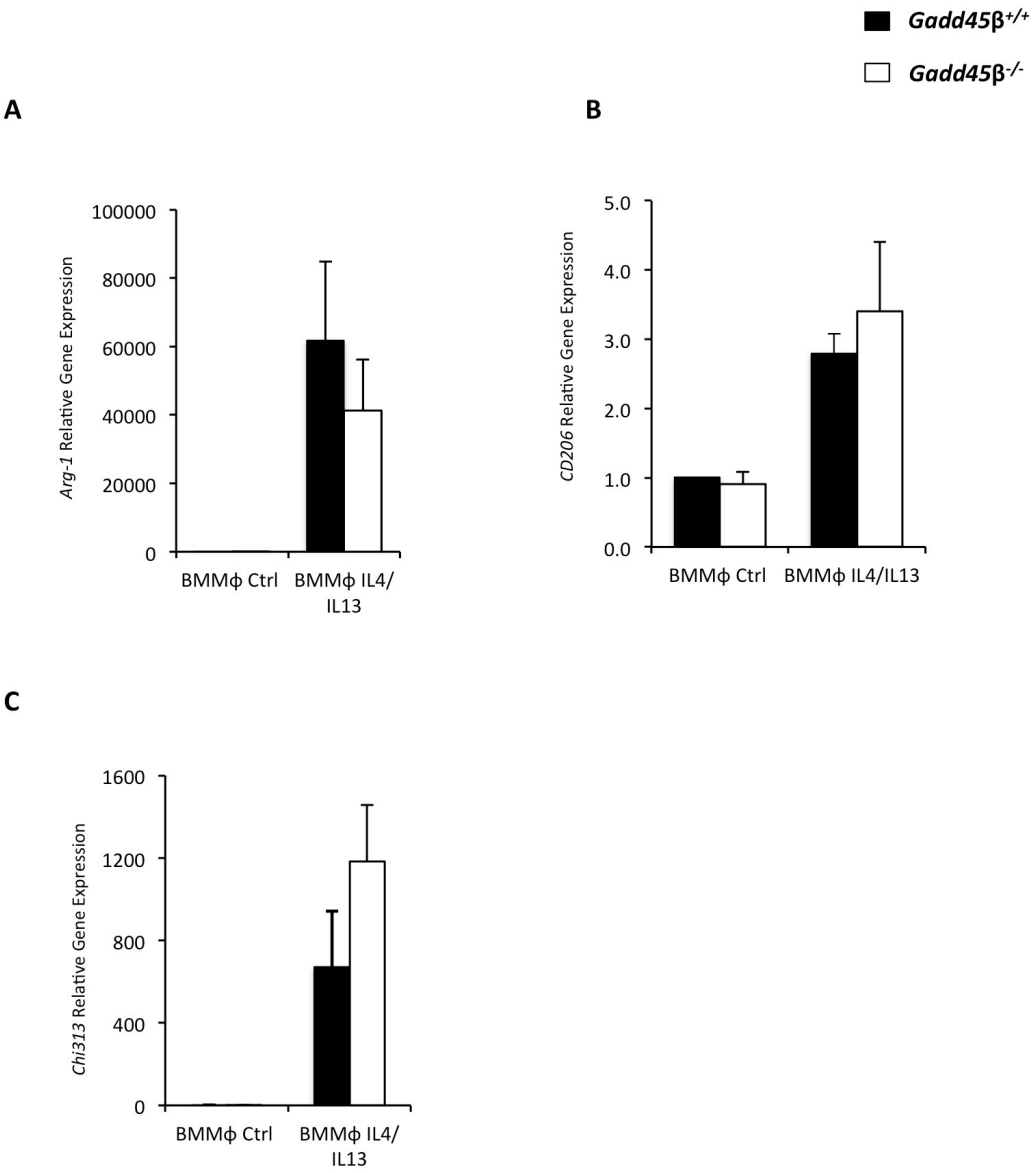


Figure 28: The *In vitro* polarisation of bone marrow- derived macrophage towards the M2 phenotype. Bone marrow-derived macrophages from *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ mice were stimulated with IL-4/IL-13. (A, B and C) Quantitative real-time PCR analysis showing the expression levels of M2 macrophage associated genes. Values denote mean ± SEM (*Gadd45β*$^{+/+}$, n=3 and *Gadd45β*$^{-/-}$, n=3).

These data suggest that *Gadd45β* activation within the macrophage by *in vitro* cytokine stimulation restrains the development of classically activated M1 macrophages without affecting alternatively activated M2 macrophage phenotype. Consequently, loss of *Gadd45β* promoted the inflammatory gene expression by M1 macrophages that have been shown to be essential for cellular and anti-tumour immunity. The results obtained by macrophage cytokine stimulation experimental system mirrored to those results obtained using macrophage co-culture experiment for M1 associated genes. It is important, to understand the precise mechanism by which loss of *Gadd45β* promotes specifically M1 phenotype. However, these mechanisms need to be investigated.

5: Discussion

5 Discussion

The connection between inflammation and cancer has been known for a long time, but the precise mechanisms behinds this connection are beginning to be understood. The hallmarks of cancer include self-sufficiency in growth signal, insensitivity to growth inhibitors, bypassing of apoptosis, illimitable replicative potential, tissue invasion, genomic instability, tumour promoting inflammation, avoiding immune destruction and deregulating cellular energetics, metastasis, and angiogenesis (Hanahan and Weinberg, 2011). NF-κB signalling has been implicated in many of these hallmarks, and recent studies have illuminated the mechanistic pathway by which NF-κB signalling contributes to these aspects of carcinogenesis. It was shown that the link between inflammation and carcinogenesis depends on NF-κB (Greten *et al.,* 2004; Maeda *et al.,* 2005; Pikarsky *et al.,* 2004). Many proinflammatory stimuli activate NF-κB, mostly through IκB kinase-dependent phosphorylation, ubiquitination and degradation of inhibitors of IκB proteins. Once activated, NF-κB dimers stimulate the transcription of genes encoding cytokines, chemokines, growth factors, and anti-apoptotic factors (Ghosh S and Karin M, 2002). Consistently, activated NF-κB was shown in many different tumour cell

lines and primary tumour samples (Gilmore, T.D 2006). It is also well known that inhibition of NF-κB activity in cancer cells leads to increased sensitivity to chemo and radiotherapy. (Amit S and Ben-Neriah Y, 2003). Delineating how NF-κB facilitates the survival of immune, proinflammatory and cancer cells will be important not only to understand pathogenic mechanisms of the disease, but also to identify new targets for anti-inflammatory and anti-cancer therapy.

To understand the basis for the NF-κB dependent link between inflammation and cancer, we sought to identify the NF-κB target genes that mediate this link. By using an unbiased genetic screen, *Gadd45β* was identified as a pivotal effector of this suppressive activity (De Samele *et al.,* 2001). *Gadd45β* is a member of the *Gadd45* family of inducible genes, also comprising *Gadd45α* and *Gadd45γ* (Abdollahi *et al.,* 1991; Zhang *et al*., 1999; Nakayama *et al.,* 1999). *Gadd45* proteins have been implicated in several biological functions, including growth control and DNA repair (Yang *et al.,* 2000; Vairapandi *et al.,* 2000; Zhan *et al.,* 1999; Wang *et al.,* 1999; Smith *et al.,* 2000). *Gadd45β* however exhibits distinct biological activities. *Gadd45β* is transcriptionally upregulated by inflammatory stimuli through a mechanism that requires NF-κB and is essential to antagonise TNF-α induced killing, and abrogates sustained JNK activation and programmed cell death in NF-κB null cells. Using *Gadd45β*$^{-/-}$

mice it has been reported that *Gadd45β* is required for liver regeneration after partial hepatectomy (PH). Loss of *Gadd45β* profoundly impairs hepatocyte survival and proliferation in response to PH (Papa *et al.,* 2008). The mechanism by which NF-κB inhibits PCD during inflammation driven tumourigenesis is poorly understood. Here to identify the precise role of *Gadd45β* in NF-κB afforded protection in cancer and to examine the role of *Gadd45β* in hepatocellular carcinogenesis DEN induced HCC model was used.

5.1 *Gadd45β* promotes DEN induced HCC development

DEN is a chemical carcinogen, its exposure causes hepatocyte cell death, and the release of products by these cells activates NF-κB within Kupffer cells. Activation of Kupffer cells induce the synthesis of TNF-α, IL-6 and other cytokines that stimulate the proliferation of surviving hepatocytes, ultimately leading to the development of HCC (Maeda *et al.,* 2005). Because, compensatory hepatocyte proliferation is critical for tumour growth, causing initial hepatocytes to enter the cell cycle and transmit the oncogenic mutation (Fausto *et al.,* 1999). Thus, NF-κB orchestrates the inflammatory crosstalk between dying hepatocytes and Kupffer cells, the two essential cell types that play an important role in DEN induced carcinogenesis (Sakurai *et al.,* 2006).

The results described in this thesis and other previous studies have indicated that the inflammatory crosstalk between dying hepatocytes and immune cells is central to the HCC development (Maeda *et al.,* 2005). The carcinogen used in this study, DEN, is metabolised into an alkylating agent that induces DNA damage by ROS accumulation and mutations, as well as a hepatocyte death. However, mutations alone are probably not sufficient for the hepatocellular carcinogenesis. Due to the reduced toxicity and reduced hepatocyte proliferation, in adult mice DEN is a weak carcinogen that requires assistance from a tumour promoter such as phenobarbital to induce hepatocyte proliferation (Sarma *et al.,* 1986). Some promoters such as carbon tetrachloride (CCL4) or agonistic anti-Fas antibody (Hara *et al.,* 2000) are cytotoxic. Others, such as phenobarbital (PB) act through various mechanisms, mostly unknown; those eventually induce hepatocyte proliferation (Sarma *et al.,* 1986). Recent studies have also shown that feeding rats with a high-fat diet may also act as a tumour promoter (Stemmer *et al.,* 2012). Furthermore, gene expression profiling studies has indicated that there is an overlap between DEN/PB induced mouse tumours and their human counterparts. Thus, supporting the usefulness of chemical carcinogenesis studies in mouse models (Lee *et al.,* 2004; Stahl *et al.,* 2005).

Previously, it has been shown that NF-κB is instrumental for tumour promotion in the CAC model (Grenten *et al.,* 2004), DEN induced HCC model (Maeda *et al.,* 2005), and inflammation associated liver cancer in Mdr2$^{-/-}$ mice model (Pikarsky *et al.,* 2004). The results described in this thesis are compatible with the previous findings, underlying the NF-κB dependent link between inflammation and cancer. Here, we show that *Gadd45β*, a downstream target of NF-κB is required for liver carcinogenesis induced by DEN. Indeed, most of the *Gadd45β*$^{+/+}$ mice treated with DEN at two weeks of age developed typical HCC by ten months without help of tumour promoter PB. In contrast, liver of *Gadd45β*$^{-/-}$ mice presented with only very few tumours by this time compared to the control mice. Notably, loss of *Gadd45β* has also reduced average HCC surface area and inhibits metastasis formation in the lung (data not shown). It should be noted that, in some cancers, inhibition of NF-κB promotes tumour growth. For example, inhibition of NF-κB in keratinocytes promotes squamous cell carcinoma (Dajee *et al.,* 2003), suggesting that the impact of NF-κB on cancer development may be cell-type or model specific. The model specific nature of NF-κB has become apparent even with its downstream target genes. Indeed, the results shown here by using DEN model are in marked contrast to Qiu W *et al.* observation who found that *Gadd45β* was downregulated in most of human HCC (Qiu *et al.,* 2003).

In the next step, to examine the molecular basis for the reduced susceptibility to DEN induced HCC in *Gadd45β*$^{-/-}$ mice. We examined the early effect of carcinogenesis.

It has been previously shown that after PH, in *Gadd45β*$^{-/-}$ mice livers there was an impaired hepatocyte proliferation (Papa *et al*., 2008). Based on the previous studies using PH model and DEN model (Papa *et al.,* 2008; Maeda *et al.,* 2005), the effect of loss of *Gadd45β* on tumourigenesis are thought to be associated with impaired hepatocyte proliferation and reduced hepatic production of inflammatory cytokines (e.g. TNF-α, IL-1β and IL-6) after DEN injection. Because, DEN induced hepatocyte proliferation was shown to be dependent on the production of hepatic inflammatory cytokines by Kupffer cells, including TNF-α, IL-6, IL-1β and HGF (Maeda *et al.,* 2005). Indeed, in *Gadd45β*$^{-/-}$ mice livers shortly after the DEN exposure there was a reduced hepatocyte proliferation (Figure 5) and inflammatory cytokine expression by Kuppfer cells (Figure 6), suggesting that *Gadd45β* might have played an additional pro-tumourigenic role in these cells. Strikingly, however, *Gadd45β*$^{-/-}$ mice displayed similar frequency of hepatocyte apoptosis after the acute DEN injection when

compared to *Gadd45β*$^{+/+}$ mice that were similarly treated. Indeed, appearance of ALT and by performing TUNEL assay revealed that there were no significant differences between both the genotypes (Figure 4). Next we examined JNK activity shortly after the DEN injection, because JNK activity has been shown to control DEN induced PCD and carcinogenesis in few mouse models (Sakurai *et al.,* 2006). In contrast to the previous findings using PH mode, the magnitude and consistency of JNK activation in both *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ mice were both similar and sustained shortly after the DEN injection (Figure 8). Although, the reasons for this contrasting results could be explained by the differences in the model system used. However, in some experimental models it has been shown that, an elevated JNK expression in *Gadd45β*$^{-/-}$ mice may not contribute to the increased apoptosis (Yu *et al*., 2013).

Together, these data uncovered the previously unknown role of *Gadd45β* in hepatocellular carcinogenesis. In the next step, we precisely elucidated the cell types that are responsible for *Gadd45β's* tumour promoting role in HCC.

5.2 *Gadd45β* acts within proinflammatory cells during tumour progression

An important step was to determine in which cell types *Gadd45β* exercises its pro-tumourigenic action, whether in hepatocytes or in proinflammatory cells (e.g. Kupffer cells). To address this, a bone marrow chimeric mice model were established with mice harbouring all four possible combinations of $Gadd45\beta^{+/+}$ and $Gadd45\beta^{-/-}$ parenchymal and haematopoietic cells, referred to as "protocol A" (Figure 9). Injecting with clodronate liposome depleted endogenous tissue resident macrophages within the livers and spleen of the bone marrow chimeric mice. Because, the rate of replacement of resident Kupffer cells with the engrafted BM-derived cells is normally very low (Kennedy *et al.,* 1997). Recent studies have shown that tissue resident macrophages have dual origin, approximately half of the population being renewed from the local stem cell production and the remaining half from circulating precursor (Schulz *et al.,* 2012; Van Furth *et al.,* 1984). Kupffer cells have been shown to be able to renew and proliferate independently of the bone marrow. Of note, bone marrow chimeric experiments have demonstrated that following lethal

irradiation there are two distinct Kupffer cell populations that share the same morphology and phagocytic capabilities (Klein et al., 2007). Klein *et al.* has shown that depletion of endogenous tissue resident macrophages by injecting clodronate liposomes four weeks after the irradiation resulted in only bone marrow-derived Kupffer cells (Klein *et al.,* 2007). Furthermore, the bone marrow-derived Kupffer cells have been shown to be important in inflammatory response (Klein *et al.,* 2007). Consistent with previous studies, in mice from "protocol A" following clodronate treatment, endogenous tissue resident macrophages were largely replaced by donor bone marrow-derived cells (Figure 12). Mechanistically, clodronate exposure causes an irreversible damage to macrophages, which subsequently undergo apoptosis due to the formation of harmful ATP analogues (Mantovani *et al.,* 2004; Van Rooijen *et al*., 1994; Milner *et al.,* 2004). The free clodronate have a very small lifespan in blood circulation. Because of their short lifespan clodronate was encapsulated in liposomes.

After successful replacement of Kupffer cells by donor bone marrow-derived cells, mice were injected with DEN (100mg/kg) to induce carcinogenesis and then analysed (Figure 9). All the *Gadd45β*$^{+/+}$ recipients with *Gadd45β*$^{+/+}$ bone marrow developed typical HCC. *Gadd45β*$^{-/-}$ recipients with *Gadd45β*$^{-/-}$ bone

marrow developed very few visible tumours, tumour number, tumour surface area, maximum tumour size, and tumour incidence was significantly lowered compared to *Gadd45β*$^{+/+}$ recipients with *Gadd45β*$^{+/+}$ bone marrow. *Gadd45β*$^{-/-}$ recipients with *Gadd45β*$^{+/+}$ bone marrow developed HCC mirroring *Gadd45β*$^{+/+}$ mice reconstituted with *Gadd45β*$^{+/+}$ BM. From the above data, we concluded that *Gadd45β* exerts its tumourigenic action primarily within the BM-derived inflammatory cells. Reconstitution with *Gadd45β*$^{+/+}$ bone marrow cells increased the HCC load in *Gadd45β*$^{-/-}$ mice, suggesting that the carcinogenic activity lies primarily within inflammatory compartment. However, this model cannot distinguish between roles of *Gadd45β* during tumour initiation or during progression.

To distinguish between these roles, we, therefore, adopted "protocol B". In this mode, *Gadd45β*$^{-/-}$ and *Gadd45β*$^{+/+}$ mice were first given DEN and then mice were given PB in their drinking water. Three months after the DEN injection, mice were lethally irradiated and adoptively transferred with *Gadd45β*$^{-/-}$ or *Gadd45β*$^{+/+}$ BM, leading to four possible combinations of *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ parenchymal and haematopoietic cells. Remarkably, the results obtained with this experimental model mirrored those obtained with "protocol A". Thus confirming that *Gadd45β* exercises its tumourigenic role primarily

within the proinflammatory cells. Interestingly, this model also showed that *Gadd45β* plays this role specifically during tumour progression rather than during tumour initiation stage. Accordingly, *Gadd45β* deficiency diminished tumourigenesis in *Gadd45β*$^{+/+}$ recipients that were adoptively transferred with *Gadd45β*$^{-/-}$ bone marrow cells. These chimeric mice contain wild-type tissue only at the time of DEN injection and *Gadd45β*$^{-/-}$ bone marrow during tumour progression. Remarkably, this deficiency found to have a lesser impact on oncogenesis during tumour initiation.

The data presented here uncovered previously unknown roles for *Gadd45β* in promoting hepatocellular carcinogenesis, and showed that this tumourigenic role tracts primarily within proinflammatory cells rather than hepatocytes. From the above data, we propose that *Gadd45β* promotes liver cancer development by affecting mainly during tumour progression within the inflammatory compartment. Altogether, in DEN injected mice, *Gadd45β* ablation had no effect on hepatocyte survival, whereas subsequent compensatory proliferation was slightly and significantly reduced in *Gadd45β*$^{-/-}$ mice. Furthermore, deletion of *Gadd45β* in BM-derived cells during tumour progression led to reduced HCC burden. These data identifies *Gadd45β* as a

pivotal component of the NF-κB dependent link between inflammation and cancer. The data also suggest that *Gadd45β* may act as a potential therapeutic target for treating HCC and other liver pathologies.

5.3 *Gadd45β* promotes MCA-203 tumour progression by restraining tumouricidal cells

In general, almost all the tumours contain both malignant and non-malignant cell populations. A number of non-malignant cell types that are derived from bone marrow have been shown to dictate the tumour cell invasion and progression. These non-malignant cell types are inflammatory cells. The presence of inflammatory cells in all the solid tumours has long been suggested that, cancers arise at the site of chronic inflammation (Balkwill *et al.,* 2001; Coussens *et al.,* 2002; Balkwill *et al.,* 2005). This hypothesis has received a molecular confirmation in inflammation-associated cancer models, providing *in vivo* evidence that support the relationship between NF-κB mediated inflammation and tumourigenesis (Greten *et al.,* 2004; Maeda *et al.,* 2005; Pikarsky *et al.,* 2004).

To further characterise the tumourigenic activity *Gadd45β* has in the inflammatory compartment *in vivo*. We used an allograft tumour model using established murine tumour cell line. On injection of MCA-203 fibrosarcoma cells, tumour grew rapidly in *Gadd45β*$^{+/+}$ mice as reported previously (Marigo *et al.,* 2013). By contrast, the tumour growth was significantly reduced in *Gadd45β*$^{-/-}$ mice. Similarly, there was a reduced spleen size in *Gadd45β*$^{-/-}$ mice, relating to its reduced tumour growth in these mice. To better characterise the cell-type responsible for the tumourigenic activity of *Gadd45β*. We examined the tumour inflammatory infiltrates in both *Gadd45β*$^{-/-}$ and *Gadd45β*$^{+/+}$ tumours using immunohistochemistry. These analyses revealed an increased accumulation of CD3^{+} T cells in *Gadd45β*$^{-/-}$ tumours compared to *Gadd45β*$^{+/+}$ tumours. The high proportion of activated intra-tumour CD3^{+} T-cells within the *Gadd45β*$^{-/-}$ tumours suggests the presence of immune stimulatory macrophages in these tumours. Accumulation of activated CD3^{+} T-cells within the tumours secrete proinflammatory cytokines that exhibit an inflammatory microenvironment and that is strongly associated with prolonged cancer patients survival (Matsuzaki *et al.,* 2005; Tosolini *et al.,* 2011; Ryan *et al.,*

2014). In addition to $CD3^+$ T-cell, there was increased number of M1 macrophages that express high levels of iNOS and Cox-2 located within the tumours in *Gadd45β*$^{-/-}$ mice. In contrast, there was no difference in the levels of M2 macrophages in *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ tumours. Interestingly, the results obtained in allograft tumour model are consistent with those obtained using macrophage co-culture system (Figure 24 and 25).

However, the findings reported in this thesis by using MCA-203 allograft model are in contrast to Songguang *et al* 's observation. Songguang *et al* has shown that *Gadd45β* suppress tumour growth in B16-melanoma allograft tumour model and also shows that in *Gadd45β*$^{-/-}$ mice there was an increased tumour growth at most of the time points that were observed. Tumour analysis revealed that the increased tumour growth in *Gadd45β*$^{-/-}$ mice was due to reduced cytotoxic $CD8^+$ T-cell accumulation within the *Gadd45β*$^{-/-}$ tumour. However, there was no apparent effect of $CD4^+$ T-cells on the tumour growth (Songguang *et al.,* 2009). The reason for the contrasting results could be because of cell-type specific role of *Gadd45β* during tumour progression, suggesting the complex nature of NF-κB and its down-stream targets in tumourigenesis.

5.4 *Gadd45β* plays an important role in macrophage differentiation and polarisation *in vitro*

The attention towards macrophage in cancer was because of its tumour-promoting role, abundance in tumour stroma, and also its interaction between inflammation, immunity, and cancer (Pollard J.W, 2004: Coffelt *et al.,* 2009; Lewis *et al.,* 2006). Generally, M1 macrophages are considered as proinflammatory cells capable of killing tumour cells by producing nitric oxide and several inflammatory mediators, whereas, M2 macrophages are considered as anti-inflammatory cells (Guiducci *et al.,* 2005; Klimp *et al.,* 2002). The signalling pathways that regulate these phenotypes *in vivo* are not very well understood. Although the phenotype of the macrophage is always in a very fine balance, when activated appropriately either they promote tumour growth or act as tumouricidal cells. Thus, explaining their enormous plasticity. Recent studies using genetically manipulated mouse models revealed, IKKβ deletion within the macrophages resulted in the reduced tumour number and tumour size in colitis-associated cancer model (Greten *et al.,* 2004) and similar results were also seen in HCC after Iκβ super-repression in Kupffer cells in $Mdr2^{-/-}$ mice (Pekarisky *et al.,* 2004). All of these studies supported the role of NF-κB in macrophages as a tumour promoting transcription factor.

Despite the evidence suggesting a pro-tumour role for NF-κB in macrophages, some of the genes regulated by NF-κB may lead to anti-tumoural role. These observations suggest that the role of NF-κB in macrophages is more complicated. The dual function of NF-κB within the macrophages was evidenced by a recent study, where it has been shown that the activation of NF-κB in macrophages resulted in a reduced lung tumour metastasis. The reduced metastasis was due to the increased expression of M1 macrophage markers and the increased apoptosis of tumour cells (Connelly *et al.,* 2011). This contrasting result could be explained by the differences in the model systems used and also the NF-κB function in macrophage could be both context and gene specific. Indeed, in this lung tumour metastases model, the anti-tumour effect of NF-κB activation was seen only during the seeding phase. This observation suggests that the activation of NF-κB, in a specific window, could contribute to anti-tumour effect (Connelly *et al.,* 2011).

Accumulated evidence shows that immune cells, in particular, macrophages have a key role in the tumourigenesis. In some experimental models, successful depletion of macrophages resulted in tumour growth reduction in teratocarcinoma, rhabdomyosarcoma, lung cancer, and melanoma (Hiraoka *et al.,* 2008; Zeisberger *et al*., 2006; Gazzaniga *et al.,* 2007). However, in other studies it has been shown that, tumour growth is exponential even after a massive amount of tumouricidal macrophage infiltration into the tumour (Evans R, 1982; Milas *et al.,* 1987; Duff *et al.,* 2007). Furthermore, in an experimental model, deletion of Kupffer cells by clodronate liposome resulted in an enhanced liver tumour growth and almost all the liver tissue was replaced by tumours. Suggesting that, Kupffer cells in their normal state have a tumouricidal role in some cases. The underlying mechanisms in Kupffer cell as a tumouricidal cell were not clearly known. (Heuff *et al.,* 1993). However, the apparent discrepancy in some studies may reflect the different tumour systems investigated (spontaneous versus transplanted or carcinoma versus sarcoma), and also with the differential involvement of different polarised inflammatory reactions. These contrasting results highlight that further understanding is required to know the precise role of TAMs in tumour growth, progression, and invasion.

To further characterise the cell types responsible for the tumourigenic role of *Gadd45β,* we focused on macrophages because of (1) high number of M1 macrophages expression in *Gadd45β*$^{-/-}$ tumours (2) the highly inducible expression of *Gadd45β* in these cells (3) their importance in DEN induced HCC and other cancers (4) the recent discovery of an important role of NF-κB in the differentiation and function of TAM. We found that *Gadd45β* restrains the development and differentiation of M1 macrophage. Here, we describe a novel role for *Gadd45β* within the macrophages. Indeed, co-culture experiments with 410.4 mammary adenocarcinoma cells revealed that, bone marrow-derived *Gadd45β*$^{-/-}$ macrophages exhibited an increased polarisation towards "classically" activated (M1) macrophage phenotype. On the other hand, in *Gadd45β*$^{-/-}$ macrophages there was a reduced polarisation to "alternatively" activated (M2) macrophages that resembled TAMs. Similar results were obtained upon BM- derived macrophage co-culture with MN/MCA-1 fibrosarcoma cells. Co-cultured macrophages resembled a TAM phenotype that is found in tumours, and this is attained through chemical communication between the tumour cells and macrophages (Hagemann *et al.,* 2006). Based on these observations, we concluded that *Gadd45β* plays an essential role in restraining macrophage M1 polarisation driven by tumour cell co-culture *in vitro.*

To further investigation the role of *Gadd45β* in macrophages polarisation driven by cytokine stimulation, bone marrow-derived macrophages from *Gadd45β*$^{+/+}$ and *Gadd45β*$^{-/-}$ mice were polarised to either M1 or M2 macrophage phenotype. All the polarised macrophages showed some key features like cytokines and chemokine production. (Mantovani *et al.,* 2004). Indeed, compared to *Gadd45β*$^{+/+}$ BM-derived macrophages, *Gadd45β*$^{-/-}$ BM-derived macrophages polarised more towards M1 phenotype without affecting M2 phenotype.

In macrophage co-culture system, loss of *Gadd45β* promoted M1 macrophage, while inhibiting M2 phenotype. The results obtained from macrophage polarisation experiments suggest that *Gadd45β* restrains the development of the classically activated M1 macrophages, without affecting alternatively activated M2 macrophages. The reason for the differences in the results obtained from co-culture system to cytokine-driven polarisation system could be explained by a recent observation, suggesting, there exist an alternative pathway that polarise M2 macrophages independent of IL-4 and IL-13 signalling. These observations suggest IL-4 and IL-13 signalling are not critical

for TAM phenotype in few cancers. One other possible mechanism that promotes TAM phenotype independent of IL-4 and IL-13 signalling is lactic acid. A recent study has found that lactic acid produced by tumour cells, during hypoxic conditions, was sufficient to induce M2 phenotype (Colegio *et al*., 2014). These observations suggest that it is possible multiple pathways exist for communicating between tumour cells and macrophages for the induction of M2 phenotypes independent of IL-4 and IL-13 signalling. These studies, however, suggest that further studies are necessary to understand the precise mechanism of M2 macrophage polarisation.

Based on the results obtained from *in vitro* macrophage studies, we conclude that targeting *Gadd45β* in TAMs reverse their tumour promoting activity and drive tumour regression. Thus, loss of *Gadd45β* has the potential to re-educate the tumour promoting macrophage population to tumour killing population, and this approach have the potential in therapeutic use that could complement the existing therapies.

In summary, our data suggest that *Gadd45β* is necessary for efficient HCC induction. The initial DEN injection experimental data indicated that *Gadd45β* upregulation had only a small effect on hepatocyte proliferation and production of factors that promote compensatory hepatocyte proliferation. More interestingly, *Gadd45β* was found to play an essential role in tumourigenesis primarily within the inflammatory cell compartment of the tumour. Finally, allograft tumour model and *in vitro* macrophage studies suggests loss of *Gadd45β* promoted the accumulation of anti-tumour macrophages within the tumour and enhanced proinflammatory cytokine expression by M1 macrophages without affecting M2 macrophages. However, the mechanism by which *Gadd45β* promoted M1 macrophage phenotype needs to be investigated. Altogether, these data suggests that *Gadd45β* might act as an important component of the NF-κB mediated link between inflammation and cancer.

6: Future works

6 Future works

The research study reported here is only an initial observation for the roles of *Gadd45β* in promoting the tumour development by modulating macrophage polarisation both *in vitro* and *in vivo*. Following are the proposed future works that need to be carried out in order to help strengthen the data presented in this thesis.

6.1 Developing conditional *Gadd45β*$^{-/-}$ mice

To further characterise tissue-specific functions in macrophage polarisation and tumourigenesis, we generate *Gadd45β* conditional knockout mouse models. As such models are essential to establishing the safety and specificity of the *Gadd45β* targeting drugs. Using this model, we will test our hypothesis that *Gadd45β* promotes tumourigenesis by regulating macrophage function. Specific gene deletion in myeloid cells will be achieved by crossing *Gadd45β*$^{F/F}$ mice (harbouring intact loxP-flanked *Gadd45β* alleles) with LysM-Cre mice. We will analyse macrophage-restricted *Gadd45β* activities for the MCA-203 model, where host's *Gadd45β* ablation impacts tumourigenesis. These analyses will provide unique insights into *Gadd45β's* tissue-specific roles in TAMs during tumourigenesis and a potential rationale for targeting TAMs *via Gadd45β* inhibitors in cancer.

6.2 Mechanisms for *Gadd45β* macrophage function.

How *Gadd45β* regulates macrophage differentiation is not clearly known. Next, to test the idea that the *Gadd45β*-afforded regulation of macrophage polarisation involves modulating JNK signalling that is suppressed by *Gadd45β* and induced in IKKβ$^{-/-}$ macrophages respectively. To facilitate our biochemical analyses, *Gadd45β*$^{-/-}$ and *Gadd45β*$^{+/+}$ BM-derived macrophages will be polarised *ex vivo* using cytokines (IFN-γ, LPS [M1]; IL-4, IL-13 [M2]). Polarisation will be verified by qRT-PCR. JNK activation will be assessed in Western blots, using phospho-specific antibodies. p38, ERK, and IKK/NF-κB will be examined as controls. Increased JNK and/or STAT1 activity in *Gadd45β*$^{-/-}$ macrophages will be functionally investigated by attenuating JNK with pharmacological inhibitors (e.g. SP600125). These studies will provide early, but important mechanistic insights into *Gadd45β's* functions in macrophages and potentially tumourigenesis.

6.3 Targeting TAMs with *Gadd45β* inhibitors

We will investigate the therapeutic application of *Gadd45β* blocking peptide, namely DTP3 that was developed in the Franzoso's laboratory (Tornatore *et al.,* 2014). MCA-203 tumour-bearing mice will be treated with DTP3 or PBS, to determine whether DTP3 reduces tumour burden, *in*

vivo. If therapeutic effects occur, to verify that these involve activity on TAMs, we will then ascertain that DTP3 impacts macrophage profile/function and lacks toxicity in tumour cells, *in vitro* and *in vivo*. This approach will provide mechanistic insights into the roles exercised by *Gadd45β* in TAMs and validate *Gadd45β*-targeting agents as powerful therapeutic tools in cancer.

In Summary, in this thesis for the very first time we reported the role of NF-κB targeted genes in promoting cancer growth specifically by modulating tumour infiltrated macrophage phenotype. These future works have potential to gain new and important insights into mechanisms of tumourigenesis and inflammation *in vivo.* Understanding the mechanisms underlying the roles of *Gadd45β* and NF-κB in macrophages has fundamental therapeutic significance.

7: References

7 References

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