Amelioration of Ionizing Radiation Induced Cell Death in Lymphocytes by Baicalein

by

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Mr. R. S. Patwardhan

DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

Mr. R. S. Patwardhan

List of publications arising from thesis

Publications in refereed journal

1. "Involvement of ERK-Nrf-2 signaling in ionizing radiation induced cell death in normal and tumor cells", **Patwardhan RS**, Checker R, Sharma D, Sandur SK, Sainis KB **PLoS One**. 2013;8(6):e65929
2. "Mitigation of radiation-induced hematopoietic injury via regulation of cellular MAPK/phosphatase levels and increasing hematopoietic stem cells", **Patwardhan RS**, Sharma D, Checker R, Sandur SK **Free Radical Biology & Medicine** 2014;68:52-64
3. "Spatio-Temporal Changes in Glutathione and Thioredoxin Redox Couples during Ionizing Radiation Induced Oxidative Stress Regulate Tumor Radio-resistance", **Patwardhan RS**, Sharma D, Checker R, Thoh M, Sandur SK **Free Radical Research** 2015;26:1-15
4. "Baicalein exhibits anti-inflammatory effects via inhibition of NF-κB transactivation", Raghavendra S. Patwardhan, Deepak Sharma, Maikho Thoh, Rahul Checker, Santosh K. Sandur **Biochemical Pharmacology** 2016;108:75–89

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1. "Baicalein induced cell death in murine T cell lymphoma via inhibition of thioredoxin system" **Patwardhan RS**, Sharma D, Sandur SK

Conference proceedings

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2. "Baicalein protected against IR induced hematopoietic injury via regulation of MAPK phosphatase and augmenting hematopoietic stem cells" **Patwardhan RS**, Sharma D, Checker R, Sandur SK Society for Free Radical Research-India 2014, Lonavala, January, 2014

3. “Baicalein offered protection against WBI induced mortality to mice” **Patwardhan RS**, Sharma D, Checker R, Sandur SK Society for Free Radical Research-India 2013, Lucknow, January, 2013
4. “Compartmental changes in cellular redox parameters after exposure to ionizing radiation” **Patwardhan RS**, Sharma D, Checker R, Maikho Thoh, Sandur SK International Conference on Radiation Biology-India 2012, ACTREC, Navi Mumbai November, 2012.
5. “Involvement of ERK-Nrf-2-ARE signaling in regulating tumor radioresistance of EL4 cells” **Patwardhan RS**, Checker R, Sharma D, Sandur SK, Sainis KB Society for Free Radical Research-India, Kolkata, January, 2012
6. "Baicalein protected murine splenic lymphocytes against IR induced cell death via activation of ERK and NF-κB” **Patwardhan RS**, Checker R, Sharma D, Sandur SK Society for Free Radical Research-India, Chennai, 2011

DEDICATION

Dedicated to Science …

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CONTENTS

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SYNOPSIS OF Ph. D. THESIS

1. Name of the Student: R. S. Patwardhan

2. Name of the Constituent Institution: Bhabha Atomic Research Centre, Trombay, Mumbai.

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SYNOPSIS

PREAMBLE

Planned or unplanned exposure to Ionizing Radiation (IR) can occur for variety of reasons in human life. Peaceful use of atomic energy for diagnostic and therapeutic purposes, food irradiation, sterilization of medical equipment and sanitary products, sludge treatment,

power production and strategic purposes etc is becoming more popular and hence the chances of accidental or occupational exposure have also increased. Presently, our unpreparedness to provide concrete medical countermeasure and inability to cover risk of bulk damage to population has severely compromised risk/benefit ratio for use of IR [1]. IR is known to alter the functions of cellular macro-molecules leading to tissue damage. The deleterious effects of ionizing radiation (IR) are mediated both through the direct deposition of energy to critical biomolecules, and indirectly through the generation of highly reactive free radicals. Approximately 75% of the damage to cells is accounted through indirect effect by low LET ionizing radiations. This cellular damage is manifested through system specific syndromes categorized based on the dose range of exposure.

Immune system is vulnerable to IR mediated damage leading to subsequent fatal consequences. Protection or reconstitution of the hematopoietic system is a critical area of research in the development of radioprotectors and therapeutic agents. Exposure of animals to radiation doses above 1 Gy leads to hematopoietic syndrome resulting in leucopenia. Hematopoietic recovery after radiation exposure is dependent on the presence of spared hematopoietic stem and progenitor cells in the bone marrow.

Currently there are no radioprotectors or mitigators available that are approved by Food and Drug Administration (FDA) for general use in humans for the prevention or treatment of IR induced hematological toxicity. Amifostine (EthyolR) is the only drug approved by the FDA to reduce the toxicity of radiation therapy for limited clinical indications [2].

Recent reports on several phytochemicals have demonstrated their radiomodulatory activity in experimental animal systems. Some of them viz. genestein and gamma tocotrienol analog are at advanced stages of drug development as radioprotector [3, 4]. Some of these potential radioprotective phytochemicals are plant secondary metabolites synthesized by phenylpropanoid pathway that include a variety of plant polyphenols viz., alkaloids, terpenoids, quinones, flavonoids etc. They primarily serve to protect plants against variety of biotic and abiotic stresses such as UV irradiation, ozone, pollutants, wound, oxidative stress, infection and herbivores.

Baicalein, 5,6,7-trihydroxyflavone isolated from dried roots of Chinese herbal medicinal plant *Scutellaria baicalensis* has been shown to inhibit 12-lipoxygenase and it induces apoptosis in

several cancer cells such as breast carcinoma cells, colon carcinoma cells, and leukemia cells [5-7]. Baicalein exhibits free radical-scavenging activity and attenuates oxidative stress [8].

Previous studies from our laboratory have demonstrated anti-oxidant and radioprotective properties of baicalein in cell free system. Structurally, it is similar to soy isoflavone geneistein (4, 5, 7-trihydroxyflavone) which is being tested as a radioprotector [9]. In the present study, efficacy of baicalein as a radioprotector and immunomodulator was investigated using mouse model.

AIMS OF THE PRESENT STUDIES:

- To investigate the potential of baicalein to offer protection to murine splenic lymphocytes in vitro against ionizing radiation induced damage and elucidate underlying molecular mechanism.
- To examine in vivo radioprotective ability of baicalein monitored in terms of changes in bone marrow cell viability, functionality of splenic lymphocytes, survival and body weight changes in mice.
- To study the effect of baicalein on T cell responses in vitro and in vivo
- To examine the effect of baicalein on murine T cell lymphoma EL4 cells in vitro and in vivo.

ORGANIZATION OF THE THESIS:

The work embodied in this thesis is divided into five chapters:

(1) Introduction

(2) In vitro and in vivo radioprotective effects of baicalein

(3) In vitro and in vivo anti-inflammatory effects of baicalein

(4) Anti-tumor effects of baicalein

(5) Summary and Conclusions

CHAPTER 1: Introduction

This chapter describes about the present developments in the field of radioprotection and highlights the overall need for continued pursuit in this area. This chapter provides reasonable information about current findings and approaches used to deal with the challenges posed in this field. It encompasses a list of drugs currently undergoing clinical or pre-clinical trials, their mode of action, potency and window of protection and type of tissue being protected against IR induced damage.

In light of the current research findings in this area, it is perceived that overall requirements of an ideal radioprotector may not be completely fulfilled by a single agent. Hence, researchers are exploring an alternative approach of combining two or more radioprotectors of different class and potency depending upon the need to enhance efficacy and reduced toxicity [10]. Researchers are employing target based design of small molecule inhibitors or activators of critical proteins in cells. In this class, critical targets inside cells are identified that regulate cell survival in stress and subsequently small molecules are designed for acting on these targets. Inhibitors of p53 namely pifithrin-alpha [11], flagellin derived activator of NF-kB [12], CDK4/6 inhibitor PD [13], Nrf2 activators [14] are some of the examples based on this approach. Other approach includes screening of phytochemicals and repurposing of certain FDA approved drugs to test their radioprotective ability. Several drugs have been tested with this approach and have been shown to possess radioprotective potential with low or moderate efficacy.

In this chapter, existing information about current literature on phytochemicals being used as radioprotector is provided to get bird's eye view about intense investigations in this field. Still continued search for newer plant derived radioprotector is in progress to overcome the drawbacks of currently available phytochemical radioprotectors.

Cells of the hematopoietic system are susceptible to radiation induced damage due to their high proliferation index. Sublethal doses of radiation kill a majority of cycling hematopoietic progenitor cells. Literature reports and previous studies from our laboratory indicated potential of baicalein to offer protection against IR induced death of cells from hematopoietic origin. This chapter describes rationale behind choosing baicalein to test for its radioprotective ability in light of current radioprotectors.

Radiation is a known immune suppressor and inducer of inflammation. Inflammatory response initiated post-radiation injury further complicates management of human health. Several findings have reported baicalein as a bonafide anti-inflammatory agent. This chapter takes an account of current studies pertaining to management of inflammation by baicalein and its probable relevance in the context of this thesis.

The very first requirement of a radioprotector is not to offer survival advantage to tumor cells when used during radiotherapy. This section summarizes current knowledge about anti-tumor activity of baicalein and study plan designed for this thesis.

CHAPTER 2: In vitro and in vivo radioprotective effects of baicalein

This chapter describes radioprotective potential of baicalein using murine splenic lymphocytes and mouse model. Baicalein scavenged IR induced free radicals in murine splenic lymphocytes at 5μM but significant prevention of radiation induced apoptosis was evident at 50μM indicating that antioxidant activity of baicalein may not be solely responsible for radioprotection. Interestingly, pharmacological inhibitors of ERK or Nrf2 abrogated baicalein mediated radioprotection. Baicalein induced phosphorylation of ERK via inhibition of corresponding dual specificity phosphatase MKP3 leading to activation of Ets1 which is involved in regulation of cell survival under oxidative stress.

Baicalein induced nuclear translocation of redox sensitive transcription factors Nrf-2 and NF-kB that was abolished upon treatment with ERK inhibitor. Nrf-2 is involved in regulating redox homeostasis under conditions of oxidative stress. There was a time dependent increase in Nrf-2 dependent genes viz. catalase, Mn-SOD, GCLC, GCLM, and hemoxygenase-I (HO-1). Administration of baicalein (10mg/kg bw i.p) to Swiss albino male mice for three consecutive days offered up to ~70% protection against whole body irradiation (WBI 7.5Gy dose) induced mortality. It was observed that baicalein administration modified LD50/30 of mice exposed to radiation from 6.5Gy to 7.5Gy resulting in DMF of 1.153.

BM-MNC isolated from mice administered with baicalein and exposed to WBI (4Gy or 7.5Gy) exhibited significantly higher recovery as compared to WBI group. Interestingly, owing to its antioxidant ability baicalein administration led to suppression of cellular ROS levels in BM-MNC induced by WBI exposure. Stimulation of splenic lymphocytes with Con A after isolation from baicalein administered mice exposed to WBI (4Gy) exhibited significantly higher proliferation as compared to that in lymphocytes isolated from WBI exposed group.

It was observed that baicalein administration led to significant increase in hematopoietic stem / progenitor cells (HSPC) as evinced from Hoechst side population assay at 24h and day 5. To confirm these results, the frequency of $lin^{-}CD244\text{-}CD150+$ cells (phenotype of HSPC using signaling lymphocyte activation marker analysis) in bone marrow of mice administered with baicalein was enumerated and observed a significant increase on day 5. Further, baicalein administration led to augmented levels of pNrf-2 in lin^{-}BM-MNC. Whereas, survival of mice

exposed to WBI was significantly reduced when they were given with all-trans retinoic acid (ATRA) (an inhibitor of Nrf-2) prior to baicalein. These results confirmed the role of Nrf-2 in baicalein mediated radioprotection.

CHAPTER 3: Baicalein suppressed mitogen induced T cell responses in vitro and in vivo:

Results from previous chapter on in vivo experiments have shown suppressive effect of baicalein on Con A induced proliferation in splenic lymphocytes. In vitro studies have shown that, baicalein activates ERK/NF-κB in murine splenic lymphocytes. Since, Con A stimulation also leads to activation of ERK/NF-κB in lymphocytes, it was interesting to see how lymphocytes respond to Con A stimulation in presence of baicalein.

It was observed that, baicalein treatment suppressed Con A induced T cell proliferation as well as cytokine secretion. Lymphocyte activation is controlled by the ubiquitous transcription factor NF-κB and its DNA binding is regulated by a dithiol disulfide, thioredoxin. Thioredoxin reduces critical cysteine residue (Cys 62) in p65 subunit o NF-κB thereby allowing its DNA binding. After Con A stimulation, binding of transcription factor to DNA requires increased thioredoxin (Trx) activity in nuclear compartment. Hence, effect of baicalein treatment on thioredoxin activity in nuclear compartment in presence and absence of Con A was studied. Interestingly, baicalein per se did not influence thioredoxin activity but it suppressed Con A induced increase in thioredoxin activity in the nuclear compartment. It was observed that, baicalein treatment did not suppress Con A induced DNA binding of NF-κB.

This raised another question that, whether suppression of thioredoxin activity has really any role to play in observed anti-inflammatory effect of baicalein. To address this question, cells were treated with sodium selenite, an activator of thioedoxin reductase (TrxR), prior to baicalein and Con A treatment. It was observed that, sodium selenite treatment could significantly reverse baicalein mediated suppression of Con A induced proliferation and thioredoxin activity. This suggested that, Trx inhibition may be the mechanism of anti-inflammatory activity of baicalein.

In order to ascertain the in vivo anti-inflammatory activity of baicalein, purified CD4+ T cells were treated with baicalein and transferred to autologus lymphopenic host. Transient exposure of purified CD4+ T cells to baicalein did not inhibit the homeostatic proliferation of these cells in mice. These findings suggested that baicalein treatment did not pose any interference to

homeostatic proliferation of T cells and its anti-inflammatory effects are limited to only mitogen induced proliferation. To study the in vivo anti-inflammatory efficacy of baicalein, splenic lymphocytes from C57BL/6 mice (allogeneic donor) were incubated with baicalein in vitro and adoptively transferred to immunocompromised Balb/c mice. The mice that received untreated control cells developed graft-versus-host disease (GVHD) that led to 80% death within 10 days. However, in mice that received baicalein treated cells showed 40% mortality and lower morbidity with better health for 30 days of experimental period. On day 5 post allo-transplantation, the levels of pro-inflammatory cytokines (IL-6, IFN-γ and IL-2) were significantly higher in the serum of mice which received vehicle treated allogenic lymphocytes as compared to that in serum of mice which received baicalein treated allogenic lymphocytes. This observation clearly shows potent anti-inflammatory activity of baicalein in vivo.

CHAPTER 4: Differential effect of baicalein on lymphocytes and lymphoma cells is due to differential modulation of thioredoxin reductase:

It was found that baicalein induced concentration dependent death in T cell lymphoma EL4 cells. Studies on anti-inflammatory effects of baicalein showed that it suppressed mitogen induced increase in Trx activity in lymphocytes. Actively dividing cells and many tumor cells overexpress TrxR. TrxR functions as oxidoreductase and serve the function of reducing critical cysteine residues in some key proteins inside cells that include ribonucleotide reductase, methionine sulfurtransferase and several transcription factors. Oxidation of Trx1 facilitates its dissociation from ASK1 (apoptosis signaling kinase) that subsequently leads to activation of apoptotic machinery. Further, inhibitors of TrxR are being explored as potential anti-tumor drugs because of their critical role in cell survival. Using specific inhibitors and shRNA based knockdown approach, it was deduced that thioredoxin system is critical for survival of mouse as well human lymphoma cells. It was observed that impairment of thioredoxin system lead to enhancement of tumor radiosensitivity.

Since baicalein suppressed mitogen induced increase in Trx activity in lymphocytes, levels and activity of Trx and TrxR in EL4 cells treated with baicalein was measured. It was observed that, baicalein treatment led to significant suppression in Trx and TrxR activity suggesting it may be responsible for its anti-tumor activity. Treatment of EL-4 cells with baicalein altered the activity of redox regulatory enzyme thioredoxin reductase. Baicalein significantly suppressed the activity

of TrxR which may be responsible for the observed anti-tumor activity of baicalein. Baicalein treatment led to increase in phosphorylation of ASK1 and activity of caspase-3.

Baicalein induced cell death in EL4 cells was significantly reduced, when Trx over expressing EL4 cells were used. These findings suggest involvement of thioredoxin system in mediating observed anti-tumor effects of baicalein.

CHAPTER 5: Summary and conclusions

This chapter describes about the implications of the studies carried out in the current thesis. In the present study, promising radioprotective properties exhibited by baicalein have been elucidated. A novel mechanism of radioprotection via inhibition of phosphatase and augmenting hematopoietic stem cell abundance is demonstrated. Baicalein has been shown to activate pro-survival transcription factors NF-kB, Ets-1 and Nrf-2. This study has outlined pleiotropic targets of baicalein in murine splenic lymphocytes as well as in other hematopoietic cells in mice. This study has generated novel insights and improved understanding about strategies for manipulation of cellular response IR to achieve better survival of cells and animals.

Further, another very important aspect about regulation of inflammatory response is also highlighted. This data provides novel mechanistic details about anti-inflammatory activity of baicalein independent of NF-kB-DNA binding. In vitro and in vivo studies on models of inflammation have underscored the fact that, baicalein suppressed mitogen and alloantigen induced activation / proliferation of T cells without affecting their homeostasis driven proliferation in lymphopenic environment.

One of the important attributes of a radioprotector is not to offer survival advantage to tumor cells during IR induced oxidative stress. This thesis has identified that thioredoxin system plays an important role in regulating tumor response to IR exposure. Our study has also provided inputs about anti-tumor activity of baicalein on EL4 cells which are tumor counterpart of normal murine T cells. This study highlights inhibition of thioredoxin system as an important strategy to curb inflammation as well as cancer.

The major conclusions drawn from this study are:

1. Baicalein protected murine splenic lymphocytes but not lymphoma cells against radiation

induced cell death

2. Baicalein activated ERK through inhibition of MKP3 activity
3. Baicalein activated pro-survival transcription factors NF-κB and Nrf-2 via ERK
4. Administration of baicalein exhibited protection against WBI induced mortality in Swiss and C57BL6 mice
5. Baicalein administration enhanced hematopoietic stem cell abundance
6. Administration of ATRA, a pharmacological inhibitor of Nrf-2 abrogated baicalein mediated in vivo radioprotection
7. Baicalein did not suppress Con A induced NF-κB activation but inhibited activation of murine splenic lymphocytes in vitro
8. Baicalein mediated suppression of T cell responses is via inhibition of mitogen induced thioredoxin activity
9. Pharmacological activator of thioredoxin reductase partly abolished baicalein mediated suppression of T cell responses
10. Baicalein induced cell death in EL4 and Jurkat cells in a dose and time dependent manner
11. Trx is a vital regulator of cellular radiosensitivity in EL-4 lymphoma tumor cells.
12. Baicalein treatment led to significant suppression in Trx and TrxR activity and over-expression of Trx in EL4 cells prevented baicalein induced cell death

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Publications in Refereed Journal

a. Published

1. **Patwardhan RS**, Checker R, Sharma D, Sandur SK, Sainis KB, Involvement of ERK-Nrf-2 signaling in ionizing radiation induced cell death in normal and tumor cells; **PLoS One**. 2013 Jun 11;8(6):e65929.

2. **Patwardhan RS**, Sharma D, Checker R, Sandur SK, Mitigation of radiation-induced hematopoietic injury via regulation of cellular MAPK/phosphatase levels and increasing hematopoietic stem cells; **Free Radical Biology & Medicine** 2014 Mar;68:52-64

3. **Patwardhan RS**, Sharma D, Checker R, Thoh M, Sandur SK, Spatio-Temporal Changes in Glutathione and Thioredoxin Redox Couples during Ionizing Radiation Induced Oxidative Stress Regulate Tumor Radio-resistance; **Free Radical Research** 2015 June 26:1-15. [Epub ahead of print]

c. Communicated: "Activator of NF-κB exhibits anti-inflammatory and anti-tumor effects via inhibition of thioredoxin system" **Patwardhan RS**, Sharma D, Thoh M, Checker R, Sandur SK Free Radical Biology & Medicine (Under Review)

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Abbreviations

8-OH dA : 8-hydroxy deoxyadenosine

ACE : Angiotensin converting enzyme

AKI : Acute kidney inflammation

ANOVA : Analysis of variance

APC-cy7 : Alophycocyanin-cy7

ARE : Antioxidant response element

ARP : Acute radiation pneumonitis

ARS : Acute radiation syndrome

ASK1 : Apoptosis signaling kinase 1

ATM : Ataxia telangiectasia mutated

ATRA : *all-trans* retinoic acid

BAFF : B-cell activation factor

BCR : B-cell receptor

BFU-E : Burst forming unit-Erythroid

BM : Bone marrow

BM-MNC : Bone marrow mononuclear cells

BRIT : Board of Radiation Isotope and Technology

BSO : Buthionine sulfoximine

CBP : CREB (cAMP response element) binding protein

CCE : Counterflow centrifugal elutriation

cdk-2 : cyclin dependent kinase-2

cDNA : complementary deoxyribonucleic acid

CDNB : 1-chloro,2,4 dinitrobenzene

CFSE : Carboxy fluoresceindiacetate succinimidyl ester

CFU-E : Colony forming unit-Erythroid

CFU-GEMM : Colony forming unit-granulocyte erythrocyte megakaryocyte monocyte

CFU-S : Colony forming unit-spleen

CLP : Common lymphoid progenitor

CMP : Common myeloid progenitor

CNS : Central nervous system

ConA : Concanavalin A

COPD : Chronic obstructive pulmonary disease

COX-2 : Cyclooxygenase-2

CsA : Cyclosporin A

CTL : Cytotoxic T- lymphocyte

CTLA4 : Cytotoxic T- lymphocyte antigen-4

DABCO : Diazabicyclo[2.2.2]octane

DHE : Dihydroethidine

DHR123 : Dihydrorhodamine 123

DMSO : Dimethylsulfoxide

DNA : Deoxyribonucleic acid

DNA-PK : Deoxyribonucleic acid protein kinase

DPPH : 2,2-diphenyl-1-picrylhydrazyl

DRF : Dose reduction factor

DTNB : 5,5'-dithiobis-(2-nitrobenzoic acid)

DTT : Dithiothreitol

dUTPs : deoxy-uridine triphosphate

ED : Effective dose

EDTA : Ethylenediamine tetraacetic acid

EGF : Epidermal growth factor

EGFR : Epidermal growth factor receptor

EGR2 : Early growth response 2

ELISA : Enzyme-linked immunosorbant assay

EMSA : Electrophoretic mobility shift assay

Eo-CFC : Eosinophil-colony forming unit

ERK : Extracellular signal-regulated kinase

FACS : Fluorescence activated cell sorting

FBS : Fetal bovine serum

FGF : Fibroblast growth factor

FITC : Fluorescein isothiocyanate

GCLC : Glutamate cysteine ligase catalytic subunit

GCLM : Glutamate cysteine ligase modifier subunit

GCSF : Granulocyte colony stimulating factor

GI : Gastrointestinal

GMCSF : Granulocyte monocyte colony stimulating factor

GMP : Granulocyte macrophage progenitor

GPx : Glutathione peroxidase

GSH : Glutathione (reduced)

GSK3β : Glycogen synthase kinase 3β

GSSG : Glutathione (oxidase)

GST : Glutathione S- transferase

GVHD : Graft versus host disease

H_2DCFDA : 2',7'-dichlorodihydrofluorescein diacetate

HCl : Hydrochloric acid

HDP : Homeostasis driven proliferation

HEPES : 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HGF : Hepatocyte growth factor

HNSCC : Head and neck squamous carcinoma cells

HO-1 : Hemoxygense-1

HPCs : Hematopoietic progenitor cells

HS : Hematopoietic syndrome

HSC : Hematopoietic stem cells

HT : 5-hydroxy tryptamine

IBD : Inflammatory bowel disease

ICAM-1 : Intercellular adhesion molecule 1

IFN-γ : Interferon- γ

IKK : IκB kinase

Ikβ : Inhibitory kinase beta

IL-1 : Interleukin-1

IL-1βR : Interleukin-1β receptor

IL-3 : Interleukin-3

IL-6 : Interleukin-6

IMDM : Iscove's Modified Dulbecco's Medium

IMP dehydrogenase : Inosine monophosphate dehydrogenase

iNOS : inducible nitric oxide synthase

IP : Immunoprecipitation

IR : Ionizing radiation

IRP : Iron regulatory protein

JNK : c-Jun N-terminal kinase

KEAP1 : Kelch like ECH associated protein 1

KI DTPA : Potassium iodide diehtylenetriamine pentaacetate

LD50 : Lethal dose 50

LET : Linear energy transfer

LFA1 : Lymphocyte function associated antigen 1

LPS : Lipopolysaccharide

LRP : Lineage restricted progenitor

LTβ : Lymphotoxin beta

LTRC : Long term reconstituting cells

mAb : Monoclonal antibody

Mast-CFC : Mast colony forming cell

M-CFC : Monocyte colony forming cell

MCP-1 : Monocyte chemoattractant protein

Meg-CFC : Megakaryocyte colony forming cell

MEK1/2 : Mitogen-activated protein kinase kinase ½

MEP : Megakaryocyte erythrocyte progenitor

MHC : Major histocompatibility complex

MKP-3 : MAPK phosphatase 3

MMP : Mitochondrial membrane potential

MMR : Mismatch repair

Mn-SOD : Manganese superoxide dismutase

MPP : Multipotent progenitors

MTD : Maximum tolerated dose

mTOR : Mammalian target of rapamycin

NADPH : Nicotinamide adenine dinucleotide phosphate reduced

NVD : Nausea, vomiting, diarrhea

NEMO : NF-κB essential modulator

NF-κB : Nuclear factor kappa B

NIK : NF-κB inducing kinase

NK cells : Natural killer cells

Nrf-2 : Nuclear factor erythroid 2 related factor 2

NSAID : Non-steroidal anti-inflammatory drugs

PBMC : Peripheral blood mononuclear cells

PBS : Phosphate buffered saline

PCR : Polymerase chain reaction

PE : Phycoerythrin

PF : Pulmonary fibrosis

PGE2 : Prostaglandin E2

PHA : Phytohemagglutinin

PI : Propidium iodide

PI3K : Phosphoinositide 3 kinase

PKC : Protein kinase C

PMA : Phorbol myristate acetate

PNPP : Para-nitrophenylphosphate

PSSG : Glutathionylated protein

PTEN : Phosphatase and tensin homolog

PTP : Protein tyrosine phosphatase

PUMA : p53 upregulated mediator of apoptosis

RAG : Recombination activating gene

RANKL : Receptor activator of nuclear factor kappa-B ligand

Ref 1 : Redox factor 1

RHD : Rel homology domain

RNA : Ribonucleic acid

RNS : Reactive nitrogen species

ROS : Reactive oxygen species

RPMI : Roswell Park Memorial Institute

RTK : Receptor tyrosine kinase

Sca-1 : Stem cell antigen 1

SCID : Severe combined immunodeficiency disease

SDS : Sodium dodecyl sulphate

SEM : Standard error of mean

SLAM : Signaling lymphocyte activation molecules

SP : Side population

STATs : Signal transducers and activators of transcription

STRCs : Short term reconstituting cells

TAD : Trans-activation domain

TBI : Total body irradiation

TCR : T cell receptor

TGF-β1 : Transforming growth factor beta

TNFR : Tumor necrosis factor receptor

TNF-α : Tumor necrosis factor alpha

Treg : Regulatory T cells

Trx : Thioredoxin

TrxR : Thioredoxin reductase

TUNEL : Terminal dUTP nick end labelling

UGT : UDP glucuronosyl tranferase

USFDA : United States Food and Drug Administration

UV : Ultraviolet

VEGF : Vascular endothelial growth factor

VLA4 : Very late antigen-4

VOD : Venoocclusive disease

WRARI : Walter Reed Army Research Institute

CHAPTER 1

INTRODUCTION

1.1 Ionizing Radiation (IR)

Ionizing radiations (X-rays and gamma rays) belong to right end of the electromagnetic spectrum that possesses enough energy to dislodge an electron from atoms or molecules **Fig. 1.1**. They can be in the form of wave or particulate matter (photons). Apart from this, there are charged (beta, alpha and protons) or neutral sub-atomic particles (neutrons) which are capable of causing excitations and ionizations in the medium. Environmental radiation is mostly non-ionizing type, including ultraviolet (UV) rays, visible light, radiowaves and microwaves.

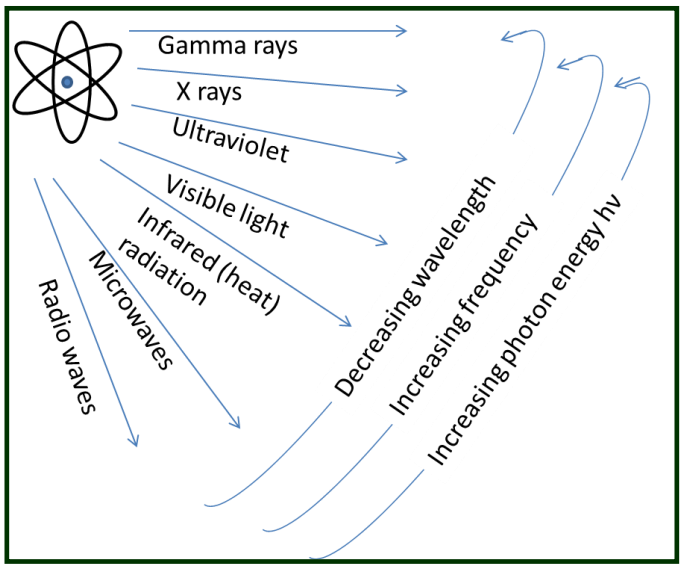


Fig. 1.1 Electromagnetic Spectrum **(Hall, E., 2005)**

Living organisms are constantly exposed to background ionizing radiation (IR) from both natural sources and also from human activities. Natural sources e.g. cosmic rays, and terrestrial sources that come from radionuclides in the earth's crust, air, food and water and the human body itself contribute to ~90% of background IR exposure [15] . Whereas man-made sources include medical uses of radiation and radioisotopes in health care, generation of electricity from nuclear power reactors, industrial uses of nuclear energy and so on.

1.1.1 Energy deposition by IR

IR loses energy while travelling through matter along the length of the path which is called as Linear Energy Transfer (LET). It is defined as the average energy deposited per unit length of track and the unit is keV/μm [16]. LET is used as a measure to indicate the quality of different types of IR. Biological effect of IR is directly proportional to LET. Energy deposition along the track is function of charge, mass and nature of radiation [17]. Based on the energy deposition, IR are categorized into low and high LET radiations and γ rays are considered as low LET radiations because photons are mass less and charge less and they cause sparse ionizations. However, high LET radiations like α, β and protons deposit most of the energy in very short distances due to charge and mass [18]. Radiation is mostly measured as absorbed dose that is amount of energy deposited per unit mass with S.I. unit of gray (1 Gy = 1 J/ kg).

1.1.2 Direct and indirect action of IR

IR can lose energy in the biological material by directly causing ionizations to the cellular macromolecules like DNA, proteins and lipids resulting in damage. IR can also lose energy by causing indirect damage through water radiolysis as it is the most abundant chemical in biological systems. Radiolysis of water generates different free radicals such as hydroxyl radical, e_{aq}, superoxide radicals, hydrogen peroxide etc. Hydroxyl radical is a highly reactive and oxidizing in nature with biological half-life in the range of nano seconds. It can diffuse very short distances before reacting with the cellular substrates. In oxygenated solutions, hydrogen atoms can react with oxygen to give hydroperoxyl free radicals ($HO2^{\bullet}$). Different products of water radiolysis are shown in ***Fig. 1.2***. The relative yields of the water radiolysis products depend on the pH and LET of the radiation. These free radicals are highly reactive and react with critical biomolecules like lipids, proteins and DNA in cells thereby influencing cellular metabolism, cell

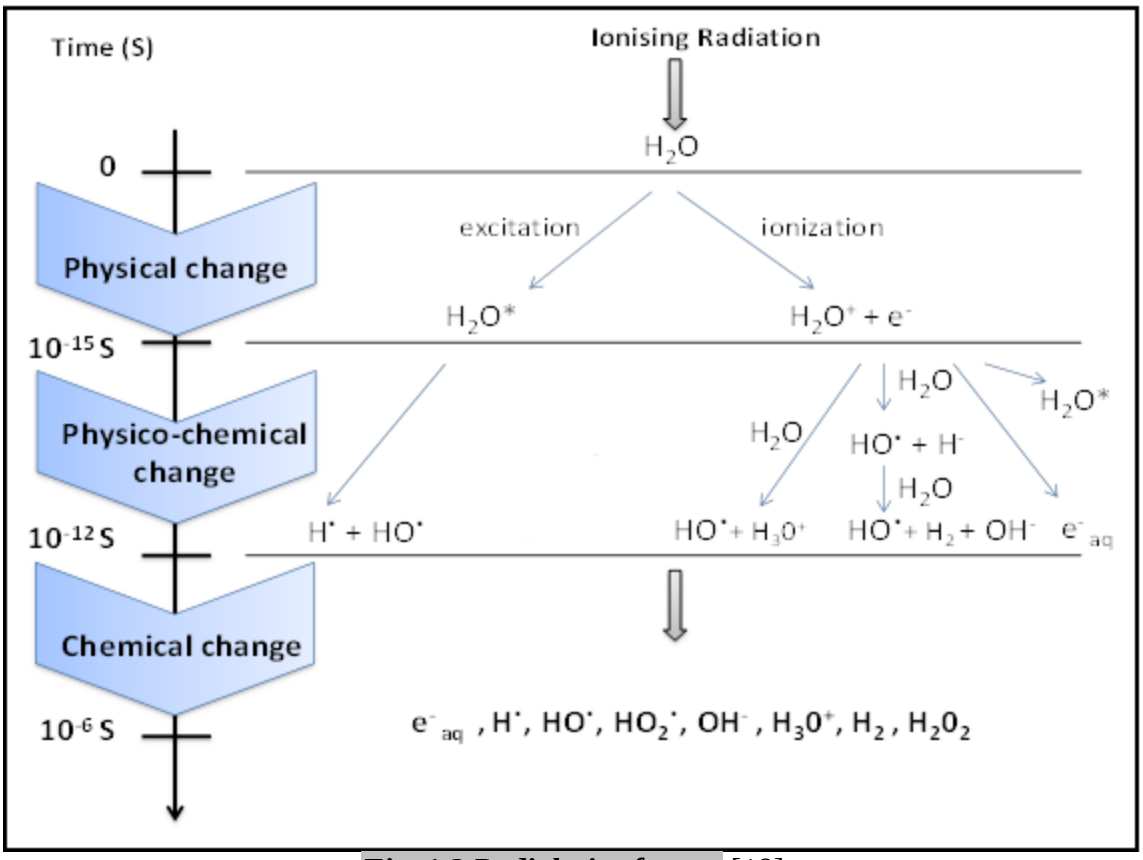


Fig. 1.2 Radiolysis of water [18]

cycle and survival. The component of indirect action is more predominant with low LET radiation mediated damage as against high LET radiation ***Fig. 1.3.***

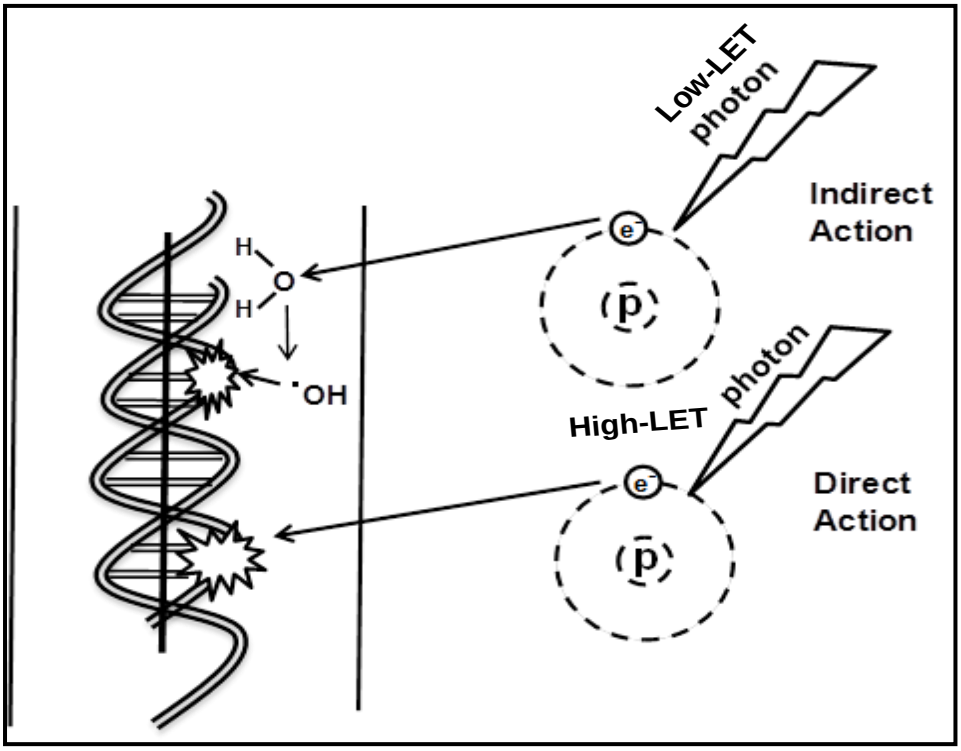


Fig. 1.3 Direct and indirect action of IR on DNA **(Hall, E., 2005)**

1.1.3 ROS as mediators of IR induced damage

Reactive oxygen species (ROS) consist of free radicals and prooxidants that are generated as a result of water radiolysis. Free radicals are chemical species having one or more unpaired electrons. ROS are also generated as byproducts of cellular metabolism [19]. **Fig. 1.4** shows four electron reduction of molecular oxygen leading to generation of water and intermediate free radicals. **Table 1.1** lists different free radicals and their half-life, whereas **Fig. 1.5** shows endogenous sources of ROS generation and **Fig. 1.6** shows role of ROS as signaling mediators. **Fig. 1.7** shows effect of level of oxidative stress on cellular functions whereas **Table 1.2** shows apoptotic pathways activated by ROS.

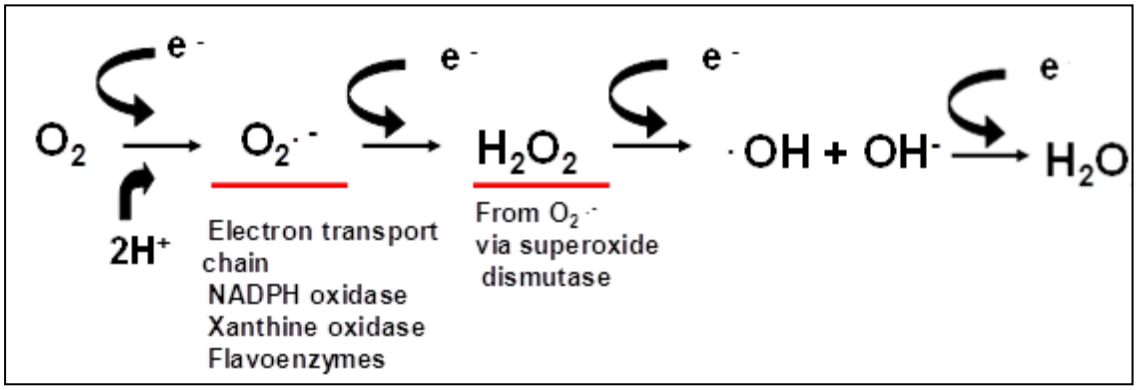


Fig. 1.4 Four electron reduction of water

Table 1.1. Different reactive oxygen species and their half-life[20]

Radical	Half-life
H_2O_2, ROOH	~minutes
peroxyl radicals, nitric oxide	~seconds
peroxynitrite	~milliseconds
superoxide anion, singlet oxygen, alkoxyl radical	~microseconds
hydroxyl radical	~nanoseconds

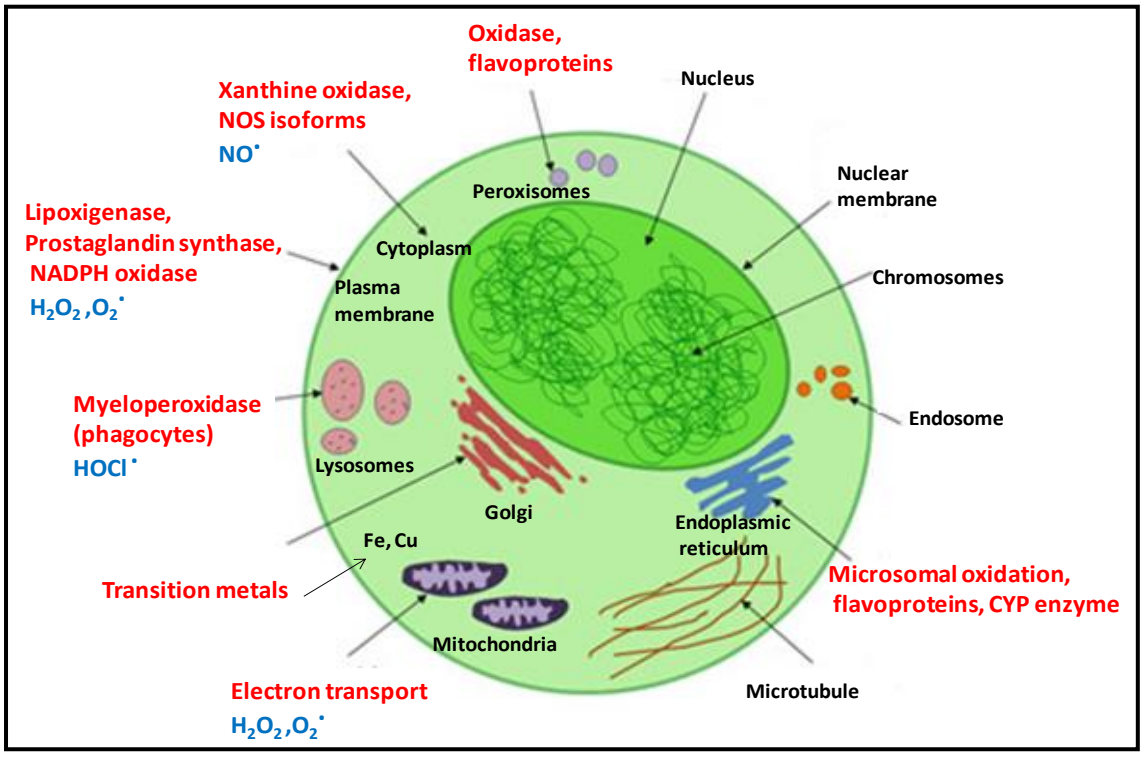


Fig. 1.5. Endogenous sources of ROS

ROS as signaling mediators

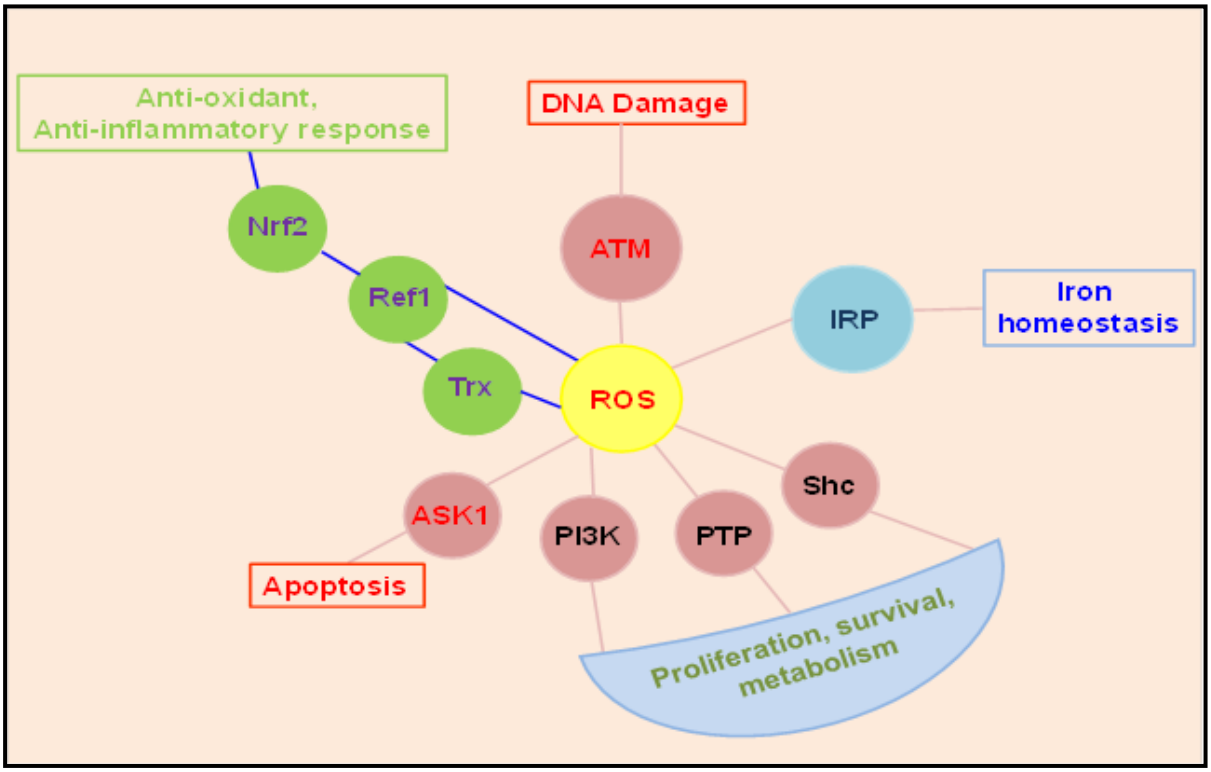


Fig. 1.6 Cellular signaling pathways regulated by ROS [21]

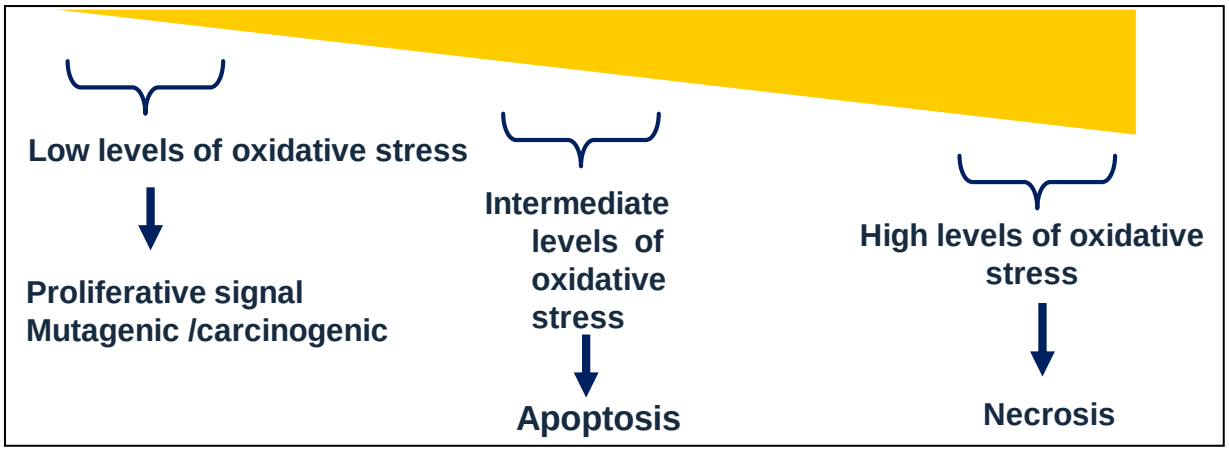


Fig. 1.7 Extent of oxidative stress and consequences on cells

Table 1.2. Appoptotic signaling pathways activated by ROS

Event	Modification
Caspase activation	H_2O_2 inactivation of caspases [22] denitrosylation of caspases [23]
Mitochondrial function	Induction of permeability transition pore, loss of membrane potential, Cytochrome c release [24] Aalteration of critical thiol groups on ANT and Peroxidation of cardiolipin [25]
Phosphatidylserine exposure	Alteration of critical thiol residue on phospholipid translocase [26]

1.2. Antioxidants couter the effects of ROS

Cells are equipped with substances that can take care of cellular load of oxidants called as anti-oxidants. They are capable of inhibiting or delaying oxidation of other subtrates by neutralizing the free radicals.Antioxidants are classified based on their mechanism of action:

Prevention of pro-oxidant formation: Inhibitors of nitric oxide synthase, NADPH oxidase, xanthine oxidase and metal chelators such as metallothionein, transferrin, lactoferrin act at the source of ROS generation. They block ROS generating machinery and hence prevent oxidant generation.

Interception of pro-oxidants: This class of agents include classical antioxidants. Cells contain pool of enzymatic (catalase, superoxide dismutase, peroxidase) and non-enzymatic antioxidants (glutathione, cysteine) that can neutralize free radicals.

Chainbreaking antioxidants: Fat soluble antioxidants like tocopherols can act as chain breaking antioxidants in the lipids thereby preventing the propogation radical mediated reactions.

Repair of damage caused by pro-oxidants: DNA repair proteins, cell cycle regulatory molecules, protein disulfide reductase (thioredoxin) etc. can account for restoration of loss of functionality of proteins. All these pathways are operative to curb oxidant stress in cells. Out of

all these strategies enzymatic anti-oxidants are more specific in action and also higly coordinated inside cells (**Table 1.3)**. They play pivotal role in regulating cellular redox homeostasis.

Table 1.3. Enzymatic antioxidants and their functions

Enzyme	Function	Location
Catalase	Reduces hydrogen peroxide to water and oxygen [27]	Peroxisomes
Superoxide Dismutase	Dismutates superoxide anion to hydrogen peroxide and O_2 [28]	Mitochondria and cytosol
Glutathione Peroxidase	Reduces hydrogen peroxide and lipid hydroperoxidesusing GSH as a substrate [29]	Cytosol
Glutathione Reductase	Reduces oxidized form of glutathione[30]	Cytosol
Peroxiredoxin	Reduces peroxides to corresponding alcohol [31]	Mitochondria and cytosol
Thioredoxin (Trx)	Reduces protein disulfides using NADPH [32]	Cytosol, mitochondria and nucleus
Thioredoxin Reductase (TrxR)	Reduces oxidized thioredoxin [33]	Cytosol, mitochondria and nucleus
Glutathione S transferase	Transfers glutathione to protein or non-protein moiety [34]	ER, cytosol
Glutaredoxin	Controls deglutathionylation of proteins [35]	Cytosol, mitochondria and nucleus

1.3. Biological effects of IR

Systemic effects after radiation exposure are seen at wide temporal scale (**Fig. 1.8)**. Biological effects of IR exposure are attributed to chemical changes in critical biomolecules that originate after energy absorption. There is a latent period between energy absorption and manifestation of biological effects which may vary from days to years depending upon confounding factors [36]. Even at low doses there is sufficient chance of modification in critical biomolecule like DNA leading

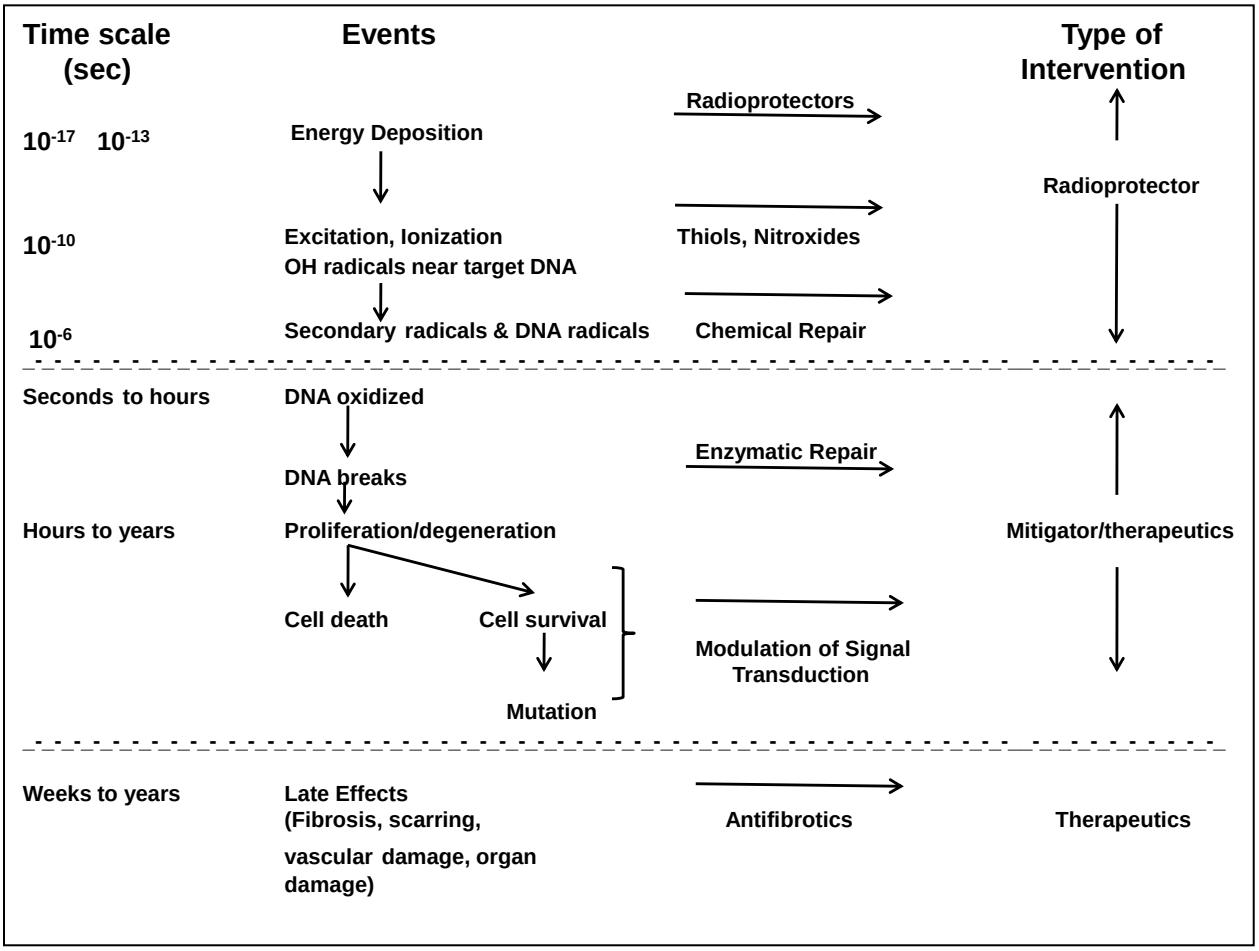


Fig. 1.8 Timescale of effects of IR on cell **[37]**

to mutagenic lesions that are apparent after few years of radiation exposure [37].

Such effects are termed as "stochastic effects". At high doses severity of radiation damage is visible in very short time leading to death or irreparable damage in exposed cells. Such effects are termed as "deterministic effects".

1.3.1 Molecular targets of radiation damage in cells

As discussed above, ROS are principal mediators of low LET IR induced damage to DNA, proteins and lipids.

DNA as primary target of IR injury

The important characteristic of IR is energy dissipated per ionizing event which is about ~33eV that is more than enough to break a covalent bond [15]. IR can induce DNA damage by single strand breaks, double strand breaks, base damage, sugar damage, apurinic/apyrimidinic sites, intra-molecular cross links, DNA-protein cross links, hydrogen bond breakage etc. [38, 39]. The presence of histones and DNA in a 1:1 weight ratio makes histones prime candidates for crosslinks. **Table 1.4** shows damage in cells exposed to 1Gy of low-LET IR.

Table 1.4. Damage in mammalian cell nucleus [40]

Event	Frequency
Ionizations directly in DNA	**~2000**
Single strand breaks	**~1000**
8-OH-dA	**~700**
DNA-protein cross links	**~150**
Double strand breaks	**~40**

This damage if unrepaired or mis-repaired causes significant toxicity at the tissue and organ level. Alteration in DNA structure results in loss of viability and hereditary changes [40]. Apart from these damages, exposure to IR also leads to induction of chromosomal aberrations [41] wherein broken chromosomes tend to rejoin and in the process incorrect reunions give rise to lethal chromosomal alterations [42]. Although many different types of aberrations are possible but out of these incorrect reunions like dicentrics, ring formation and anaphase bridge formation are potentially lethal [43]. Other than these, translocations and interstitial deletions are two non-lethal chromosomal aberrations that lead to development of malignancies.

Chromosomal aberrations scored in peripheral blood lymphocytes obtained from exposed individuals serve as biomarkers to estimate the dose received by these individuals [18].

Lipid peroxidation of biological membranes

Membrane lipids are highly susceptible to radiation induced damage primarily due to presence of poly-unsaturated fatty acids. Peroxidative decomposition of membrane lipids and oxidation of proteins results in loss of membrane bound enzyme activity. Oxidation of lipids leads to structural changes in cellular membranes like altering fluidity and channels, altered membrane-bound signaling proteins, increased ion permeability and disruption in membrane dependent signaling [44].

1.3.2 Effect of IR on cell cycle

Cells can repair radiation induced damage to critical biomolecules. However, if the repair is incomplete cells can undergo apoptosis or necrosis or mitotic catastrophe or senescence. The accepted gold standard for measuring the radiosensitivity of a dividing cell population is their ability to retain the reproductive integrity. Survival curves are best shown as a semi-log plot of survival against radiation dose, generally in the dose range of 1–10 Gy for single cells. Renewing cells in a growing population (e.g. skin, gut, bone marrow, tumor cells or cells in culture) participate in the cell cycle. Replication of the genome occurs in S-phase and mitotic propagation to daughter generations occurs in G2/M phases. There are checkpoints at the G1/S and G2/M boundaries that monitor the fidelity of genomic processing. Radio sensitivity differs throughout the cell cycle, late S-phase being most radio-resistant, G2/M being most radiosensitive and G1 phase taking an intermediate position. Following table describes radio sensitivity profile of cells in different phases **Table 1.5.**

Table 1.5. Radio sensitivity of cells in different phases of cell cycle **[45]**

Cell Cycle Phase	Sensitivity	Reason
Late S	Most Radio resistant	Greater proportion of repair by HR pathway
G1	Intermediate	Open chromatin, accessibility for repair proteins
G2/M	Highly radiosensitive	Chromatin compaction, poor repair competence

1.3.3 Acute effects of IR

Acute Radiation Syndrome (ARS) is an acute illness caused by irradiation of the whole body by a high dose of IR in a very short period of time. Depending on the anatomic site treated acute effects may include: nausea and vomiting, tiredness, fatigue, diarrhea, headache, as well as normal tissue swelling, skin erythema, cough, difficulty in swallowing and difficulty in breathing [46-48]. Both direct and indirect effects influence the magnitude and duration of acute side effects. In cells that repair irradiation damage and survive, release of inflammatory cytokines can induce cell killing [49, 50]. Inflammatory cytokine released act on sub-lethally irradiated or unirradiated cells leading to cell death through the apoptosis, autophagy and necrosis [51, 52].

1.3.4 Late effects of IR

Chronic effects are delayed toxic effects of IR exposure. Besides the organ exposed, dose and volume of irradiation also determine the severity of chronic effects [53]. Some of the chronic effects are hair graying, skin thinning and dryness, formation of cataracts, early myocardial fibrosis, myocardial infarction, neurodegeneration etc. [54, 55]. IR exposure hampers endocrine function by inducing cell death [56]. One of the most prominent chronic effects of ionizing irradiation is induction of cancer [57]. Prolonged production of ROS even months and years after irradiation can potentially induce genetic change in cells [58, 59]. Migration of stem cells into an irradiated environment can expose them to oxidative stress due to ROS released from stromal

cells causing mutations [60]. These mutations may lead to malignant transformation in stem or non-stem cell population resulting in radiation induced secondary cancers [61-63].

1.3.5 Systemic effects of IR

Systemic effects are response to partial body or local irradiation such as a thoracic, abdominal or pelvic region as well as whole body irradiation (WBI) [64-66]. A collection of health effects that appear within 24 hours of exposure to high amounts of IR is called as acute radiation syndrome [47, 67]. Acute effects of radiation are predominantly due to death of the dividing cells in the body present in bonemarrow, gut cells, skin and hair roots [68, 69]. **Table 1.6** describes various syndromes and its medical management.

Table 1.6. Acute radiation syndromes **[70]**

Dose (Gy)	Radiation syndrome	Symptoms & Consequences	Medical Management
1 - 2	**Nausea, vomiting, diarrhea (NVD) syndrome**	Nausea, vomiting, diarrhea, anorexia, giddiness, and loss of appetite	Symptomatic treatment, antacid, sucralfate, anti-emetics
2 - 6	**Hematopoietic syndrome**	Loss of cellularity in bone marrow, spleen and thymus. The individual may die between 10-30 days without medical intervention.	Antibiotics, cytokines, bone marrow transplant, stem cell therapy
8 - 15	**Gastrointestinal (GI) syndrome**	Damage to intestinal crypt cells, loss of absorption of nutrients, dehydration, loss of weight, severe electrolyte imbalance and low blood pressure. Death occurs usually within 3–5 days without medical intervention.	Antibiotics, anti-emetics, replacement of fluids and electrolytes, stem cell therapy, bone marrow transplant
> 25	**Central Nervous System (CNS) syndrome**	Irritability, hyper excitability response, epileptic type fits and coma. Symptoms are irreversible. Death usually occurs within 48 h.	No treatment available

1.3.6 Hematopoietic syndrome

The signs and symptoms of radiation sickness after an acute total body exposure are predominantly the consequences of radiation injury to the hematopoietic cells in the bonemarrow [71, 72]. Bone marrow progenitor cells and stem cells decrease their proliferative activity after radiation exposure [73]. Consequently, fewer cells are available for differentiation and maturation to white blood cells, red blood cells and platelets. Thus, the kinetics of cell production and migration from bonemarrow is disturbed thereby depleting the peripheral pool of immune cells [74].

Since mature granulocytes have a life span of only one day, the radiation-induced decrease of supply of granulocytes occurs followed by decrease in the number of platelets [75]. Then further development of the hematopoietic radiation syndrome depends on the number of bone marrow stem cells that have survived radiation exposure [76]. The surviving stem cells can reconstitute the bonemarrow compartment [77]. If the number of surviving bone marrow stem cells is insufficient for regeneration of hematopoietic system, fatal consequences of leukopenia and thrombopenia are observed [78]. Individuals experience tiredness associated with anemia (low red cell count), propensity for bleeding (associated with low platelets), and inability to fight infections [72, 79]. The production of inflammatory cytokines including TNFα, TGFβ1 and IL-1 correlates with the severity of suppression of hematopoiesis [80].

The severity of the hematopoietic damage increases with increasing dose of radiation [81, 82]. Death occurs at the time of the nadir of granulocyte depletion in the blood, i.e. in the third week after acute radiation exposure [47, 74]. Bacterial contamination of gut, oropharynx, depletion in platelets and infection with multiple pathogens such as fungal, herpes and septic infections lead to death of the exposed individual [73, 83-85].

Past efforts to stimulate hematopoiesis in myelosuppressed animals have involved administration of components of microbial cell walls or their synthetic analogs [86, 87] and natural factors such as cytokines, prostaglandins, and peptides or their synthetic analogs. Many cytokines including vascular endothelial growth factor I (VEGF-1), hepatocyte growth factor (HGF), fibroblast growth factor (FGF) and epidermal growth factor (EGF) facilitate the repair and replenishment of irradiated tissue [88]. It would be advantageous to develop a small-molecule that would ameliorate hematopoietic radiation injury with least toxicity.

1.4 Radiation exposure scenarios

1.4.1 Planned exposures Since the discovery of X-rays in the early 1900s, radiation therapy has been used for treating various skin conditions along with cancers of the head, neck, and lymph nodes. Reports as early as 1902–1904 document the application of radium in treating pharyngeal carcinomas and delivering radiation through glass tubes placed in close vicinity of the tumors through interstitial brachytherapy. Different types of radiation therapies (e.g., external beam radiation therapy, brachytherapy, and systemic radioisotope therapy) and fractionation regimens (e.g., hypofractionation, hyperfractionation, andaccelerated fractionation) are currently tested in a variety ofcancers under small and large clinical settings [89]. It is estimated that radiotherapy is main line treatment for ~40% of the cancers [63, 90]. It is often used along with chemotherapy to reduce tumor burden [91]. For patients with incurable cancers it is one of the preferred treatment options to control the symptoms. It can be used before the surgery to reduce the tumor size so that it is easier to remove (neoadjuvant therapy) or after surgery to eradicate small mass of tumor cells that may be remaining after surgery (adjuvant therapy) depending on the type of cancer [90, 92, 93].

Limitations of radiotherapy IR cannot discriminate between normal and tumor cells thereby exerting its harmful effects on normal cells surrounding the tumor tissue. This limits the amount of dose that can be delivered to tumor cells thereby compromising the therapeutic output of radiotherapy. Since tumor cells proliferate rapidly, they overgrow and create anoxic and hypoxic zones thereby reducing efficacy of radiotherapy [94]. To achieve similar therapeutic index, higher radiation doses are needed but vulnerability of normal tissue seated next to the tumor (owing to well perfused, vascularised and oxygenated environment) [57] makes it infeasible. Under these circumstances, to obtain optimum therapeutic output a judicial balance between total dose that can be delivered and threshold tolerance limit of surrounding normal tissue needs to be considered. However, reduction in total dose or dose per fraction during radiotherapy enhances the risk of induction of radio-resistance in tumors and also increases the relapse. Improvements in targeted radiotherapy treatments with better technological control and high precision analytical modeling or antibody therapy have resulted in achieving better tumor control. But the use of such techniques or treatment options is limited due to unclear margins between normal and tumor tissue and also because of high cost of the treatment. An alternate approach to increase the total dose or dose per fraction is to employ agents that can differentially modulate the responses of normal and tumor tissues to radiation. These are called as radiomodulatory agents that can protect normal cells against radiation induced cell death without compromising the tumor tissue toxicity.

The other known planned exposure scenarios is astronauts getting exposed to high energy protons, α-rays, heavy ions and cosmic rays during space flight. These high LET radiations can cause clustered damage because of direct effect. Radiation workers in the nuclear industry may

have to undertake emergency operations in power plants also fall under planned exposure category.

1.4.2 Unplanned exposures Radiation accidents such as those that occurred in Fukushima, Japan (2011), Tokaimura, Japan (1999), Goiânia, Brazil (1988), Chernobyl, Russia (1988), and Three Mile Island nuclear power station, United States (1979), all serve as warning signs of the potential hazards associated with catastrophic nuclear/radiological events [95-99]. In addition, threats from exposure to high doses of radiation due to terrorist attacks have become serious risk factor in recent years [100, 101].

1.5 Radioprotectors To counter both planned and unplanned exposures there is a need for radioprotective agents that can prevent or reverse the damage caused by IR. Radioprotector is defined as "any medicinal agent or device applied prior to or during radiation exposure that actively prevents or limits injury, whether that injury may be at molecular, cellular, tissue, organ or system level" (Seed 2005) [102]. Radioprotectors can be classified into three categories based on their time of administration. i) prophylactic agents that need to be administered prior to irradiation ii) mitigators are the agents that need to be administered during exposure or immediately after the exposure iii) therapeutics agents are administered post IR exposure.

1.5.1 Prophylactic agent Prophylactic agents will find multiple applications in planned exposure scenarios like radiotherapy of cancers and protection of astronauts during spaceflight against protons and high-energy particles. The radioprotector can be administered to the individual prior to the exposure depending on its time window of protection so that it can protect normal tissues but not tumors, during radiotherapy. These prophylactic agents may also find use during radiological accidents to select groups, such as military personnel and first responders,

who need appropriate protective agents to be administered prophylactically prior to entry into contaminated areas.

1.5.2 Mitigators These can be used in emergency conditions like soon after exposure to IR or ingestion/ inhalation of radionuclides.

1.5.3 Therapeutic agents

Therapeutic agents that are effective even after several hours of radiation exposure are required to treat victims of radiological accidents or incidents. The clinical scenarios like radiotherapy, radiological accidents and credible risks warrant the need for developing radiation countermeasures for protecting against exposure-related morbidity and/or mortality [103]. Extremely limited numbers of safe and effective medical countermeasures have been approved by the United States Food and Drug Administration (USFDA). None of these drugs have been approved to counter specifically "acute radiation syndrome" (ARS) or as a general radioprotector. This situation has intensified the research on identifying a new generation radiation countermeasure. The success in the development of radiation countermeasures will mainly depend on the understanding of the damage resulting from radiation exposure.

Attributes of Ideal Radioprotector

- Globally effective
- Protects against early and late effects
- Efficacious in protecting radiosensitive tissues
- Nontoxic
- Performance should be non-decrementing with time
- Safe with repeated doses
- Self-administered pill, inhaler, swab, skin patch
- Broad time window of protection
- Rapidly effective

- Chemically stable, long shelf life
- Simple to produce / manufacture
- Inexpensive

The mechanistic/biological basis for development of a radioprotective strategy necessitates an understanding of the molecular biology underlying the cellular, tissue and organ specific radiation damage. Some of the events that occur in an irradiated cell are generation of primary and secondary ROS, DNA strand breaks, communication of nuclear stress responses through the cytoplasm to mitochondria, mitochondrial response to nuclear signaling, and mitochondrial initiation of apoptosis [104-106]. Several strategies have been adapted by targeting these cellular processes that follow radiation exposure which are enlisted below and representative examples are provided in **Table 1.7** whereas list of radioprotectors given different types of approval by USFDA is provided in **Table 1.8.**

1.6 Strategies used for design and development of putative radioprotectors

1. Suppression of formation of ROS / free radicals (sulfhydryl compounds)
2. Induction of intracellular anti-oxidants e.g. SOD mimics, glutathione, Mn-SOD liposomes
3. Enhancement of DNA repair and DNA binding agents (Selenium, Hoechst 33342)
4. Delay in cell division (p53/p21 inhibitors)
5. Cytokines and growth factors (IL3, GCSF, VEGF, FGF)
6. Activation of pro-survival/anti-apoptotic pathways (NF-κB, Nrf-2, Bcl-2)
7. Inhibitors of pro-apoptotic molecules (p53, PUMA, Bax)
8. Management of post irradiation inflammation (NSAIDS)
9. Immune and hematopoiesis stimulators (stem cells, antibiotics)
10. Chelating agents (KI, DTPA)

Table 1.7. Representative examples of radioprotective agents

Radioprotector /Mitigators/ Therapeutics	Representative	Proposed Mechanism of Action/Use
Sulfhydryl compounds	cysteine, cysteamine, glutathione, amifostine [2, 107]	Free radical scavenging, induction of hypoxia and DNA repair
Selenium compounds	selenomethionine, sodium selenite [108] [109]	Antioxidant upregulation
Nitroxides	tempol, tempol-H, tempace [110] [111]	Free radical scavenging
Natural antioxidants	vitamin A, C, E, phytochemicals, melatonin [112-114]	Free radical scavenging, anti-mutagenic
Immuno-modulators	cytokines (IL3, GCSF), LPS, steroids (5-androstenediol, oxymetholone) [115-117]	Increased cytokine production, immune stimulation, myelopoiesis
NF-κB activators	LPS, CBLB502, CBLB613, DMA [12, 118, 119]	Induction of anti-oxidant and anti-apoptotic genes
Nrf-2 activators	synthetic triterpenoids, trolox, caffeic acid phenethyl ester, [120-122]	Free Radical Scavenging enzymes, augmentation of hematopoiesis and cytoprotective genes
Non-steroidal anti-inflammatory drugs	indomethacin, diclonefac, flurbiprofen [123, 124]	Enhanced hematopoiesis, increased GCSF
HIF-1α activator	dimethyloxalylglycine [125, 126]	Induction of DNA damage response, HSC quiescence
Cell cycle regulators	CDK4/6 inhibitor (PD0332991),VRX0466617 (Chk2 inhibitor) [127, 128]	Protection of HSC
mTOR inhibitor	rapamycin [129, 130]	Suppression of p53 and PUMA

Inhibitor of CD47	thrombospondin-1 [131, 132]	Increased NO signaling
Molecular hydrogen	Hydrogen gas [133, 134]	Selective reduction of hydrogen, hydroxyl radical and peroxynitrite
p53 inhibitors	pifithrin-α, pifithrin-μ [135-137]	Blockade of apoptotic pathways; inhibition of caspases
PUMA inhibitors	[106, 138]	Protection of HSCs and HPCs, protection against GI syndrome
GSK3β inhibitors	SB216763, SB415286 [139, 140]	Inhibition of p53, activation of Nrf-2
HMG-CoA reductase inhibitor	statins (lovastatin, simvastatin, pravastatin) [141, 142]	Protection against radiotherapy induced inflammation and fibrosis
Stem cell based therapy	mesenchymal stem cells, cord blood stem cells, myeloid progenitor cells, [143, 144]	Release of pro-survival factors
Antibiotics	tetracyclines, fluoroquinolones, ciprofloxacine [145, 146]	Increase in histone acetyl transferase activity and increase in DNA repair
DNA binding ligands	hoechst 33342, proamine, methylproamine and netropsin [147] [148]	Electron/hydrogen donation to damaged DNA
ACE inhibitors/ receptor agonists	captopril, enalapril, rampril, penicillamine, pentoxyfylline [149] [150]	Suppression of chronic oxidative stress
Ca- antagonists and Zn salts	nifedipine, nimodipine, Zn aspartate [151]	Inhibition of calcium influx
TGF-β signaling inhibitors	ID11, SM16 [36] [152] [153]	Antifibrotic

Table 1.8. List of currently FDA approved radiation countermeasures **[1, 102]**

	Trade Name	Mode of Action	Use
USFDA Approved Countermeasures	Amifostine (WR-2721)	Cytoprotectant, free radical trap	Mitigation of radiation induced xerostomia in head & neck cancer patients
	Potassium Iodide	Blocking agent	Blocking uptake of radioactive Iodine by thyroid
	Ca/Zn DTPA	Chelation and elimination	Reduction of body burden of plutonium, americium, curium etc
	Ferric III hexacyanoferrate II (Radiogardse)	Chelation and elimination	Reduction of body burden of cesium and thallium
	Granisetron	Anti-emetic; 5-HT3 inhibitor	Control of GI disturbances
Countermeasures approved for other Indications: Off Label	G-CSF, Filgrastim (Neupogen)	Stimulates hematopoiesis	Alleviates neutropenia in immune-compromised patients
	Pegylated G-CSF, Pegfilgrastim	Stimulation of neutrophil production	Highly effective in alleviating severe neutropenia
	GM-CSF, Sargramostim (Leukine)	Stimulate granulopoiesis by bone marrow	Alleviates granulocytopenia
Countermeasures that are given USFDA Investigational New Drug (IND) status	5-AED		
	BIO300 (Genistein)	Improves survival and organ system protection	Protection against system specific side effects
	CBLB502 (Entolimod)	Activates NF-κB, immunomodulator	Minimized marrow and gut damage
	ON01210 (Ex-Rad)	Kinase inhibitor	Orally and parenterally effective against LD50 dose of radiation
	HemaMax Recombinant Human IL-12	Enhances survival and systemic protection	Minimized gut injury
	OrbeShield (BDP)		
Countermeasures without USFDA IND	Myeloid progenitors (MPC/CLT-008)	Partial marrow reconstitution	Significant mitigation of ARS
	γ-tocotrienol 3		
	AEAOL 10150	Free radical quencher, mesoporphyrin mimic	Mitigates acute lung injury

1.6.1 Current radioprotective drug development initiatives

Weapons of Mass Destruction Medical Countermeasures Subcommittee was constituted in 2003 with the mission of ‘‘identification, coordination and prioritization of research, development and acquisition of medical countermeasures for radiological threat agents’’. In their report areas listed in **Table 1.9** were identified to encourage researchers to address these critical issues.

Table 1.9. Priority research areas for radiological nuclear threat countermeasures

Top	**Radioprotector: Pre-exposure agents**
Top	**Therapeutic agents: Post-exposure treatment**
Top	**Antimicrobial agents**
Top	**Cytokines and growth factors**
Top	**Better understanding of mechanisms of radiation-induced injury**
Top	**Bio-dosimetry assay automation**

[154]

Despite several efforts by different investigators none of the agents (plant based or synthetic compounds developed based on rational drug design) received FDA approval as general radioprotector to be employed to patients undergoing radiotherapy or to victims of accidental exposures. Although moderate success is achieved in identifying prophylactic radioprotectors, developing a therapeutic agent is still a challenge to the human kind. However, prophylactic agents that have shown good promise in animal models also suffer from several drawbacks starting from toxicity, bio-availability and efficacy. Presently, post-irradiation exposure cases are given appropriate supportive measures like infusion of fluids, electrolytes, blood products, antibiotics, antivirals, antifungals, analgesics, antiemetics, antidiarrheals, reverse isolation and topical treatment of radiation burns depending on the absorbed dose of radiation [155].

Based on the current knowledge of development of radioprotectors, FDA has laid down following guidelines to streamline the research efforts

1.6.2 Basic R&D strategies employed for development of radioprotector [102]

1. Large scale screening of newly identified chemical classes or natural products
2. Reformulation of old radioprotectors of good potency but unwanted toxicity
3. Using nutraceuticals with moderate radioprotective efficacy with less toxicity
4. Employing combinatorial approach
5. Accepting lower level of drug efficacy in lieu of reduced toxicity

1.6.3 Relative efficacy of radioprotection

Comparison of different radiation protective agents can best be made when a standard quantifiable method of a biological response is used in preclinical studies. The magnitude of protection against radiation damage is commonly expressed as the dose reduction factor (DRF) [156]. The DRF is calculated by dividing the radiation dose at which 50% of the animals die in presence of the radioprotector with the radiation dose at which 50% of the animals die in absence of the radioprotector. The DRF for 30-day survival (LD50/30) in the mouse quantifies protection of the hematopoietic system and probably is the most useful measure for comparative screening of agents for protection against ARS. With the loss of hematopoietic stem cells (HSC), death follows due to infection, hemorrhage and anemia. In an early study of the radioprotective effect of WR-2721, Yuhas and Storer (1969) administered the compound to mice at 500 mg/kg intraperitoneally (IP) before irradiation and obtained a DRF of 2.7 for hematopoietic death and 1.8 for GI death and no protection against central nervous system death [157]. However, the effective drug dose used was very close to LD50 dose of WR-2721 (704mg/kg) [157]. While comparing the DRF between the drugs, one must also take into consideration the toxicity of the

dose used. The therapeutic index of a drug classically is defined as the ratio of effective dose of the drug to LD50 dose of the drug [158]. The time of administration of the radioprotector relative to radiation exposure is critical and the efficacy of the drug is dependent on radiation quality, route of administration and pharmacokinetic considerations [159].

1.7 Antioxidant radioprotectors

Acute and chronic effects of IR are mediated via generation of ROS and reactive nitrogen species (RNS), depletion of antioxidant stores, induction of apoptosis and depletion of stem cells [160]. Therefore, over past five decades maximum efforts have gone into identifying free radical scavengers from plant sources. Antioxidants interfere with membrane lipid peroxidation and induction of apoptosis by ROS after IR exposure [161, 162]. First study demonstrating role of antioxidants in radiation injury was carried out by Patt et al in 1949 showing protection to rats from lethal doses of IR by thiol amino acid cysteine [163]. Thereafter, a major program was initiated by Walter Reed Army Research Institute (WRARI) and screened thousands of molecules including N-acetyl cysteine, glutathione and β-mercaptoethylamine (cysteamine, MEA) and identified amifostine as the most effective mitigator of radiation induced toxicity. Several natural compounds from edible and medicinal plants were also screened to protect mice against lethal doses of IR [164-167].

Anti-apoptotic properties of exogenous and endogenous antioxidants make them suitable candidate for radioprotection. Exogenous administration of antioxidants such as glutathione, superoxide dismutase (SOD), antioxidant vitamins (A, C, and E), the disulfide lipoic acid, as well as substances that mimic or induce activity of endogenous antioxidant systems (e.g., selenium, zinc and copper salts and metal complexes) have shown protection against radiation induced hematopoietic injury [168-171].

1.7.1 Dietary and plant derived antioxidants

Apart from cellular antioxidants another class of antioxidants that are part of our diet is referred to as dietary antioxidants. They are defined as ‘‘a substance in foods that significantly decreases the adverse effects of reactive species, such as reactive oxygen and nitrogen species, on normal physiological function in humans” (Food & Nutrition Board). Vitamin C, E & A are the most widely consumed dietary antioxidants. Presence of antioxidants prior to or at the time of exposure to high or low-LET radiation at adequate levels may offer protection against injury caused by radiation Vitamin E, vitamin A and β-carotene[168-170, 172], selenium and superoxide dismutase [109, 173].

Apart from these dietary antioxidants, several plant derived antioxidants including green tea (polyphenols), Chinese herbal medicines, Ayurvedic preparations, cruciferous vegetables (e.g., cabbage and broccoli), dithiolthiones, Panax ginseng, Shigoka extract, Gingko biloba extract (flavone glycosides and terpene lactones)[174, 175], milk thistle (silymarin), curcumin, orientin, vicenin, garlic (allicin), lycopene,caffeine [176], pentoxyfylline [177], dipyridamole [178], flavonoids such as genistein [179] luteolin and other tea components [180], vicenin-2 [181] had been investigated in different model systems in different scenario of radiation exposure for protection against specific syndromes or overall side effects of radiation[182].

1.7.2 Pros and cons of phytochemical radioprotector

In general, naturally occurring antioxidant radioprotectors offer protection at lower doses of radiation exposure when compared with amifostine (WR2721). DRF values of phytochemical radioprotectors are in the range of 1.1 to 1.3 in 30-day survival experiment [183]. However, natural antioxidants may provide benefits of low toxicity and chemoprevention [169, 170]. They exhibit a long window of protection, including post-irradiation protection against lethality and

chronic effects of radiation exposure. Organ-specific late radiation injury such as pulmonary fibrosis, renal failure, hepatic fibrosis and central nervous system damage resulting in neuro-cognitive impairment has been shown to be ameliorated by antioxidant therapy [184]. Apart from their low DRF values, other important question that needs to be addressed before employing them as radioprotectors is their ability to differentially protect normal and tumor cells. If it is possible to achieve therapeutic gain by differential protection to normal and tumor cells then antioxidants provide lot of promise to be developed as radioprotector.

1.7.3 Flavonoids as radioprotectors

Flavonoids are one of the main classes of compounds that have been examined for radioprotection from plant sources. A number of flavonoids (genistein, quercitin, luteolin) reduce the frequency of micronucleated reticulocytes in the peripheral blood of irradiated mice [185]. Procyanadins (flavan-3-ols) from grape seed extract, including rutin were radioprotective as measured by a decrease in the frequency of micronucleated erythrocytes from bonemarrow of irradiated mice [186]. Thirty-day survival of mice exposed to X-rays did not improve by rutin or other flavonoids quercitin, naringin, or morin [187]. Detailed studies have been reported by Uma Devi and coworkers on the flavonoids orientin and vicenin isolated from *Ocimum sanctum* (Indian holy basil, tulasi), which demonstrated significant protection from radiation injury when administered before irradiation. DRF for 30-day survival in mice treated 30 min before irradiation with low doses (50 mg/kg) IP were 1.30 for orientin and 1.37 for vicenin. The drugs were not very effective when administered after irradiation [188]. Genistein, a soy isoflavone, increased 30-day survival, when administered subcutaneously (SC) 24h before irradiation [189]. It was also effective when administered orally for four to seven days before irradiation. A DRF of 1.16 was obtained at a genistein dose of 200 mg/kg given SC [190]. This radioprotective dose of

genistein did not induce any adverse pathology or behavioural toxicity [189]. Protection to the bonemarrow by genistein was attributed to the transient cell cycle arrest in HSC [179]. **Based on these studies, we have chosen structural analogue of genistein i.e. baicalein to explore the radioprotective potential in murine splenic lymphocytes. The difference between baicalein and genistein is the position of the hydroxyl group i.e. baicalein is 5,6,7-trihydroxy flavone whereas genistein is 4',5,7-trihydroxy isoflavone.**

1.7.4 Pleitropic effects of antioxidant radioprotectors

Many of the antioxidant radioprotectors have been shown to elicit their action not only by free radical scavenging but also by activating multiple pro-survival transcription factors leading to changes in gene expression and antioxidant enzyme activities [191]. Exposure to thiol compounds such as WR-1065 and N-acetyl-L-cysteine (NAC), which are known to scavenge free radicals have also been shown to elevate manganese superoxide dismutase (Sod2) expression levels resulting in increased radiation resistance [37, 38]. WR-2721 given at high doses not only offered radioprotection to normal tissues but also inhibited radiation-induced malignancies in mice when injected before radiation exposure [36]. Most of the antioxidant radioprotectors are known to suppress inflammation [192] which could be an additional benefit because IR induces systemic inflammation. This IR induced inflammation further amplifies radiation injury caused by ROS. Therefore, an agent which can scavenge ROS and also exhibit anti-inflammatory activity may offer better protection against IR induced primary and secondary damage.

1.8 IR and inflammation

Radiation-induced late effects are caused by systemic inflammation leading to continuous production of ROS [193-195]. This chronic oxidative stress leads to further increase in lipid

peroxidation, oxidation of DNA and proteins, as well as activation of pro-inflammatory transcription factors nuclear factor kappa B (NF-kB) and signal transducers and activators of transcription (STATs) [196, 197]. IR induced DNA double strand breaks can activate NF-kB via the Ataxia Telangiectasia mutated protein (ATM) or DNA-PK [198].

NF-kB has a central role in immune and inflammatory responses as it can regulate the expression of many pro-inflammatory cytokines and chemokines such as tumour necrosis factor alpha (TNF-α), IL2, IL1, IL6 [199]. STAT3 also up-regulates many pro-inflammatory genes such as cyclooxygenase COX-2, IL-1β, IL-6, and IL-8 [200-202]. These cytokines are mediators of IR induced inflammation.

1.8.1 Advantages of anti-inflammatory radioprotector

RT has been extensively employed as a curative or palliative intervention against cancer. Targeting IR induced inflammatory pathways may improve the radiation therapy clinical outcomes by enhancing radiosensitivity [203]. The anti-inflammatory effect of radioprotective molecule is an addendum to the long list of desirous attributes that a radioprotector is supposed to possess. An anti-inflammatory radioprotector would not only protect cells against IR induced immediate damage but it is also expected to prevent radiation induced secondary tissue injury. Such molecule can offer double layered protection to cells against IR induced primary as well as secondary tissue injury. In cancer patients undergoing radiotherapy, such compounds have significant implications as they reduce the risk of IR induced abscopal effects and may also improve quality of health by suppressing tumor associated inflammation. It is reported that antioxidant radioprotectors curb inflammation through activation of an anti-inflammatory transcription factor Nrf-2.

1.8.2 Nrf-2 in oxidative stress and inflammation

Nuclear factor erythroid-2–related factor 2 (Nrf-2) is encoded within a 2.2-kb transcript and codes for a 66-kDa protein with a basic leucine zipper DNA binding domain. It was first isolated from hypersensitive site 2 located in the beta-globin locus control region [204]. Nrf-2 regulates cytoprotective response to counteract the deleterious effects of ROS and confers protection against oxidants [205, 206]. Nrf-2 dissociates from its inhibitor Kelch-like ECH-associated protein 1 (KEAP1) and binds to antioxidant response element (ARE) in mediating transcriptional activation of its target genes that include multiple antioxidant enzymes and electrophile detoxification enzymes [207]. Some synthetic as well as natural antioxidants are known to activate Nrf-2 by oxidative stress dependent or kinase dependent mechanism [208].

Nrf-2 also exerts a protective role toward several inflammatory disorders induced in murine models [209]. Nrf-2 knockout mice are susceptible to the development of inflammation-related diseases, such as sepsis [210], atherosclerosis [211], chronic obstructive pulmonary diseases [205], asthma [212] and autoimmunity [213].

Augmentation of Nrf-2 in T cells is essential to mitigate oxidative stress during IR-induced acute kidney inflammation (AKI) [214]. Adoptive transfer of Nrf-2 activated T cells into wild type mice improved the outcome from AKI. T cell activation, proliferation and immunological responses are regulated by NF-κB. Hence, regulation of inflammatory response largely relies on suppression of NF-κB and its dependent gene expression. Nrf-2 and NF-κB are known to antagonize each other although they share common effectors and regulatory points [215]. Several anti-inflammatory or anti-carcinogenic phytochemicals suppress NF-κB signaling and activate the Nrf-2-ARE pathway [216, 217]. Recently, it was reported from our laboratory that schisandrin B (SB), a dibenzocyclooctadiene derivative isolated from *Schisandra chinensis*,

increased the expression and nuclear translocation of Nrf-2 and its downstream target genes such as HO-1. SB inhibited the activation and nuclear accumulation of NF-κB by preventing IκBα degradation. Furthermore, a causal role of the Nrf-2/HO-1 pathway in the observed anti-inflammatory effects of SB was demonstrated by inhibiting Nrf-2 and HO-1 [218]. In the nucleus, p65 promotes recruitment of the co-repressor HDAC3 to ARE, facilitating the interaction of HDAC3 with either CBP or Maf leading to local histone hypo-acetylation, blocking chromatin decondensation and suppressing Nrf-2/ARE gene expression [219, 220]. Lee et al., has reported that Keap-1 dissociated from Nrf-2 ubiquitinates IKK-β leading to its degradation [221]. IKK-β is the upstream kinase that phosphorylates p65 thereby degrading the IκBα [220] resulting in nuclear translocation of NF-κB [222].

As discussed above, curbing inflammation could lead to radioprotective environment, we have investigated the anti-inflammatory activity of baicalein in terms of suppression of T cell responses.

1.8.3 Anti-tumor effects of radioprotector

An ideal radioprotector should only ameliorate damage to the normal cells but should not protect tumor cells. If the radioprotector can elicit anti-tumor activity, it would further enhance its applicability in clinic. Since antioxidant radioprotectors exhibit anti-inflammatory activity, it is possible that they may also show anti-tumor activity because inflammation and cancer are very closely linked [223]. There are number of reports showing direct and indirect evidences that inflammation precedes cancer induction [224]. Inflammatory responses can favor cancer cells invasion, providing a favorable environment for tumor promotion and metastasis [225, 226]. IR induced IL-1β expression can favor cancer cell invasion [227]. Both IL-8 and IL-6 are involved

in IR induced inflammatory response, enhancing cancer cell invasiveness [228]. Another key pro-inflammatory enzyme COX-2 which converts arachidonic acid to prostaglandins, has been shown to be overexpressed in patients with various types of cancers [229-231].

IR can activate NF-κB and many tumor cells usually express high levels of constitutive NF-kB [232-234]. NF-κB provides a mechanistic link between inflammation, carcinogenesis, and tumor radioresistance. All of these mediators act together in perpetuating and amplifying the inflammatory cascade. They suppress DNA repair mechanisms leading to an increase in genetic instability [235]. These inflammatory mediators induce DNA double-strand breaks, affect function of cell cycle checkpoints and homologous recombination repair [203]. Recently inhibitors of NF-κB by synthetic and natural compounds have shown good promise as tumor radiosensitizers [236].

The present thesis embodies the anti-tumor activity of baicalein using T cell lymphoma model.

1.9 Baicalein

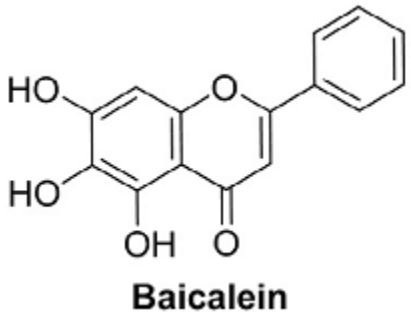


Fig. 1.9 Baicalein

Baicalein **Fig. 1.9** is highly cell permeable and poorly water soluble, which is classified as a class II compound according to Biopharmaceutical Classification System (BCS) [237]. After oral administration of baicalein, it is subjected to the extensive first pass metabolism in liver and

small intestine [238] and therefore, glucuronides/sulfates of baicalein including baicalin are found in the plasma [239]. Therefore, the conjugated metabolites may be responsible for the in vivo effects. Various formulations have been developed to improve the oral bioavailability of baicalin and baicalein. Baicalein nanocrystal [240], baicalein-hydroxypropyl-β-cyclodextrin inclusion complex [241], baicalein self-microemulsifying drug delivery system [242], and baicalein solid dispersion [243] have been developed to improve dissolution and oral bioavailability of baicalein. Besides this, changing the administration route of baicalein may be employed to avoid the first-pass metabolism of the gastrointestinal tract or liver and enhance its bioavailability. For example, pulmonary administration of baicalein nanocrystal form can obtain similar pharmacokinetic parameters as that of intravenous injection of baicalein solution [244]. Several studies regarding the pharmacokinetic profiles of baicalein have been reported. In order to calculate the absolute bioavailability, intravenous pharmacokinetic study was also carried out after administration of 10 mg/kg baicalein. The absolute bioavailability of baicalein in different doses was ranged from 13.1% to 23.0%. Extensive glucuronidation of baicalein occurs in the intestinal wall and liver catalyzed by UDP-glucuronosyltransferase (UGT) in rat and human [245].

The detailed biological effects reported in the literature are listed in **Table 1.10**. Based on these findings, it was proposed that baicalein may protect cells of hematopoietic system against IR induced cell death and thereby enhancing the survival of mice. Hence, we have explored detailed radioprotective properties of baicalein and presented in Chapter 2 along with the experimental design and discussion. Baicalein is a well-known anti-inflammatory agent owing to inhibition of 12-lipoxygenase [246]. Further, it can induce Nrf-2/HO-1 axis in some cell types [247, 248] thereby suppressing inflammatory responses.

Table.1.10. Protective effects of baicalein against oxidative stress injury

Stress	Cell type	Consequence	Effect of baicalein
H_2O_2	Rat glioma C6	Reduced viability	Inhibition of apoptosis by induction of HO-1 [249]
Prion proteins	Neuronal cells	Aggregation and cell death	Protection by inhibiting ROS [250]
H_2O_2	NG108-15 neuroblastoma	Apoptosis	Protection by up-regulation of Nrf-2 and inhibition of 12-LOX [251]
H_2O_2	H9c2&cardio myocytes	Apoptosis	Protection by up-regulation of Nrf-2 pathway [252]
6-OHDA	PC-12 (adrenal)	Oxidative damage	Protection by up-regulating Nrf-2/HO-1[248]
IR	Hippocampal neurogenesis	Necrotic cell death	Protection by scavenging ROS
aggregation of alpha-synuclein	Neuronal cells	Parkinson's disease	Prevents fibrillation and promotes disaggregation [253]
IR	Kidney	Inflammatory response	Prevented IR induced inflammation by modulation of NF-κB [254]
UV-B	Skin	Increased skin thickness, MMP9 & VEGF	Inhibited skin damage by inhibiting NF-κB expression [255]

In the context of its radioprotective properties on lymphocytes, study on modulation of T cell responses by baicalein becomes essential. In the present thesis, murine splenic lymphocytes have been employed as a model system to test the efficacy of baicalein as an anti-inflammatory and immune-modulatory agent. Experimental details and results of anti-inflammatory activity of baicalein have been presented in Chapter 3.

One of the most prime attributes of radioprotector includes no survival advantage to tumors in presence of IR. In fact, anti-tumor potential of radio-protective molecules qualifies them as ideal agents to be employed during radiotherapy. The results of anti-tumor activity of baicalein in lymphoma model are presented in Chapter 4 along with the experimental design and discussion.

1.10 Scope of the Thesis

In the present thesis, attempt has been made to carry out detailed investigation about radio-protective, anti-inflammatory and anti-tumor properties of plant derived flavonoid baicalein using murine model in vitro and in vivo. Investigation of radio-modulatory properties of baicalein has been the major focus of this study and studies pertaining to its effect on inflammation and cancer have been carried out to further strengthen observed radioprotective effects. These studies have provided valuable insights about other molecular targets influenced by baicalein and its possible implication towards development of a radioprotector. Our study plan includes following:

A) Investigation of radioprotective properties of baicalein

i) Evaluation of in vitro radioprotective properties of baicalein using murine splenic lymphocytes as model system

ii) Validation of results by performing multiple assays for cell viability

iii) Investigation of molecular mechanism responsible for observed radioprotective action in terms of changes in cellular redox parameters and pro-survival signaling molecules

iv) Analysis of changes in pro-survival factors in a dose and time dependent manner after baicalein treatment

v) Investigation of in vivo radioprotective properties of baicalein monitored in terms of 30 days survival, changes in hematological parameters, abundance, functionality and viability of cells of hematopoietic system

vii) Identification of DRF for modification of LD50/30 in presence of baicalein

vi) Analysis of hematopoietic cells for changes in pro-survival/anti-apoptotic molecules in a time dependent manner

vii) Administration of inhibitor of key pro-survival molecule in mice to pinpoint most prominent pathway

B) Investigation of anti-inflammatory properties of baicalein

i) Evaluation of in vitro anti-inflammatory properties of baicalein using murine splenic lymphocytes as model system in terms of proliferation, cytokine secretion and surface marker expression

ii) Investigation of molecules regulating suppression of T cell responses

iii) Validation of proposed signaling pathway operative for observed anti-inflammatory effects

iv) Analysis of anti-inflammatory potential using in vivo model systems

C) Evaluation of anti-tumor potential and mechanism of action of baicalein

i) Investigation of changes in pro-survival molecules in regulating radio-sensitivity of corresponding lymphoma cells

ii) Evaluation of anti-tumor potential of baicalein in murine T cell lymphoma (EL4 cells) using different assay systems

iii) Investigation of molecular mechanism responsible for anti-tumor action of baicalein in EL4 cells

Experiments performed to unravel potential of baicalein as a radioprotector in the present thesis unfolds very novel and significant mechanistic aspects about radio-modulatory, immune-modulatory and anti-tumor properties.

CHAPTER 2

IN VITRO AND IN VIVO RADIOPROTECTIVE EFFECTS OF BAICALEIN

2.1. Introduction

Exposure to IR can cause damage to various cells, organs and vasculature system in the human body depending on type, dose, dose rate and radiosensitivity of tissue being exposed [45]. Radiation induced biochemical changes in the tissue are responsible for associated acute and/or chronic effects of IR exposure. Depending on the dose range of exposure, the response of human body is categorized into different syndromes. Besides the effects of radiation on the exposed cells and organs, bystander effects and delayed effects also contribute to the overall outcome of radiation exposure [256, 257]. Exposure to IR can lead to development of different pathological conditions due to cell death and malfunctioning in some critical organ systems in the body [46].

2.1.1 Effect of radiation on different systems in the body

Pathologic changes seen in human tissues after radiation exposure can be divided into

i) Epithelial (parenchymal)

ii) Stromal (mesenchymal) and

iii) Vascular

Fig. 2.1 summarizes common ways of radiation injury. These changes are associated with certain characteristic features and depending on the tissue exposed the respective organs are also affected (**Table 2.1** and **Table 2.2).**

Table 2.1 Effect of radiation on different systems in the body

Changes after radiation exposure	**Atrophy**	**Necrosis & Ulceration**	**Atypia**	**Dysplasia**
Epithelial Characteristics	Progressive loss of number and volume of epithelial cells	Sharply demarcated mucosal ulcers, denudation of the epithelium with an ulcer base consisting of mixed inflammatory cells, necrotic debris, and granulation tissue	Cytologic atypia, significantly enlarged nuclei with dense "smudgy" appearance	Premalignant alteration, increased nucleus-to-cytoplasm ratio
Organs affected	Skin, gastro-intestinal system, genitourinary tract, respiratory tract, breast, salivary glands		Prostate, breast, bladder, lungs, salivary glands, and squamous mucosa of the head & neck	Occurs in squamous linings
Stromal Changes Characteristics	**Fibrosis**	**Atypical Fibroblasts**	**Stromal Necrosis**	
	Delayed radiation injury, acellular acidophilic collagen	Angulated basophilic cytoplasm, enlarged and hyperchromatic nuclei with no mitotic activity, cytomegaly	Rare, delayed, can occur in any organ where stroma is present, fat necrosis of breast	
Organs affected	Skin, head & neck, breast, GI tract	GI, Respiratory tract, Urinary, Skin, Soft tissue, Breast		

Vascular Changes Characteristics	**Capillaries**	**Arterioles**	**Small and medium sized arteries**	**Large arteries and veins**
	Endothelial cells are the most radiation-sensitive in vascular structure, thrombosis obstruction, capillary destruction,telangiectasia, hematuria	Swollen endothelial cells in GI, skin and brain	Nuclear enlargement, random medial wall necrosis, mural thickening with hyalinization	Intimal fibrosis, transmural healed necrosis, intimal plaque formation with foamy macrophages

Table 2.2 Pathological findings in specific organ [258]

Organ		Pathologies
Heart and great vessels		Pericarditis, pericardial effusion, myocardial fibrosis
Lung	**Acute Radiation pneumonitis (ARP)**	Increased number of macrophages in alveolar spaces Mixed chronic inflammatory infiltration Fibroblasts in the alveolar septa Hyaline membranes (homogenous, acellular, and eosinophilic material)
	Pulmonary fibrosis (PF)	Months or years after the development of ARP, Alveolar septa fibrosis Diffuse fibrotic areas, or scarred tissue replacing alveolar spaces, bronchiolitis obliterans, unlike ARP, PF is observed as a sharply demarcated fibrous lesion within the previously irradiated site.
Liver		Venoocclusive disease (VOD), involves the central veins and afferent sinusoids of the lobules in the irradiated parenchyma. VOD is sharply limited to the exposed area.
Kidneys		Radiation-induced nephropathy: tubular atrophy, tromal fibrosis, diffuse glomerular sclerosis, vascular intimal proliferation, foamy cells

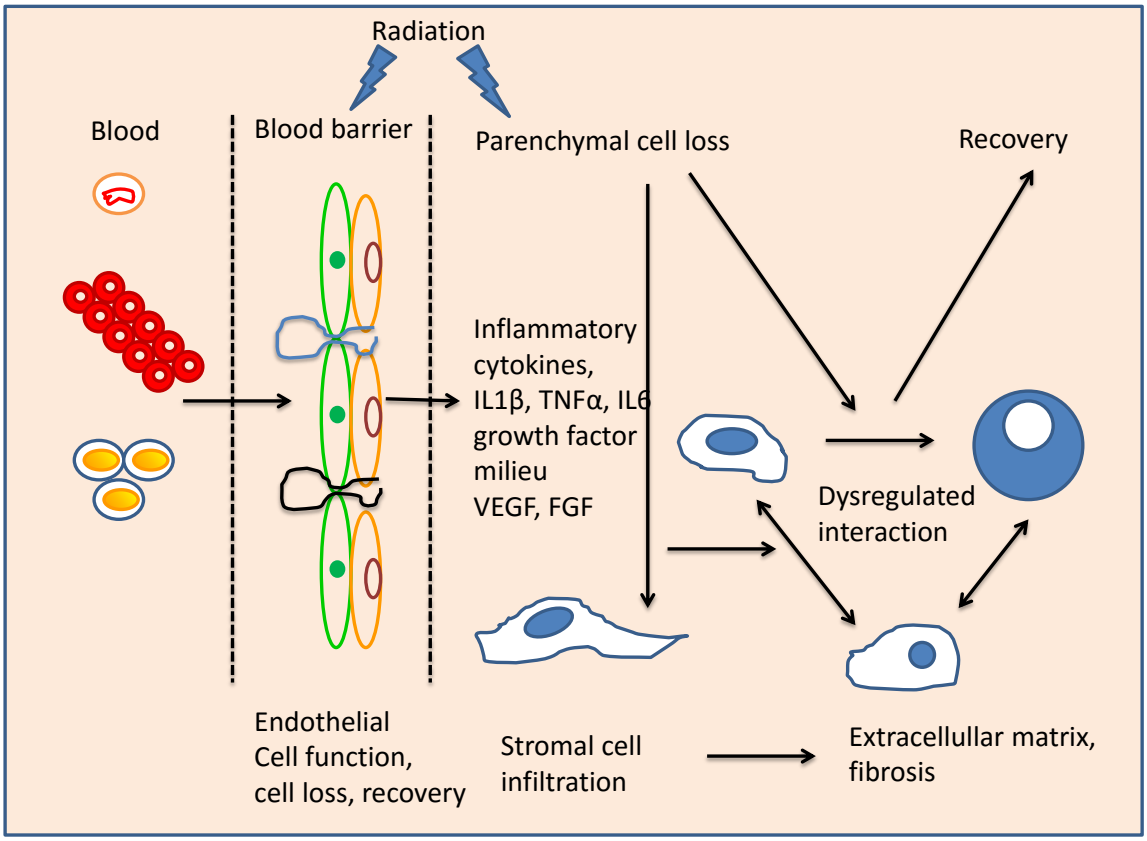


Fig. 2.1 Common ways of radiation injury *[46]*

2.1.2 Hematopoietic system

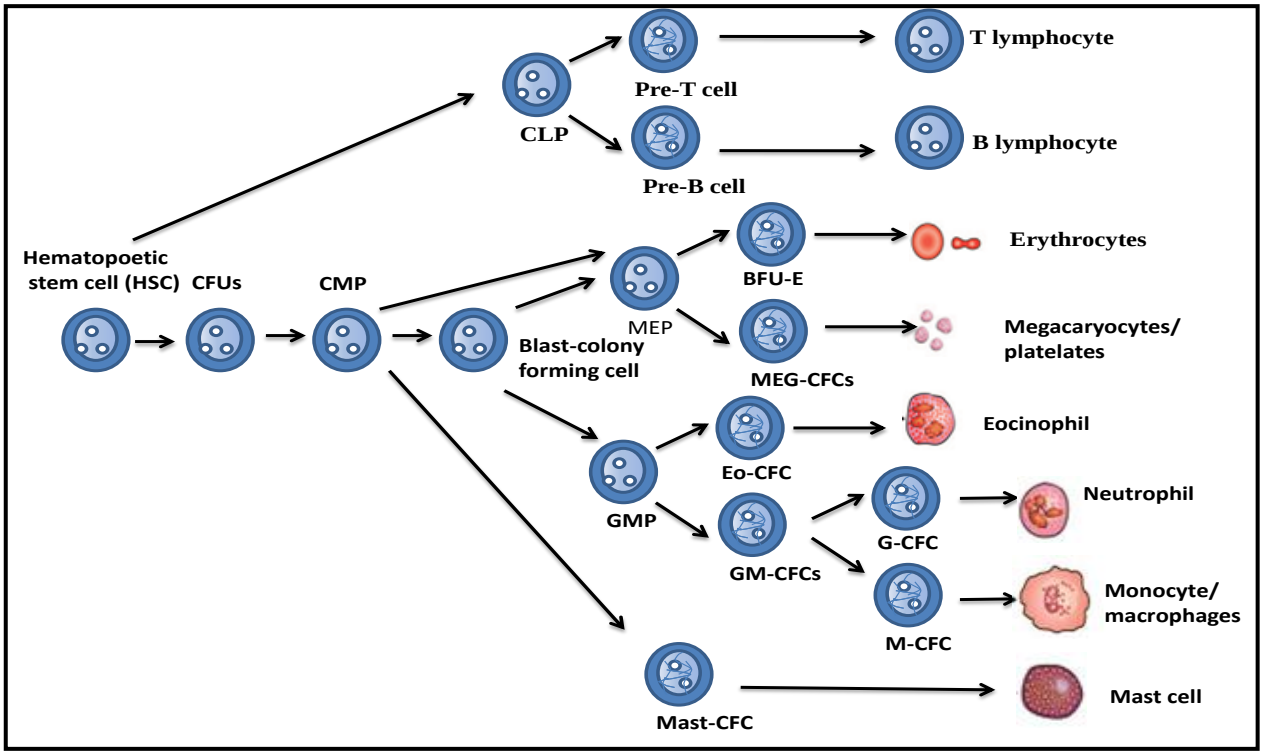


Fig. 2.2 Cells of hematopoietic system

(BFU-E, burst-forming units–erythroid; CFU-S, colony-forming units–spleen; CLP, common lymphoid progenitors; CMP, common myeloidprogenitors; Eo-CFC, eosinophil–colony-forming cells; G-CFC, granulocyte–colony-forming cells; GM-CFC, granulocyte macrophage–colony-forming cells; GMP, granulocyte-macrophage progenitors; HSC, hematopoietic stem cell; M-CFC, macrophage–colony-forming cells; Mast-CFC,mast–colony-forming cells; Meg-CFC, megakaryocyte–colony-forming cells; MEP, megakaryocyte-erythroid progenitors [259]).

2.1.3 Hematopoiesis

A lifelong process of supplying mature blood cells to the whole organism defines hematopoiesis which occurs in the bonemarrow (BM). It is estimated that approximately 10^{12} blood cells are generated every day in order to balance the loss of aging blood cells [260]. In addition external agents like infection, hemorrhage, hypoxia, foreign antigen can induce increased production of blood cells to immediately meet the demands. In order to support this high turnover, the

hematopoietic system has several feedback and intrinsic regulatory mechanisms that regulate the self-renewal, asymmetric division and differentiation of a relatively small number of HSCs and progenitor cells (1 HSC in 10^4 BM cells) residing mainly in the adult BM [261]. These rare cells that undergo proliferation and differentiation into different lineages of mature blood cells (erythroid, myeloid, lymphoid cells and platelets) and maintain HSC pool through self-renewal throughout the life [262] (**Fig. 2.2).**

2.3.4 Regulation of hematopoiesis

HSCs can undergo several fates during the lifetime of an individual viz self-renewal, differentiation and migration, quiescence and cell death [263]. In response to various types of stress (bleeding, infections), HSCs are rapidly activated to meet the increased demand of specialized blood cell production. Self-renewal is the most essential process of the HSCs as it is required to sustain hematopoiesis [264]. Stem cells can either generate an identical copy through symmetrical division or give rise to cell destined to an alternative fate via asymmetrical cell division (**Fig. 2.3).** Thus, by regulating the balance of symmetrical and asymmetrical divisions through intrinsic and/or extrinsic factors, the number of HSCs is maintained at steady state level [262]. Differentiation is defined as the sequence of events through which primitive stem cells gradually lose their self-renewal ability while undergoing a stepwise commitment process generating more mature and lineage restricted progenitors [265]. Fate decision can be mediated through extrinsic factors (cytokines, microenvironment of the BM niche) (**Fig. 2.4)** or intrinsic factors (pre-programmed in the cell genome) [266].

Two distinct subsets of hematopoietic progenitors, common lymphoid progenitor (CLP) [267] and the common myeloid progenitor (CMP) [268] have been identified in adult bone marrow. CLP differentiates into cells of the lymphoid lineage- T, B and NK cells, while the CMP generates myeloid precursors of the megakaryocyte / erythroid, granulocyte and monocyte / macrophage lineages (**Fig. 2.2).**

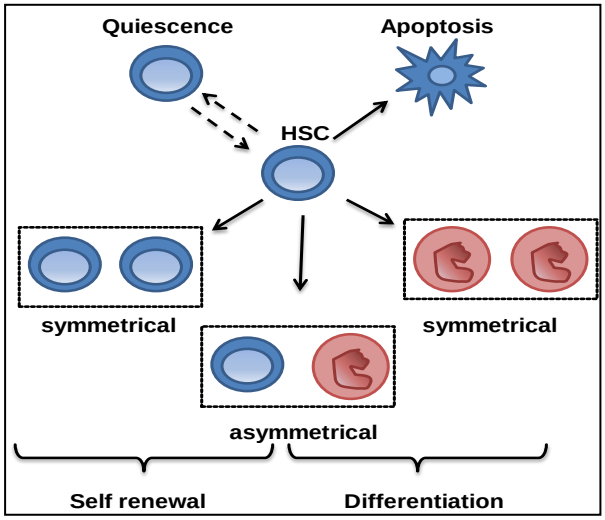


Fig. 2.3 HSC fate decisions

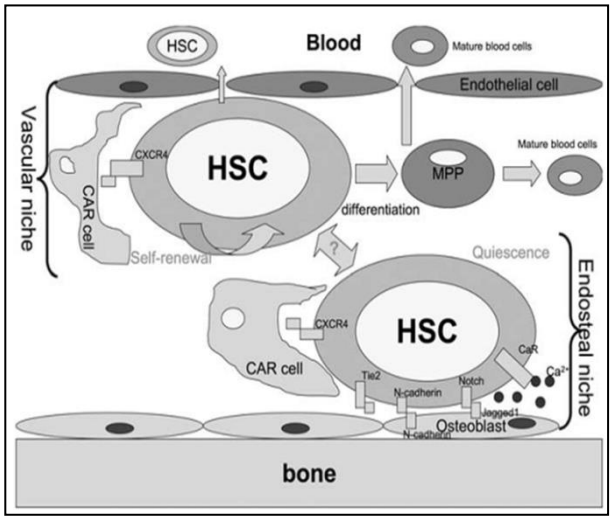


Fig. 2.4 Hematopoietic stem cell niche *[269]*

Maintaining quiescence is another highly important characteristic of the HSCs which is believed to serve two purposes: i) prevent exhaustion of HSCs by maintaining steady state hematopoiesis and ii) protect HSCs from DNA alterations (and thus cancerous transformation) more likely to occur in an activated state [270].

2.1.5 Hematopoietic stem cells

The search for HSC began when it was first recognized that animals given lethal doses of irradiation suffered bone marrow aplasia which could be reversed by injection of unirradiated bone marrow cells [271][272]. Operationally, HSC can be defined as that single cell which can generate entire hematopoietic system of a lethally irradiated animal [262]. HSCs are distinguished from other hematopoietic progenitors in the bone marrow by their unique ability to

undergo multilineage differentiation and self-renewal. Bone marrow stromal cells make up the microenvironment for hematopoiesis. The fate of the HSC is determined largely by the microenvironment (niche) [273]. The small population of HSC in the bone marrow consists of short-term reconstituting cells (STRCs) and long-term reconstituting cells (LTRCs), based on how quickly the transplanted cells can produce progeny in an irradiated recipient [274]. They can be "purified" using a combination of cell size, density, fluorescent dye uptake, resistance to cytotoxic chemicals and cell-surface markers.

Physiological characteristics

Using counterflow centrifugal elutriation (CCE), which sort cells on the basis of size and density, Jones et al. (1990) separated a bone-marrow subset (CCE25) of small, dense cells, showing delayed, but long-term repopulating ability [275].

Metabolic characteristics

The level of up-take of Rhodamine 123 by cells can be used as a means to isolate HSCs. Rhodamine 123 binds to the mitochondrial DNA, and a low level of Rhodamine 123 suggests an active outflow pump that has been shown to be highly active in HSCs. With split spectrum analysis, Goodell et al. (1996) discovered side population cells (SP cells) and demonstrated their LTRC properties [276]. Hoechst 33342, has also been used in HSC identification [277]. It appears that a low level of Hoechst 33342 results from a more activated multi drug- resistant gene product, as evidenced by the fact that verapamil will block Hoechst efflux from SP cells.

Cell surface markers

HSCs are a population of low abundance and morphologically indistinguishable cells, interspersed in the BM among large numbers of differentiated blood cells at various stages of maturation. The development of flow cytometry, fluorescence-activated cell sorting (FACS) and

wide availability of fluorochrome-conjugated monoclonal antibodies against cell surface markers account for the breakthrough in identification of HSCs [272][278][279].

A more commonly used method is to identify cells via recognition of surface-specific expressed proteins. Spangrude et al. (1988) showed that HSCs in murine bone marrow can be enriched 1000- to 2000-fold in a population of cells that express low levels of Thy-1.1(Tlo), high levels of Sca-1 (Shi), and absence of (or low) lineage markers [280]. Murine HSCs are characterized by a unique pattern of surface markers known as Signaling Lymphocyte Activation Markers (SLAM) expressed on bone marrow cells [281].

CD150 is the founding member of the SLAM family of cell surface receptors. CD150+ cells exhibit long-term multilineage cells [281]. CD244 is expressed by transiently reconstituting MPPs but not by HSCs. CD48 is expressed by restricted progenitors but not by HSCs or MPPs. SLAM family members are differentially expressed among hematopoietic progenitors in a way that correlates with primitiveness. SLAM family members are so precisely differentially expressed that HSCs are very highly enriched within the CD150+CD244-CD48- cell population [282]. HSCs can thus be characterized by CD150+CD244-CD48- cells while MPPs as CD244+CD150-CD48- and lineage restricted progenitors as CD48+CD244+CD150-.

2.1.6 Essentials and significance of hematopoietic system

The hematopoietic system plays an important part in maintaining the vitality of mammals. Functional cells of this system transport oxygen in the blood provide specific and nonspecific immune protection to the organism against foreign substances (viruses, bacteria, and so on), ensure the blood coagulates, and sustain intact blood vessels.

2.1.7 Radiation induced alterations in hematopoietic system

Actively dividing cells are highly sensitive to IR induced cell death. Cells of the hematopoietic system are continuously proliferating and hence are most radiosensitive among all the mammalian systems [283, 284]. Radiation induced injury to hematopoietic system can lead to hemorrhage, increased susceptibility to internal and external infections and anemia. Exposure to IR and certain chemotherapeutic agents not only cause acute BM suppression but also leads to long-term residual hematopoietic injury. It has been well established that acute myelo-suppression induced by IR and / or chemotherapy is the result of induction of apoptosis in the rapidly proliferating hematopoietic progenitor cells (HPCs) and to a lesser degree in the relatively quiescent HSCs [285, 286]. Management of acute myelosuppression can be significantly improved by the use of growth factors such as granulocyte-colony stimulating factor, granulocyte/macrophage-colony stimulating factor, or erythropoietin [287].

The severity of lymphopenia and thrombocytopenia generally correlate with cumulative radiation dose and dose rate [288, 289]. The primary causes of hematopoietic syndrome (HS) are radiation-induced suppression of mitosis in hematopoietic stem/progenitor cells and their progeny, resulting in hypo-cellularity and aplasia of the bone marrow and apoptosis in lymphocytes and other hematopoietic cells [290]. Hematopoietic stem / progenitor cells of the bone marrow undergo mitotic death after exposure to IR [291]. This particular in vitro measure of sensitivity to radiation correlates with the appearance of the HS that occurs in individuals whose partial-body or whole-body radiation exposure exceeds approximately 1 Gy [292] leading to significantly diminished capacity of hematopoietic stem / progenitor cells to proliferate in vivo. Depending on the dose, dose rate, and radiation quality factor, various degrees of pancytopenia develop over several weeks after whole-body or significant partial-body exposure

[293]. Factors that may exacerbate the effects of radiation include a patient's age, underlying state of health, and overall nutritional status. **Table 2.3** summarizes some of the radiation induced alterations in hematopoietic system.

Table 2.3 Radiation induced alterations in hematopoietic system

Target	Effect
Spleen	• Reduced size • Decreased cell count • Apoptosis in lymphocytes • Decreased responsiveness to mitogenic stimuli • Increased oxidative stress in cells
Bone Marrow	• Aplasia • Decreased cell count of BM-MNC • Apoptosis in BM-MNC • Cell cycle arrest • Mobilization of stem cells • Alteration in bone marrow niche (stromal cells, fibroblasts) • Increased oxidative stress
Blood	• Decline in lymphocyte count • Decreased platelet count • Chromosomal aberrations in lymphocytes • Decreased enzyme activity and accelerated ageing in cells • Increased damage to erythrocyte membranes

2.1.8 Screening of radioprotectors

A clear understanding of the research methods is essential for screening any compound for ability to offer radioprotection. These methods include cell free systems, cellular systems, animal systems, and human clinical trials. When cells are irradiated, damage is produced primarily by free radicals and a candidate radioprotector may exhibit the ability to scavenge free radicals formed during irradiation and thereby inhibit the chain propagation steps of free radicals. There are several assays for assessment of free radical scavenging, membrane damage, protein

oxidation, cytogenetic damage, DNA repair and apoptosis which are employed to test the radioprotective efficacy. Below presented is the list are some of these assays.

Antioxidant activity

- DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity
- Hydroxyl radical scavenging
- Superoxide anion scavenging
- Plasmid relaxation assay
- Metal chelation activity
- Nitric oxide scavenging activity
- Reducing potential
- Total Antioxidant capacity
- Lipid peroxidation
- Protein oxidation

Cell death, cytogenetic damage, DNA repair and other parameters

- Apoptosis
- Comet assay
- Estimation of micronuclei and dicentric frequency
- Measurement of endogenous antioxidant enzymes (catalase, superoxide dismutase, glutathione s transferase)

In vivo screening

The drug development process is a time consuming activity and needs a systematic and focused approach to achieve the goal. The preliminary in vitro assays provide an idea about basic properties and mechanism of action of the candidate radioprotector. However, it is possible that

the data obtained from in vitro assays may not necessarily corroborate with in vivo observations. The most promising radioprotectors obtained based on testing in rodents (mice, rats and rabbits) are taken to higher animals (dogs and monkeys).

(i) Survival (30 days)

Radioprotective efficacy of compounds is evaluated in the rodents by their administration via different routes like intra-peritoneal, subcutaneous, intra-venous or oral followed by exposure to lethal dose (≥7.0Gy) of WBI. Different doses and different formulations of the drug are tested at single or multiple doses to achieve maximum efficacy. Survival and health of animals is observed up to 30 days. The change in the average weights of the animals at different time intervals is also compared to that of untreated control animals.

(ii) Dose reduction factor (DRF)

DRF serves as a standard criterion for grading radioprotectors and assessing their relative efficacy of test radioprotector as compared to established radioprotector. Magnitude of modification of LD50/30 by compound of interest is a parameter of the ability of radioprotector to protect animals against lethal doses of irradiation. DRF is calculated as a ratio of LD50/30 dose of radiation in the presence of radioprotector to that of LD50/30 dose of radiation in absence of radioprotector.

(iii) Hematologic parameters

Radiation affects number (frequency and abundance), viability and functionality of cells of hematopoietic systems and induces distinct molecular changes characteristic of radiation damage. Those can be monitored by different assays like:

- Total leukocyte count
- Differential leukocyte count

- Erythrocyte count
- Hemoglobin content
- Spleen colony forming units
- Lymphocyte viability
- Lymphocyte proliferation and cytokine secretion
- Spleen phenotyping
- BM-MNC Count
- BM-MNC viability
- Colony forming cell assay
- Abundance of HSC

(iv) Toxicity testing

It is of utmost importance that beneficial effect of a radioprotector should outweigh its side effects. The maximum dose of drug which is well tolerated without significant toxicity or death in the experimental animals is considered as maximum tolerated dose (MTD). Whereas the dose that induces 50% killing is referred to as LD50 of the drug which is an important measure of drug toxicity. Dose that needs to be given to obtain optimum desired effects is called as effective dose (ED). Therpauetic index of a drug is the ratio of LD50 dose of drug (radioprotector) to its effective dose. Drugs with high therapeutic index i.e. low effective dose and/or high LD50 dose are preferable. The effective dose of radioprotector should ideally be much lower than LD50 dose (less than 1/4).

(v) Pharmacokinetics and pharmacodynamics

The understanding of absorption, tissue distribution, serum concentration, pharmacokinetics, metabolism and excretion of any externally administered substance is an essential requirement for understanding the efficacy and toxicity of the formulation.

The general strategy for screening of radioprotectors is outlined in **Fig. 2.5**.

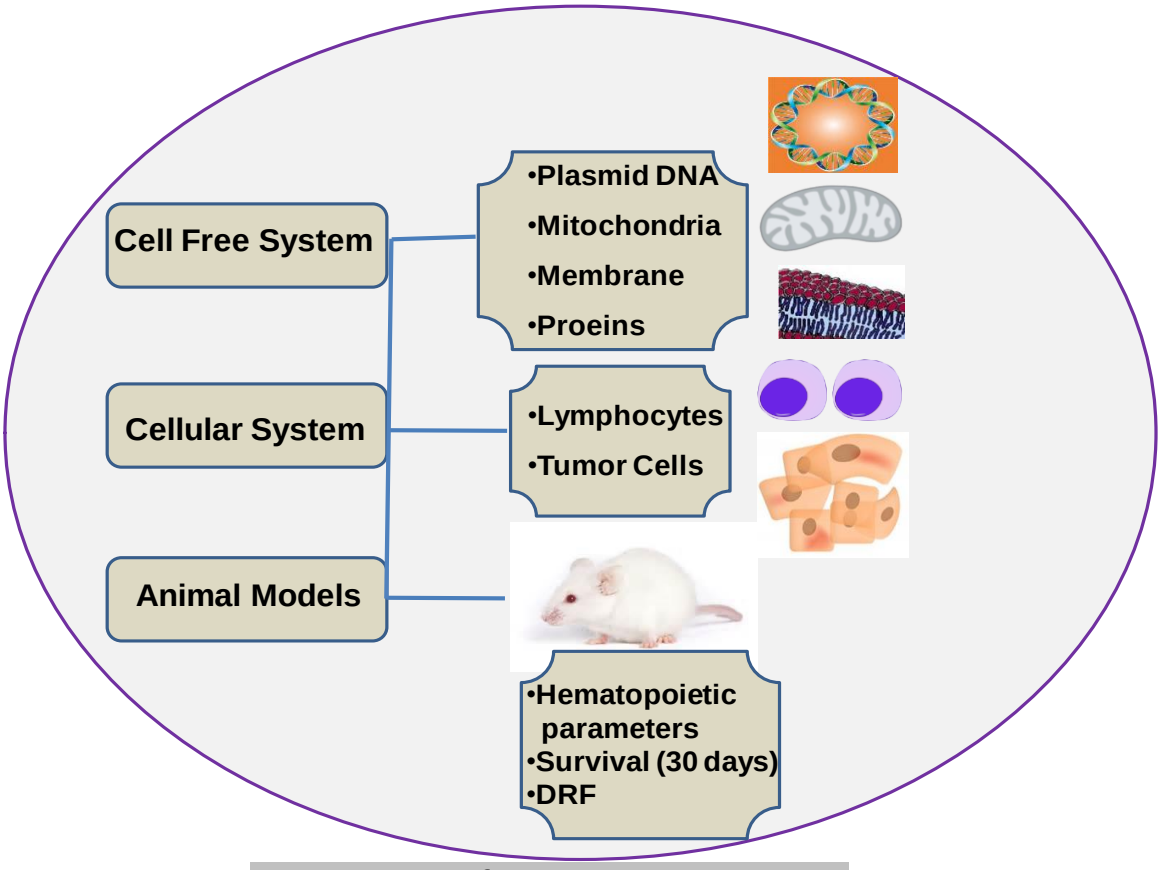
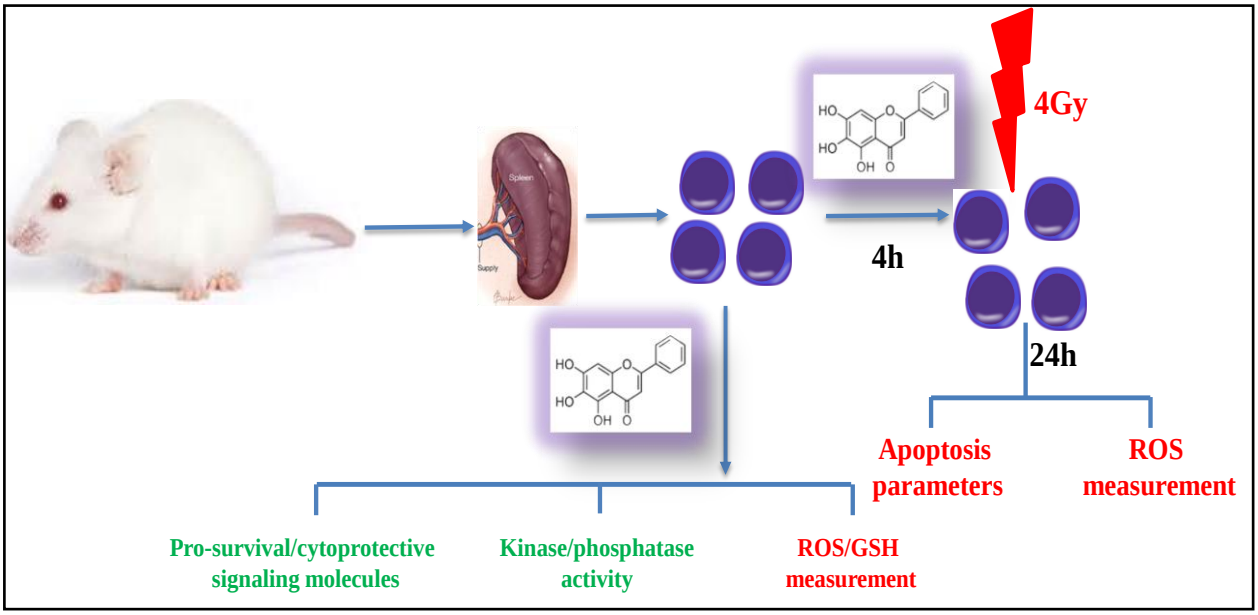


Fig. 2.5 Strategy for screening radioprotector

In vivo models are especially important as a proof-of principle in establishing the preclinical safety and efficacy data required for translating to human clinical trials. Plant products are extensively used in alternative medicines like Ayurveda and provide promising alternative of synthetic chemical agents. Large numbers of plants products have been reported for their radioprotective properties, albeit with limited success in clinic. However, considering the vast variety of metabolites found in the plants, systematic investigation using the purified active

pharmacological agents (and not crude extracts) may provide a highly potential and non-toxic radioprotector drug for human use. The study plan and experimental layout employed in current study is outlined in **Fig. 2.6 & Fig. 2.7**.

Fig. 2.6. Study plan for evaluating in vitro radioprotective potential of baicalein



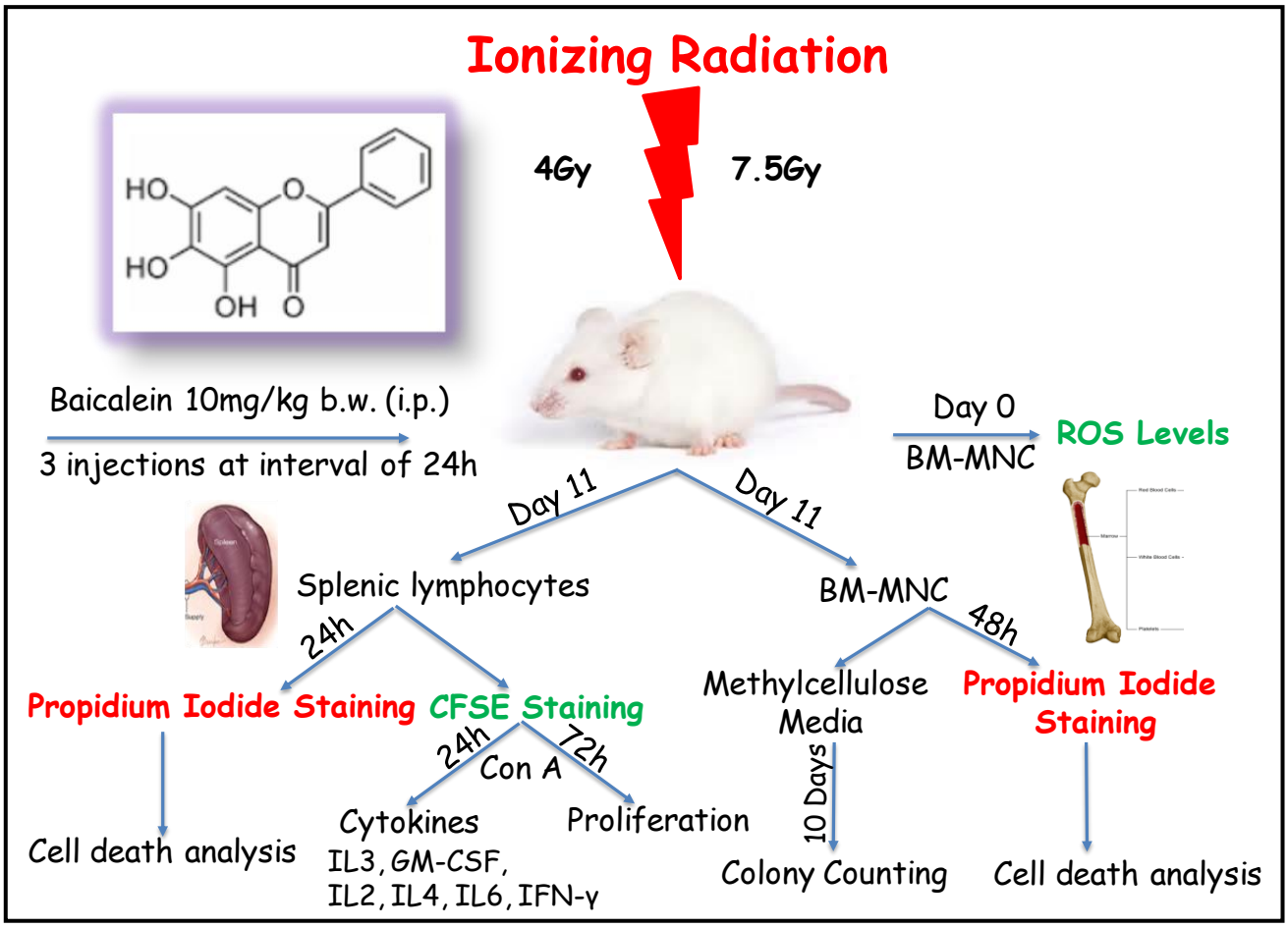


Fig. 2.7 Study plan for evaluating in vivo radioprotective ability of baicalein

2.2. Materials and Methods

2.2.1 Chemicals

Baicalein, Hoechst 33342, H_2DCFDA, HEPES, EDTA, EGTA, glycerol, DTT, NaCl, trizol reagent, JC-1 dye, DABCO reagent, tris, glycine, DMSO, para-nitro-phenylphosphate (PNPP), immunoprecipitation kit, verapamil, triton X-100, bovine serum albumin, trypan blue and Iscove's Modified Dulbecco's Medium (IMDM) were purchased from Sigma Co. USA. RPMI, fetal bovine serum (FBS), 10X PBS and sterile water were purchased from (Himedia, Mumbai, India). Homogenous caspase assay kit, in situ cell death detection kit, poly dI:dC and SYBR green PCR mix were purchased from Roche chemical Co (Indianapolis, USA). Carboxy fluoresceindiacetate succinimidyl ester (CFSE) was purchased from Invitrogen (USA). Concanavalin A (Con A) and pharmacological inhibitors of MEK, p38, m-TOR, ERK and Nrf-2 (*all-trans* retinoic acid or ATRA) were purchased from Calbiochem (CA, USA). ELISA sets for detection of cytokines (IL2, IL4, IL6, IFN-γ, IL-3 & GM-CSF) and monoclonal antibodies against HO-1 and pERK labeled with PE, CD244 labeled with PE, CD150 APC-Cy7, lineage cocktail antibodies labeled with FITC and respective isotype controls were procured from BD Pharmingen (CA, USA) and pNrf-2 antibody labeled with PE was purchased from Bioss (Woburn, MA). Polynucleotide kinase, kinase buffer and cDNA synthesis kit were purchased from New England Biolabs (Ipswich, MA). ERK kinase activity kit and antibodies against tERK, pERK, MEK, MKP3, p65, caspase 3, HO-1 and β-actin were obtained from Cell Signaling Technologies (CA, USA). Oligonucleotide probe for Nrf-2 was purchased from Santacruz Biotechnology (Indianapolis, USA). All other chemicals used were obtained from reputed manufacturers and were of analytical grade.

2.2.2 Animal maintenance

Six to eight week old inbred Swiss and C57BL/6 male mice, weighing approximately 20-25g, reared in the animal house of Bhabha Atomic Research Centre were used. They were housed at constant temperature (23 ^{0}C) with a 12 / 12 hour light / dark cycle and were given mouse chow and water ad libitum. The guidelines issued by the Institutional Animal Ethics Committee of Bhabha Atomic Research Centre, Government of India, regarding the maintenance and dissections of small animals were strictly followed.

2.2.3 Irradiation schedule

Swiss albino male mice, C57BL6 mice, splenic lymphocytes and EL-4 cells suspended in medium were exposed to IR using a ^{60}Co γ-irradiator at a dose rate of 2.19Gy/min (Gamma Cell 220; AECL, Canada).

2.2.4 Cell culture

EL-4 (Murine T cell lymphoma) cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100µg/ml streptomycin. Cells were incubated at 37°C in a 5% CO_2 humidified atmosphere, maintained in exponentially growing conditions and passaged thrice in a week.

2.2.5 Estimation of cell death

The percent cell death was estimated using propidium iodide (PI) staining followed by flow-cytometry (Cyflow, Partec) or DNA fragmentation assay as described earlier [218]. The pre G_1 population represents cell death. DNA ladder was visualized by UV-absorbance in Gel Doc (DNR Biosystem). Trypan blue exclusion test was also performed to enumerate viable cells.

2.2.6 Homogenous caspase activity assay

Homogenous caspase activity was measured in lymphocytes exposed to radiation in presence or absence of baicalein using homogenous caspase activity kit as per manufacturer's instructions.

2.2.7 Measurement of mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was assessed using the mitochondria specific fluorescent probe JC-1 (5μM) by spectroflourimetric method as described previously [294].

2.2.8 Confocal microscopy

Baicalein treated lymphocytes were harvested at various time intervals on a glass coverslip. These cells were washed once with 1X PBS, fixed with 4% paraformaldehyde, permeabilized with PBS containing triton X 100 and stained with PE-pERK antibody as described previously [120]. Further, these cells were stained with Hoechst. Slides were examined using an LSM510 confocal microscope (Carl Zeiss, Jena, Germany) with a krypton–argon laser coupled to an Orthoplan Zeiss photomicroscope using a 488-nm laser line and a 530-nm band-pass filter.

2.2.9 RNA isolation, cDNA synthesis and quantitative real time PCR

Gene specific primers Table 2.4 were used to quantify the mRNA levels in the samples by quantitative real-time polymerase chain reaction (qPCR) as described previously [295]. The threshold cycle (the cycle at which the amplification enters into exponential phase) values obtained from above runs were used for calculating the expression levels of genes by REST-384 version 2 software [296]. The expressions of genes were normalized against that of a housekeeping gene, β-actin, and plotted as relative change in the expression with respect to control.

Table 2.4. Primer sequence

Gene	Sequence
HO-1	Forward: AGGTACACATCCAAGCCGAGA Reverse :CCATCACCAGCTTAAAGCCTT
TrxR1	Forward: GGGTCCTATGACTTCGACCTG Reverse: AGTCGGTGTGACAAAATCCAAG
GCLC	Forward: CTACCACGCAGTCAAGGACC Reverse: CCTCCATTCAGTAACAACTGGAC
GCLM	Forward:AGGAGCTTCGGGACTGTATCC Reverse:GGAAACTCCCTGACTAAATCGG
Catalase	Forward: AGCGACCAGATGAAGCAGTG Reverse: AGGACATCAGGTCTCTGCGA
Mn-SOD	Forward: CAGACCTGCCTTACGACTATGG Reverse: CTCGGTGGCGTTGAGATTGTT
β-actin	Forward: GCGGGAAATCGTGCGTGACATT Reverse: GATGGAGTTGAAGGTAGTTTCGTG

2.2.10 Intracellular antibody staining by flow cytometry

Detection of proteins by intracellular antibody staining was performed as described earlier [297]. Briefly, 2.5 x 10^6 lymphocytes were blocked with medium containing 10% serum on ice, fixed with paraformaldehyde at 4^0C and permeabilized with 1X PBS containing 0.05% triton X100. Antibody staining solution (medium with 5% serum) containing 0.3μg antibody was added (20μl) to cells and incubated at RT for 30min. Cells were washed with 1X PBS and resuspended in 1ml 1X PBS. Nuclei were stained with Hoechst 33342 (10μg/ml). Unstained cells and isotype antibody stained cells were used. Cells were acquired on flow cytometer.

2.2.11 Immunoprecipitation and Western blot analysis

Lymphocytes (40 x 10^6/group) were treated with baicalein and harvested at different time intervals. Cytosolic, whole cell or nuclear extract was prepared as described earlier [298]. Vehicle treated cells served as a control. Whole cell lysates were equally loaded onto SDS-PAGE and electrophoresed at 100V. Proteins were transferred onto nitrocellulose membrane by

semi-dry transfer at 45mA. Presence of proteins on nitrocellulose membrane was confirmed by Ponceau S staining. Membranes were blocked with 5% milk in 1X tris buffer containing 0.05% triton X100 and probed with pERK, pMEK, ERK, MKP3, HO-1 and β-actin. Nuclear extracts were probed with Nrf-2 and PARP. MKP3 was immunoprecipitated using protein-G IP kit from Sigma and extracts were probed for phosphatase activity.

2.2.12 Electrophoretic mobility-shift assay (EMSA)

Lymphocytes were treated with baicalein (100μM) for different time intervals, cells were harvested and nuclear pellets were prepared as described earlier [299]. EMSA was performed by incubating 10 μg of nuclear protein with 16fmol of ^{32}P-end-labeled, Nrf-2 oligonucleotide (5'-TGGGGAACCTGTGCTGATCACTGGAG-3') in the presence of 0.5 μg of poly (2′-deoxyinosinic–2′-deoxycytidylic acid) in binding buffer (25 mM Hepes, pH 7.9, 0.5 mM EDTA, 0.5 mM DTT, 1% NP-40, 5% glycerol, and 50 mM NaCl) for 30 min at 37 °C. The DNA–protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels using buffer containing 50 mM Tris, 200 mM glycine, and 1 mM EDTA, pH 8.5. The dried gel was exposed to a phosphorimage plate and the radioactive bands were visualized using a phosphorimage plate scanner (Fuji).

2.2.13 Measurement of proliferation and cytokine secretion

Lymphocytes were isolated from mice 11 days after administration of baicalein or DMSO and WBI 4Gy treatment. The cells were stained with CFSE and stimulated with Con A. After 72h of culture they were monitored for proliferation by dye dilution on a flow cytometer. The concentration of IL-2, IL-4, IL-6, IFN-γ, IL-3 and GM-CSF in the supernatant of cells derived from these mice 24h post Con A stimulation was estimated using cytokine ELISA sets [300].

2.2.14 Side population and HSC analysis

The femur bones were dissected, and the marrow was flushed into cold IMDM, 2% FBS using a 21-gauge needle. Nucleated cells were counted using hemocytometer and trypan blue dye exclusion. Three million cells from each mouse were resuspended in pre-warmed 37^0C DMEM without phenol red. Verapamil (100µM) was added to a set of pooled cells and incubated for 10 min at 37^0C. Hoechst 33342 (5µg/ml) was added and cells were incubated at 37^0C for 90 min. At the end of incubation, cells were washed with DMEM without phenol red and acquired on flow cytometer within 2h. Cells incubated with verapamil and then stained with Hoechst 33342 were used to define side population [301]. For HSC analysis, BM-MNC were washed with 1X PBS and resuspended in IMDM with 2% serum and stained with antibody cocktail containing lineage-FITC, CD244-PE, CD150-APC-Cy7 in complete medium and 0.3µg antibody per sample was added. Single stained, unstained and FMO (fluorescence minus one) controls were used for compensation analysis. Surface staining was performed on ice as described earlier [302]. Cells were acquired on partec cyflow flow cytometer and analyzed using FlowJo 7.6.5 (Treestar, Inc. Ashland) software.

2.2.15 *In vivo* radioprotection studies

Mice (ten per group) were administered baicalein (10 mg/kg body wt) or vehicle (DMSO) intraperitoneally for 3 consecutive days with time interval of 24h. Ten minutes after last injection of baicalein, mice were exposed to WBI (4Gy/7.5Gy) and sacrificed at different time intervals or monitored for 30 days survival. BM-MNC and lymphocytes were isolated and cultured for 24 or 72h at 37°C. The cells were processed for CFSE staining, PI staining or ROS measurement [303]. Another group of mice was administered with ATRA (5mg/kg body wt) twice in a week following baicalein and WBI treatment. To determine the DRF, mice were exposed to WBI

doses between 5.5 and 10.5 Gy. Group of 10 animals each were pretreated with three consecutive i.p. injections of baicalein (10 mg/kg body wt) and 10 min after last injection they were irradiated with 5.5, 6.5, 7, 7.5, 8.5, 9.5 and 10.5Gy. Animals were monitored for 30 days after WBI for body weight changes and mortality.

2.2.16 Statistical analysis Data are presented as mean ± SEM. Statistical analysis was done using ANOVA by microcal origin 6.0 software. Statistical significance for survival studies was calculated from log-rank test using Graphpad Prism 5.0 software. *refers to $p<0.05$, as compared to control, # refers to $p<0.05$, as compared to irradiated or Con A stimulated group and $ refers to $p<0.05$ as compared to compound treated and irradiated or Con A stimulated group

2.3. Results

2.3.1 Baicalein protected murine splenic lymphocytes against IR induced cell death

Lymphocytes were incubated with different concentrations of baicalein (5, 10, 25, 50 & 100μM) for 4h and then exposed to radiation dose of 4Gy. Cells were harvested at 24 h, stained with PI and acquired on a flow cytometer. Representative flow cytometric histograms of (PI) stained cells (A) and bar chart (B) show that radiation induced cell death in ~80% cells over control. Incubation of lymphocytes with baicalein (100μM) per se did not induce any cell death. Baicalein treatment led to a concentration dependent reduction in radiation induced cell death as evinced from decrease in pre-G1 population. At 100μM, it offered complete protection against radiation induced cell death (Fig. 2.8A & B). Caspase activation is a hallmark of IR induced apoptosis. Activated caspases further activate DNases that cleave genomic DNA at the intervals of ~180bp or its multiples which is monitored by DNA fragmentation on agarose gel. To confirm radioprotective action of baicalein, DNA fragmentation assay was also performed which corroborated with results of PI staining and revealed dose dependent protection of murine splenic

lymphocytes (Fig. 2.8C). Viability of lymphocytes exposed to radiation in presence or absence of baicalein and cultured for 24h was assessed by performing trypan blue staining. Non-viable cells take up this dye owing to their compromised membrane permeability. It was observed that cells treated with baicalein and exposed to 4Gy dose of IR were viable (Fig. 2.8D).

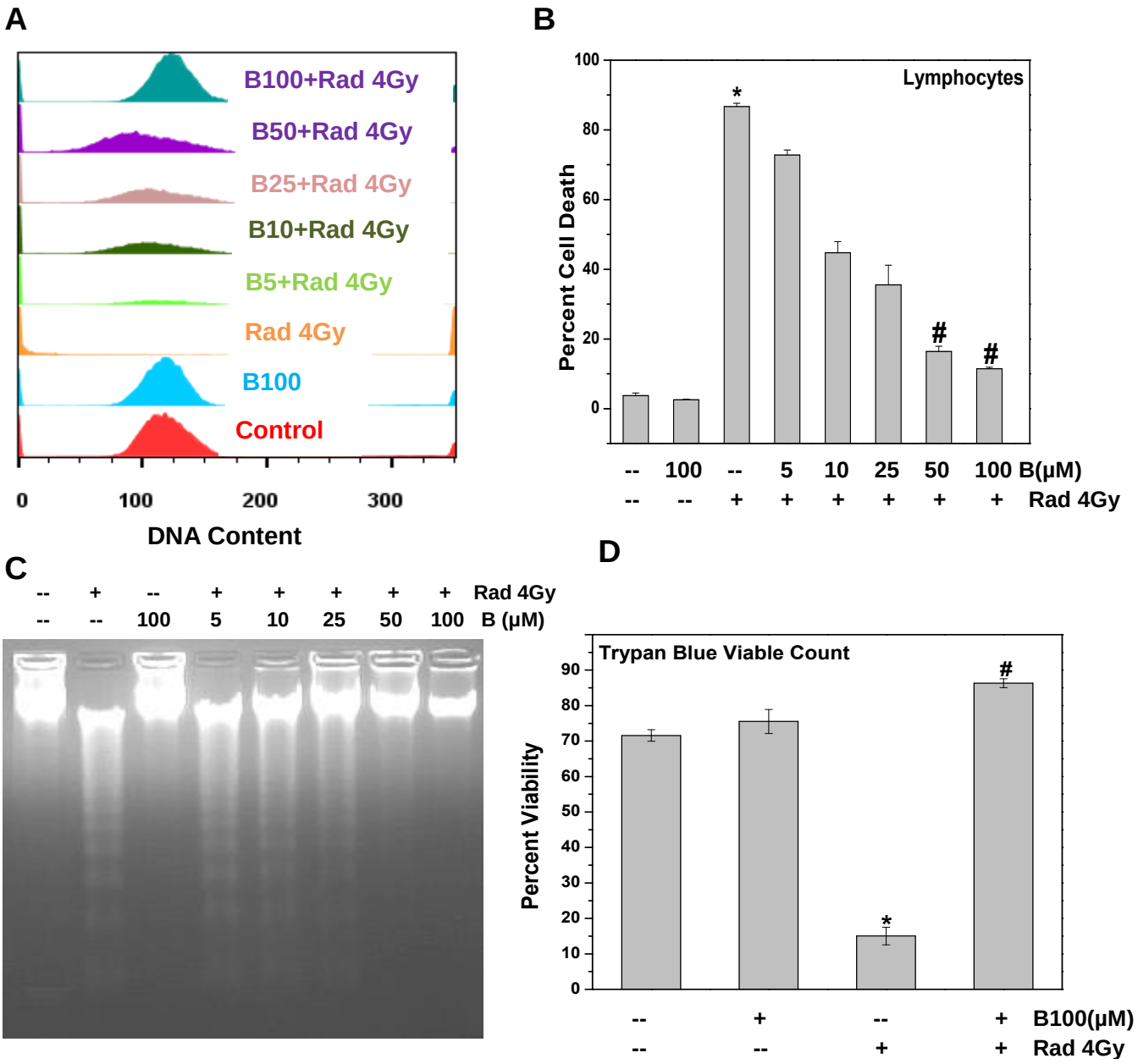


Fig.2.8 Baicalein protected murine splenic lymphocytes against IR induced cell death in vitro

*Lymphocytes were incubated with different concentrations of baicalein (5, 10, 25, 50 & 100μM) for 4h and then exposed to 4Gy IR and cultured for 24h at 37^0C in CO_2 incubator. (A) Cells were stained with PI and acquired on a flow cytometer. Overlaid flow cytometric histograms show cell death. (B) Percentage cell death is shown in graph. (C) DNA fragmentation in cells treated with baicalein and exposed to IR. DNA ladder indicates cells undergoing apoptosis. (D) Viability of lymphocytes was measured using trypan blue dye exclusion assay. Bar graph represents per cent viability. *p<0.05, as compared to control, #p<0.05, as compared to IR.*

2.3.2 Baicalein protected lymphocytes but not lymphoma against IR induced apoptosis

During apoptosis, activity of executioner caspases increases significantly leading to cleavage of proteins and ultimately cell death. Hence, homogenous caspase activity was monitored in lymphocytes in the presence and absence of baicalein. Exposure to 4Gy significantly enhanced homogenous caspase activity in lymphocytes, but radioprotective concentration of baicalein reduced it to control level suggesting that caspases are no more active in baicalein treated cells exposed to 4Gy (Fig. 2.9A). Exposure to IR leads to cleavage of caspase-3. Baicalein prevented radiation induced cleavage of caspase-3 (Fig. 2.9B). Baicalein treatment significantly prevented late events of IR induced apoptosis such as DNA fragmentation, homogenous caspase activity and viability. Exposure to IR leads to loss of mitochondrial membrane potential (MMP) as an early event which is also used as a measure of cell viability. Effect of baicalein on this early event of apoptosis was monitored by JC1 staining and it was observed that baicalein significantly prevented IR induced loss of mitiochondrial membrane potential (Fig. 2.9C).

An ideal radioprotector should not offer survival advantage to tumor cells. Hence, effect of baicalein on radiosensitivity of EL4 (murine T cell lymphoma) was monitored. Incubation of EL4 cells with radioprotective concentration of baicalein alone led to significant increase in cell death. Exposure of EL4 cells to radiation showed marginal increase in cell death over control and addition of baicalein prior to irradiation did not offer protection to these cells (Fig. 2.9D).

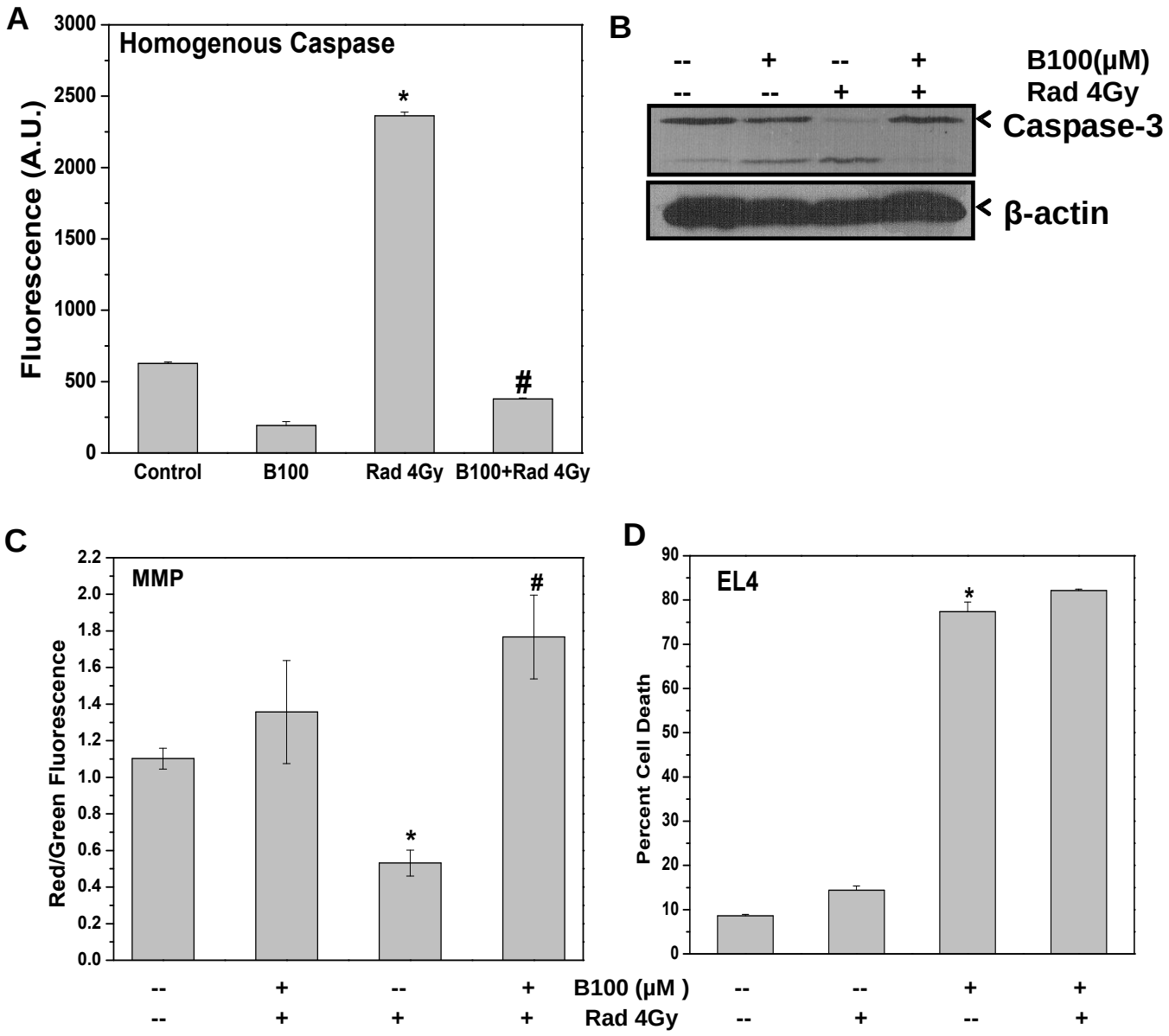


Fig. 2.9 Baicalein protected lymphocytes but not lymphoma against IR induced apoptosis *(A) Homogenous caspase activity was measured spectro-fluorimetrically. Bar graph represents homogenous caspase activity (A.U.). (B) Incubation of lymphocytes with baicalein prevented radiation induced caspase-3 cleavage. (C)Change in mitochondrial membrane potential was measured spectro-fluorimetrically. Bar graph represents ratio of red to green fluorescence. (D) Murine T cell lymphoma cells (EL4) cells were incubated with 100μM baicalein for 4h prior to irradiation and cultured for 48h.. Cells were acquired on flow cytometer after PI staining. Vehicle treated cells served as control. Percentage cell death is shown in bar graph. *p<0.05, as compared to control, #p<0.05, as compared to IR*

2.3.3 Baicalein scavenged ROS and did not affect GSH/GSSG ratio

Since baicalein is reported to be a potent anti-oxidant, experiments were performed to examine whether baicalein mediated radioprotection is through scavenging of free radicals. Baicalein scavenged radiation induced ROS at 5μM (Fig. 2.10A) as measured by H_2DCF-DA. Interestingly, baicalein did not offer any radioprotection at this concentration indicating that antioxidant action may not be playing a key role in baicalein mediated radioprotection.

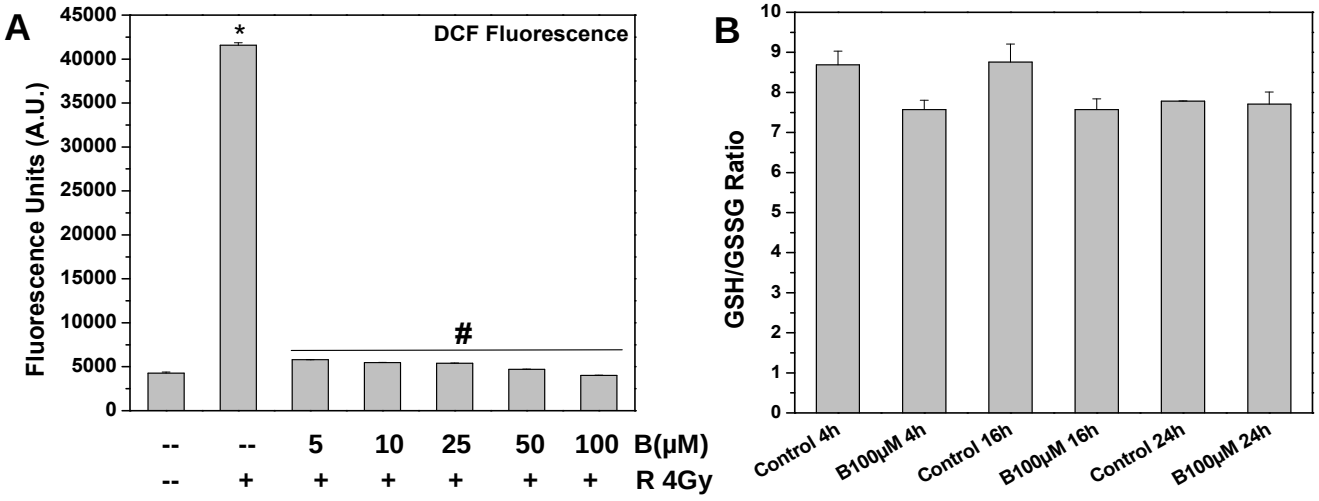


Fig. 2.10 Baicalein scavenged ROS but did not affect GSH levels in lymphocytes *(A) Cells were stained with H_2DCF-DA, incubated with different concentrations of baicalein and then exposed to 4Gy IR. ROS levels were estimated spectro-fluorimetrically. Bar graph represents arbitrary units of DCF fluorescence indicative of cellular ROS levels. (B) Cells incubated with radioprotective concentration of baicalein for different time intervals and ratio of GSH to GSSG was determined spectrophotometrically. Bar graph represents GSH/GSSG ratio. *p<0.05, as compared to control, #p<0.05, as compared to IR.*

GSH/GSSG ratio is another important measure of cellular redox status. Hence, effect of baicalein treatment on changes in GSH and GSSG was monitored. It was observed that, baicalein treatment did not lead to any change in cellular GSH or GSSG content (Fig. 2.10B).

2.3.4 ERK and Nrf-2 inhibitor abolished baicalein mediated radioprotection

Thus, it was observed that radioprotective action of baicalein is independent of its antioxidant effect. To investigate mechanism of action of baicalein mediated radioprotection lymphocytes were incubated with pharmacological inhibitors of ERK (ERKi) or MEK (PD98059) or Nrf-2 (ATRA) prior to incubation with baicalein. It was observed that, inhibitors of ERK and Nrf-2 completely abrogated baicalein mediated radioprotection suggesting the involvement of these two pro-survival molecules in baicalein mediated radioprotection (Fig. 2.11A & B).

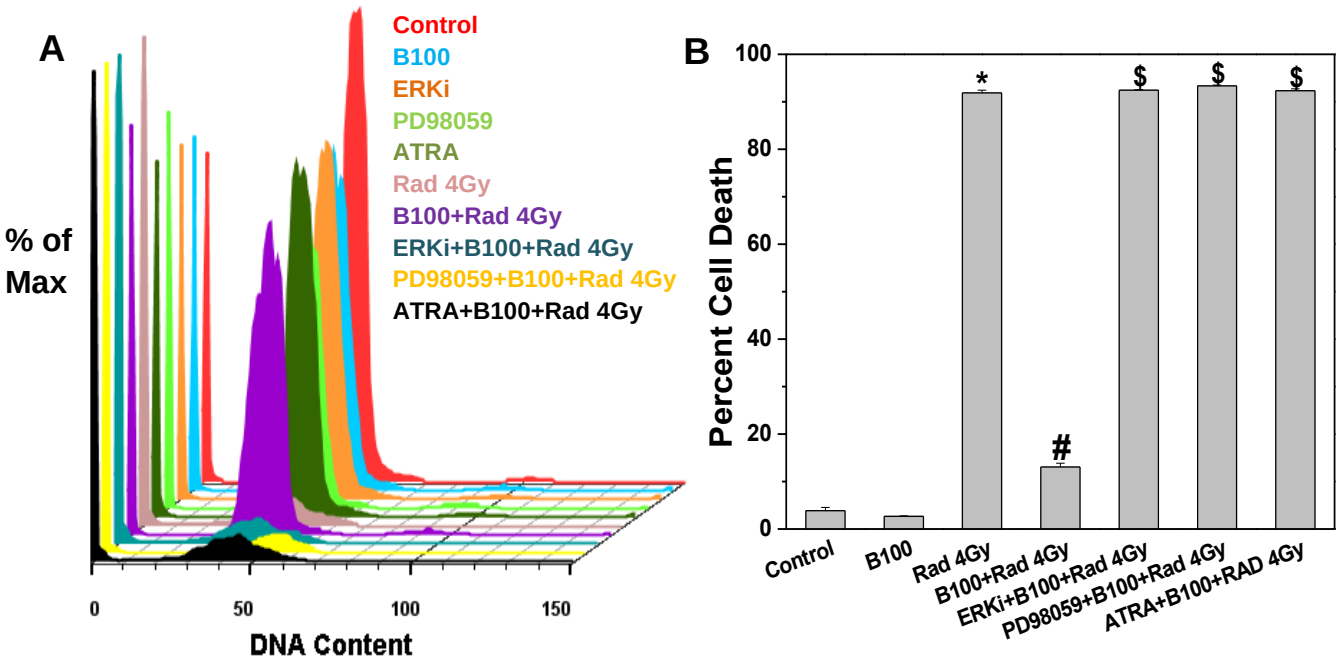


Fig. 2.11 ERK and Nrf-2 inhibitor abolished baicalein mediated radioprotection to lymphocytes *Cells were treated with pharmacological inhibitors of ERK or MEK or Nrf-2 prior and then incubated with baicalein followed by irradiation at 4Gy. Cells were cultured for 24h, stained with PI and acquired on a flow cytometer. Pre-G1 peak represents apoptotic population (A). Bar graph represents per cent apoptotic cells (B). *p<0.05, as compared to control, #p<0.05, as compared to IR and $p<0.05, as compared to baicalein treated and irradiated group.*

2.3.5 Baicalein induced phosphorylation of ERK

Since baicalein mediated radioprotection was abrogated by ERK inhibitor, the effect of baicalein on levels of phosphorylated form of ERK in lymphocytes was studied using flow cytometry and confocal microscopy. These experiments established that baicalein treatment induced ERK phosphorylation in lymphocytes (Fig. 2.12A-C).

2.3.6 Baicalein suppressed phosphatase activity

Further, experiments were performed to investigate the mechanism of activation of ERK by baicalein. Treatment of cells with baicalein led to phosphorylation of upstream kinase of ERK i.e. MEK1/2 (Fig. 2.13A). Inactivation of phosphatases is one of the mechanisms of activation of kinases. To address how baicalein activated ERK, total cellular phosphatase activity in lymphocytes after baicalein treatment was monitored. It was observed that baicalein reduced total cellular phosphatase activity. Since, baicalein treatment led to increased phosphorylation of ERK, total cellular phosphatase activity was monitored after baicalein treatment. Baicalein suppressed total phosphatase activity (Fig. 2.13B). MKP3 is ERK specific dual specificity phosphatase which is involved in dephosphorylation of ERK. Interestingly, baicalein inhibited MKP3 levels in a cyclic manner which was inversely related to levels of pERK (Fig. 2.13C). To measure the activity of MKP3, cell lysates from cells treated with baicalein or vehicle were immunoprecipitated using anti-MKP3 antibody, MKP3 was released from agarose beads and phosphatase activity was measured by para-nitro phenylphosphate method. It was observed that, baicalein treatment led to significant decrease in MKP3 activity (Fig. 2.13D). These experiments revealed that baicalein activated ERK pathway via inhibition of its corresponding phosphatase.

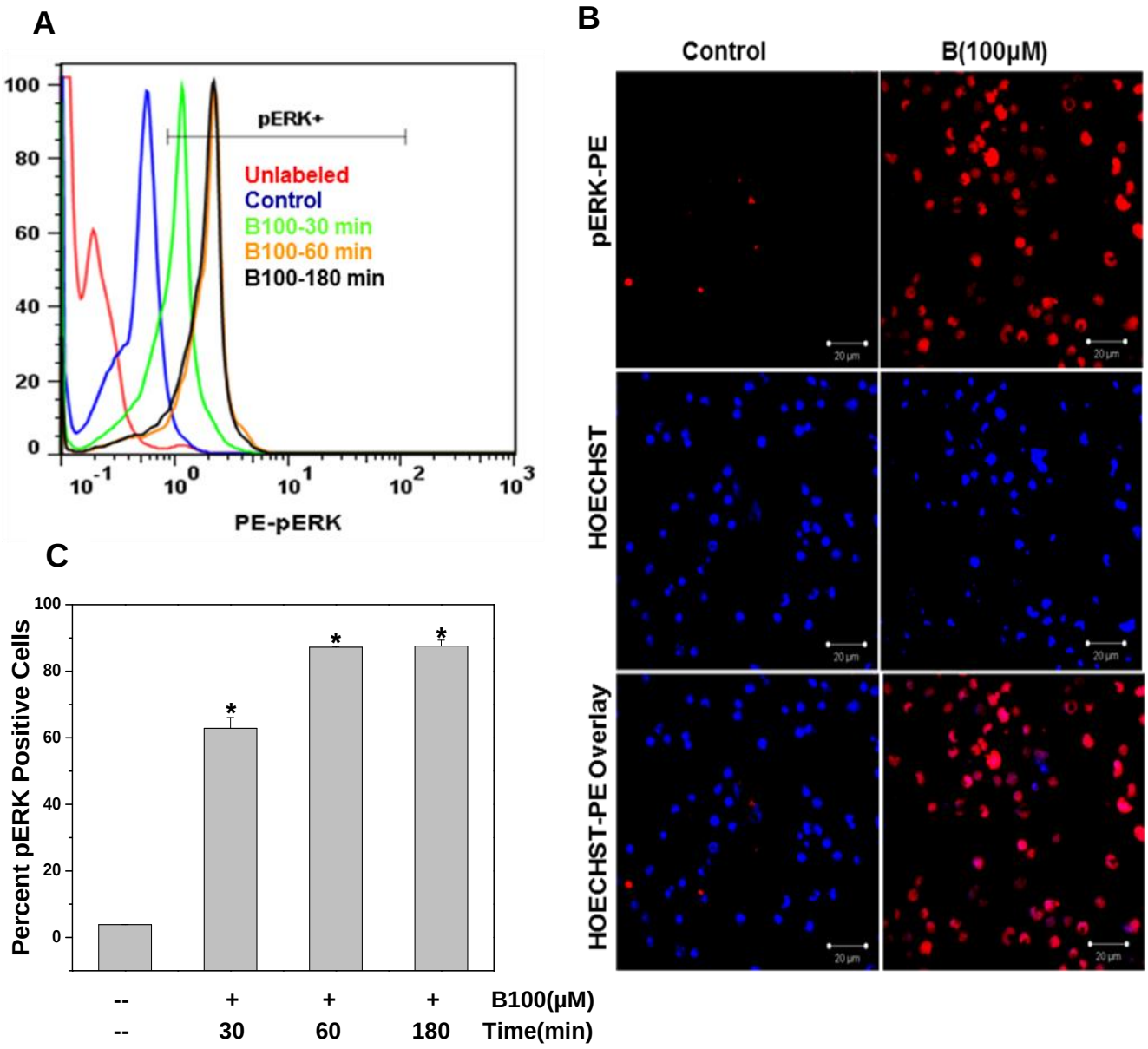


Fig. 2.12 Baicalein induced phosphorylation of ERK *Cells were incubated with baicalein (100µM) for different time intervals (30, 60 & 180 min), stained with PE-labeled pERK antibody and acquired on a flow cytometer. pERK positive cells are shown in overlaid flow cytometric histograms (A) and their percentage is shown in (B). Cells were treated with baicalein 100µM for 1h and stained with PE-labeled pERK antibody and cells were visualized by confocal microscopy (C). Hoechst staining was used to label nuclei. *p<0.05, as compared to control.*

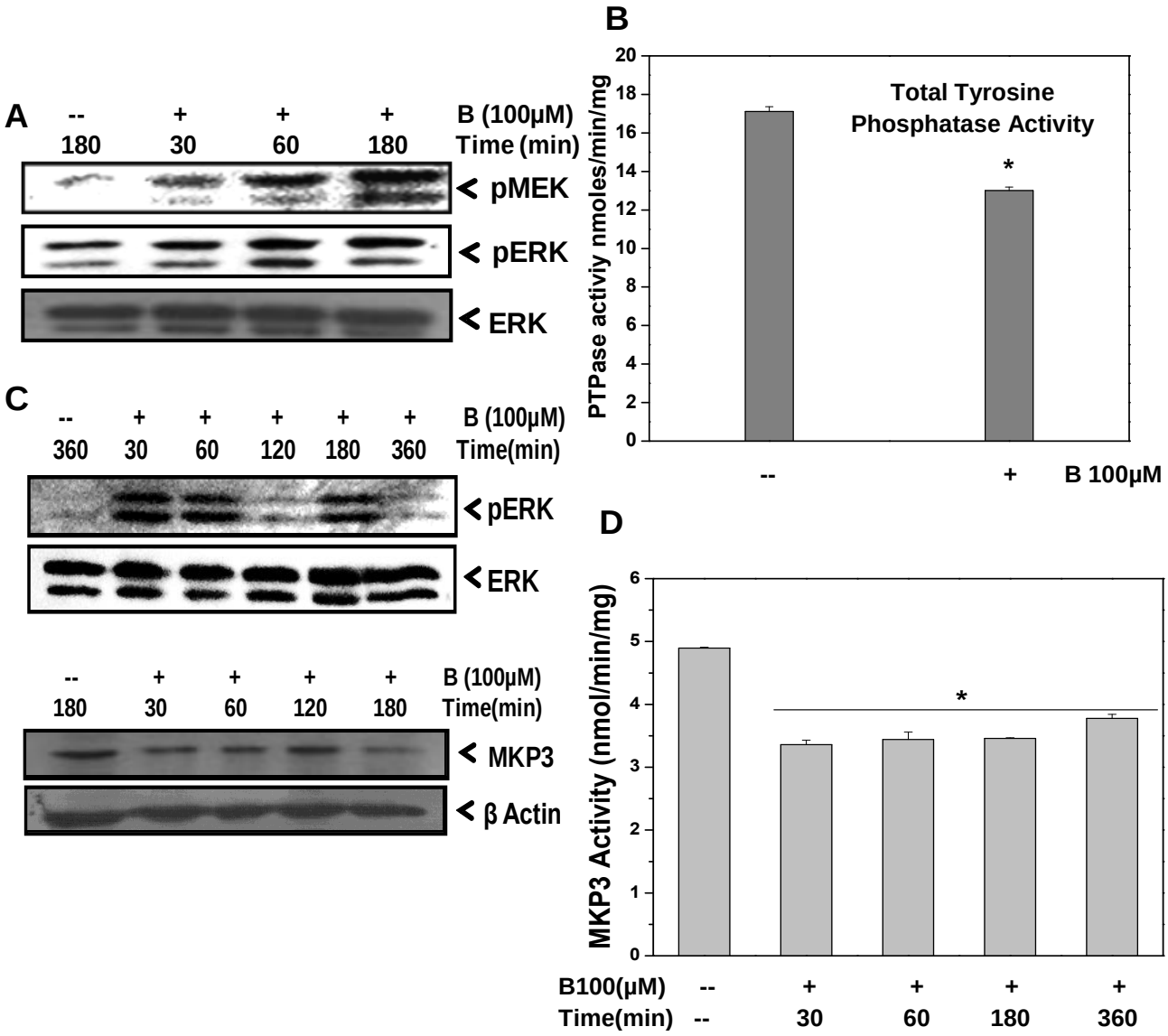


Fig. 2.13 Baicalein suppressed phosphatase activity *Cells were treated with baicalein 100μM or vehicle for different time intervals (30, 60 & 180 min) cell lysates were prepared and probed for pMEK & pERK. Western blot images are shown in (A). Whole cell extracts from baicalein or vehicle treated lymphocytes were probed for total cellular tyrosine phosphatase activity. Bar graph represents nmol/min/mg activity of phosphatase (B). Cell lysates from baicalein or vehicle treated cells (30, 60, 120, 180 &/or 360) were probed with pERK & MKP3, Western blot images are shown (C). Cell lysates from baicalein or vehicle treated cells were immunoprecipitated with anti-MKP3 antibody. MKP3 was released from agarose beads and MKP3 phosphatase activity was measured using PNPP. Bar graph represents MKP3 phosphatase activity nmoles/min/mg protein (D). *p<0.05, as compared to control.*

2.3.7 Baicalein induced phosphorylation and DNA binding of Nrf-2 Since Nrf-2 (nuclear factor erythroid 2 related factor 2) inhibitor abrogated baicalein mediated radioprotection to lymphocytes, further investigations were carried out to establish a causal role of this transcription factor in baicalein mediated radioprotection. Keap1, an inhibitor of Nrf-2 is dissociated from Nrf-2 after induction of oxidative stress. Nrf-2 is phosphorylated at Ser 40 by ERK or PKC and subsequently it translocates to nucleus and binds to ARE (antioxidant response element). Hence, phospho-Nrf-2 levels in vehicle and baicalein treated cells were monitored by immunofluorescence. It was observed that, baicalein treatment significantly increased phospho-Ser40-Nrf-2 levels at 6h as shown in flow cytometric histograms (Fig. 2.14A & B). Phosphorylation of Nrf-2 leads to its nuclear translocation and subsequent DNA binding. Since, Nrf-2 is a transcription factor its functional activity was determined by DNA binding and transcriptional activity. It was observed that baicalein treatment lead to significant increase in DNA binding of Nrf-2 at 6h in lymphocytes as revealed by EMSA (Fig. 2.14C). Interestingly, ERK inhibitor abolished baicalein induced nuclear translocation of Nrf-2 suggesting that ERK activation may be necessary upstream signaling event for its activation in lymphocytes (2.14D).

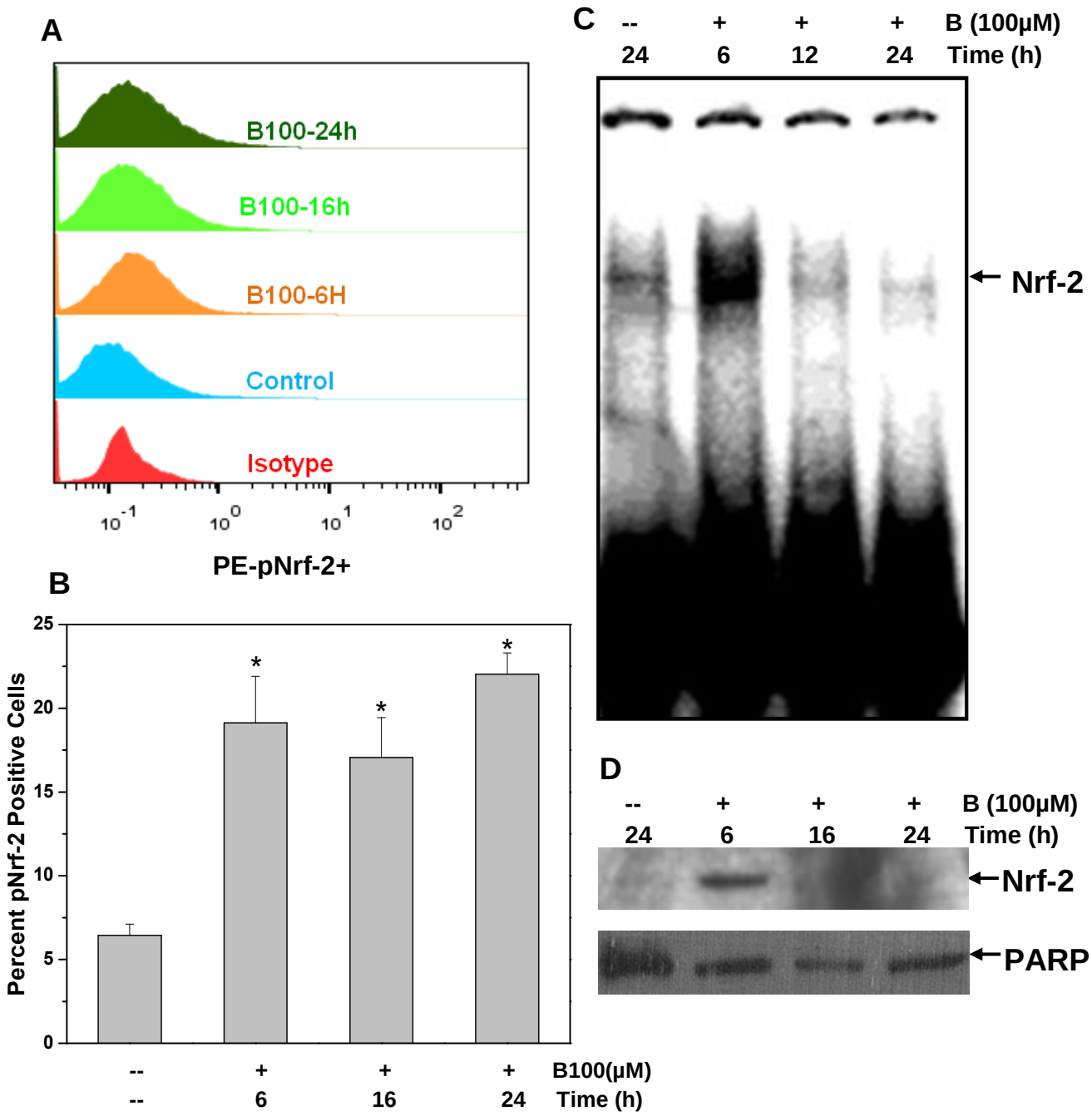


Fig. 2.14 Baicalein activated Nrf-2 *Baicalein treated cells were stained with PE-phospho-Nrf-2 antibody and analyzed by flow cytometry. Overlaid flow cytometric histograms show pNrf-2 positive cells (A). Per cent pNrf-2+ cells are shown in (B). Nuclear translocation and DNA binding activity of Nrf-2 was assessed by electrophoretic mobility shift assay using nuclear extracts from lymphocytes at different time intervals after baicalein treatment (C). Nuclear extracts prepared from cells treated with baicalein or vehicle were probed with Nrf-2 and PARP. Western blot images are shown in (D). *p<0.05, as compared to control.*

2.3.8 Baicalein activated Nrf-2 dependent antioxidant defense machinery

Gene expression analysis was carried out to monitor Nrf-2 dependent genes in lymphocytes after baicalein treatment. This analysis revealed that there was a progressive increase in Nrf-2 dependent genes involved in antioxidant defense viz. catalase & Mn-SOD, genes involved in synthesis of most abundant intracellular antioxidant GSH viz. GCLC & GCLM, gene for TrxR which is involved in reducing Trx (oxidized) and cytoprotective gene hemoxygenase-I (HO-1) (Fig. 2.15A). Corroborating with mRNA expression, there was a time dependent increase in HO-1 protein levels after baicalein treatment (Fig. 2.15B & C). Thus, it can be inferred that, baicalein treatment leads to ERK activation in lymphocytes which is upstream regulator of Nrf-2 activation and nuclear translocation, which further leads to upregulation of antioxidant defense response.

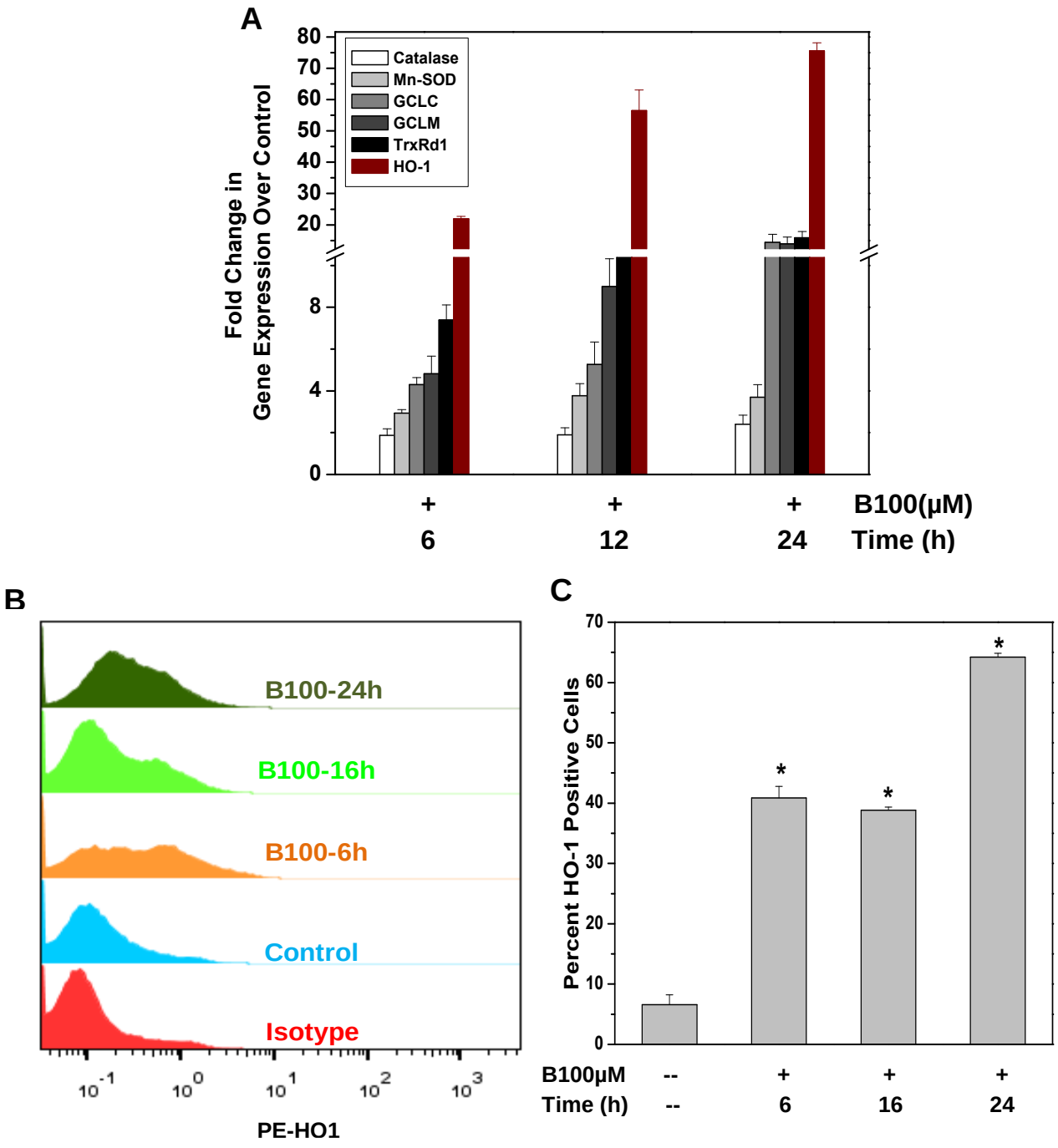


Fig. 2.15 Baicalein treatment increased Nrf-2 dependent gene and protein expression *Cells were incubated with baicalein 100μM for different time intervals (6, 12 & 24h). Total RNA was extracted and relative mRNA expression was estimated by quantitative real time PCR for catalase, Mn-SOD, GCLC, GCLM, thioredoxin reductase & HO-1 genes (A). Baicalein treated cells were stained with PE-HO-1 antibody & acquired on flow cytometer. Flow cytometric histograms show HO-1 positive cells (B). Bar graph represents per cent HO1 positive cells (C).* *p<0.05, as compared to control.

2.3.9 Baicalein protected BM-MNC against WBI induced loss of viability and functionality

More importantly, the observed in vitro radioprotection by baicalein was demonstrated in in vivo model system. The number of BM-MNC in mice administered with baicalein and exposed to WBI (4Gy / 7.5Gy) was significantly higher as compared to that in WBI group on day 11 (Fig. 2.16A). Interestingly, owing to its antioxidant ability, baicalein administration lead to suppression of cellular ROS levels in BM-MNC induced by WBI exposure (Fig. 2.16B). Survival of BM-MNC isolated from baicalein treated mice exposed to radiation (4Gy) was significantly more as compared to WBI group (Fig. 2.16C).

2.3.10 Baicalein protected splenic lymphocytes against WBI induced loss of viability and functionality

Baicalein administration protected against WBI induced apoptosis in lymphocytes (Fig. 2.17A & B), loss of mitochondrial membrane potential (Fig. 2.17C) and increase in homogenous caspase activity (Fig. 2.17D). Functionality of viable lymphocytes was determined by their responsiveness to mitogenic stimulation in terms of proliferation and cytokine secretion. Stimulation of splenic lymphocytes with Con A (T cell mitogen) after isolation from baicalein administered mice exposed to WBI (4Gy) exhibited significantly higher proliferating ability (Fig. 2.18A & B) and secretion of cytokines as compared to WBI group (Fig. 2.18C & D).

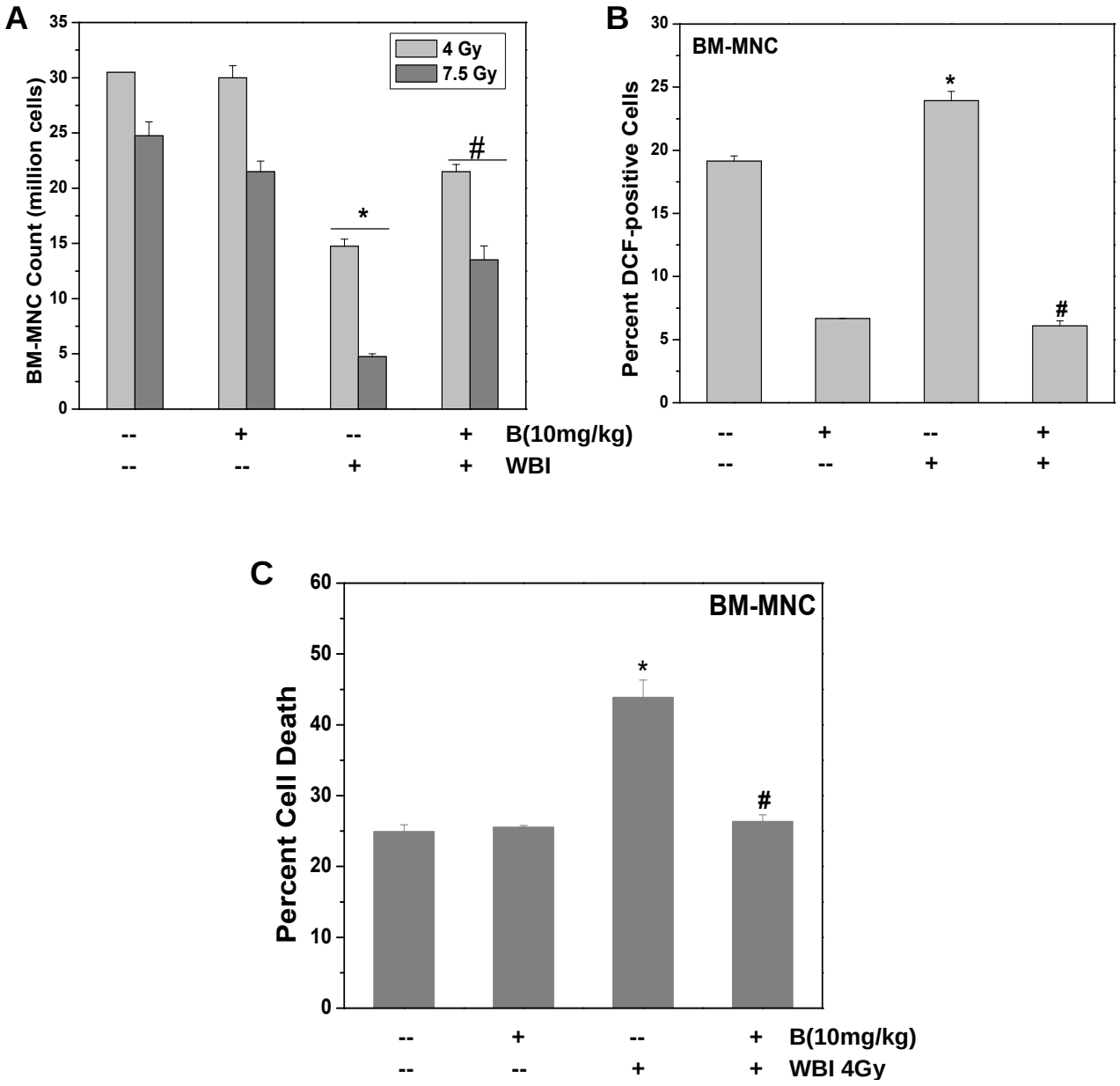


Fig. 2.16 Baicalein administration improved survival and recovery of BM-MNC *Mice were administered vehicle or baicalein (10mg/kg b.wt.) and were exposed to 4Gy or 7.5Gy IR and sacrificed 11 days after irradiation. BM-MNCs were isolated & viable counting was performed using trypan blue dye exclusion. Bar graph represents viable cell count. (A). BM-MNC from baicalein treated and control or irradiated (4Gy) mice were isolated on day 0 and processed for ROS estimation (B) or isolated on day 11 and cultured for 24h. Cell death was estimated by PI staining and flow cytometery. Bar graph represents per cent cell death (C). *p<0.05, as compared to control and #p<0.05, as compared to IR group.*

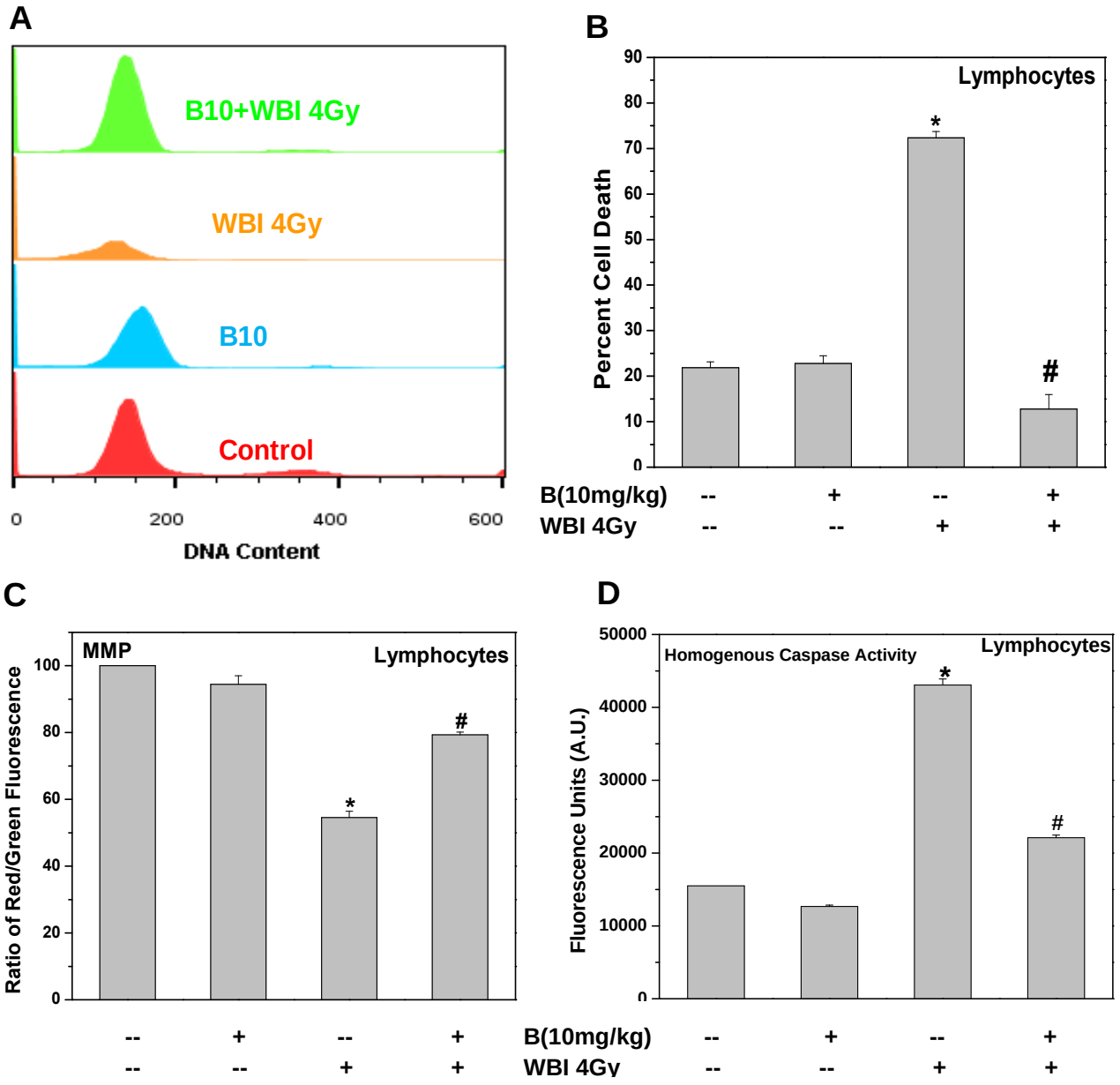


Fig. 2.17 Baicalein protected lymphocytes against WBI induced cell death *(A) Lymphocytes were isolated from mice 11 days after administration of baicalein and exposure to radiation and cultured for 24h. Cells were stained with PI and acquired on flow cytometer. Representative histograms are shown. Bar graph represents per cent daughter cells (B). Lymphocytes isolated as above were cultured for 6h and analyzed for changes in miotochondrial membrane potential (C) or these cells were cultured for 24h and analyzed for homogenous caspase activity(D). *p<0.05, as compared to control, #p<0.05, as compared to IR group.*

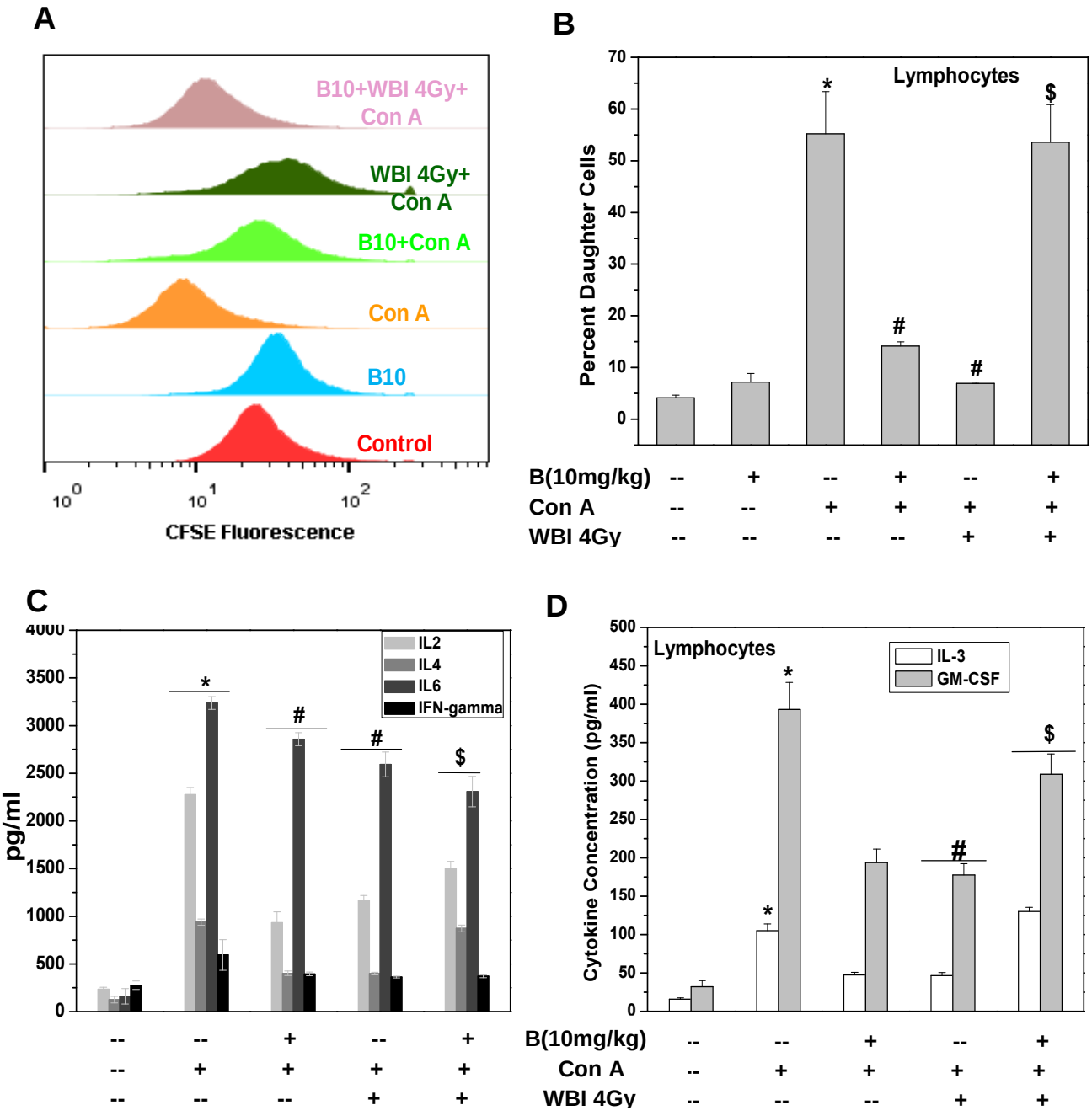


Fig. 2.18 Lymphocytes recovered from WBI induced injury were functional *(A)) Lymphocytes were isolated from mice 11 days after administration of baicalein and exposure to radiation, stained with CFSE, stimulated with Con A and cultured for 72h or 24h. Cells were acquired on flow cytometer. Representative histograms are shown. Bar graph represents per cent daughter cells (B). Supernatant harvested at 24h was analyzed for cytokines and bar graph represents cytokine concentration (pg/ml) (C & D). *p<0.05, as compared to control, #p<0.05, as compared to Con A stimulated and $p<0.05, as compared to baicalein treated and Con A stimulated cells.*

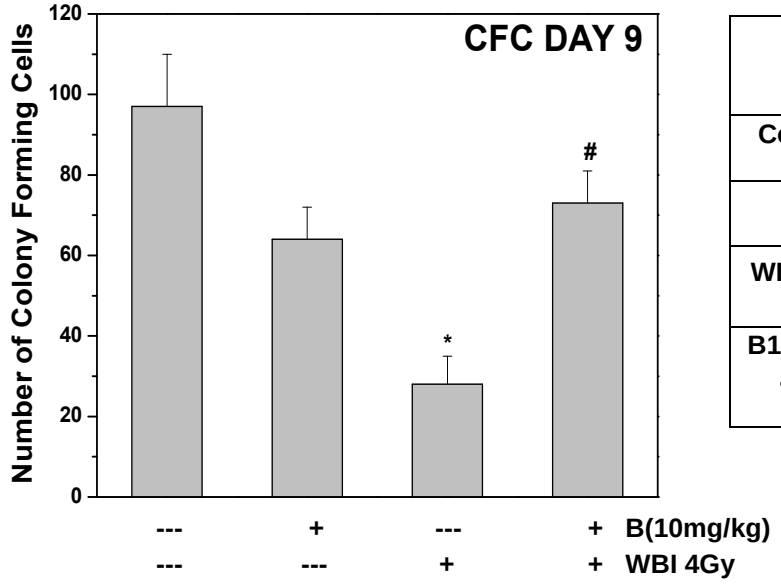
2.3.11 Baicalein augmented abundance of hematopoietic progenitors

Recovery in the number of viable BM-MNC is determined by the proliferation of hematopoietic progenitors that give rise to different subpopulations of hematopoietic system. Effect of baicalein administration on the frequency and proliferation potential of hematopoietic progenitors from colony forming ability of bone marrow cells after exposure to sub-lethal or lethal doses of WBI was monitored. Baicalein administration led to significant increase in the recovery of colony forming cells. WBI exposure at 4Gy or 7.5Gy significantly decreased number of colony forming cells (Fig. 2.19 A-D) but baicalein administration protected the bone marrow cells from WBI induced decrease in proliferative potential of hematopoietic progenitors (Fig. 2.19 A-D).

2.3.12 Baicalein administration increased HSC abundance and increased phosphorylation of Nrf-2 in lineage negative BM-MNC

Increased HSC frequency or protection of HSCs against IR induced cell death is important mechanism for radioprotection. Experiments were carried out to study the effect of baicalein administration on HSC frequency as one of the mechanisms of radioprotection. Baicalein administration lead to significant increase in Hoechst side population at 24h (Fig. 2.20A & B) and day 5 (Fig. 2.20C). To confirm these results, the frequency of lin^-CD244-CD150+ cells (which represent HSC) in bone marrow of mice administered with baicalein was enumerated and a significant increase in HSC population was observed on day 5 (Fig. 2.21A & B). Nrf-2 plays a vital role in HSC survival under normal as well as oxidative stress conditions. Baicalein administration increased the levels of pNrf-2 in lin^- BM-MNC (Fig. 2. 21C & D).

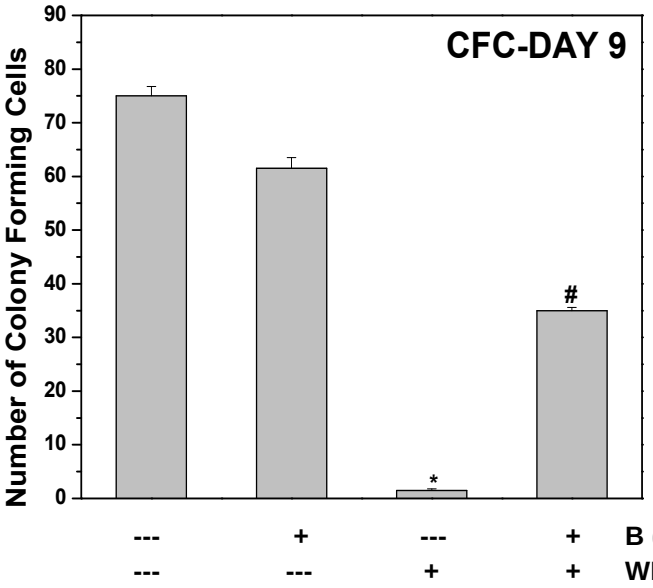
A



B

	BFU-E	CFU-G	CFU-M	CFU-GM	CFU-GEMM
Control	23	32	24	19	-
B10	23	12	19	7	-
WBI 4Gy	17	7	3	-	2
B10+WBI 4Gy	44	19	3	-	2

C



D

	BFU-E	CFU-G	CFU-M	CFU-GM	CFU-GEMM
Control	34	13	10	16	1
B10	31	16	5	17	
WBI 7.5Gy	3	-	-	-	1
B10+WBI 7.5Gy	20	5	3	5	-

Fig. 2.19 Baicalein prevented WBI induced decrease in proliferation potential of hematopoietic progenitors

*BM-MNC from mice administered with vehicle or baicalein and exposed to WBI 4Gy or 7.5Gy were isolated 9 days after exposure. Cells were seeded in mouse complete methylcellulose medium and cultured for nine days in CO_2 incubator for colony formation. At the end of incubation number of colonies were counted and their morphology was scored using microscope. Bar graph represents number of colonies and table shows differential colony count after exposure to WBI 4Gy (A) or 7.5Gy (C) in presence or absence of baicalein. Table (B) and (D) shows differential counts of colonies after respective treatments. *p<0.05, as compared to control and #p<0.05 as compared to WBI group.*

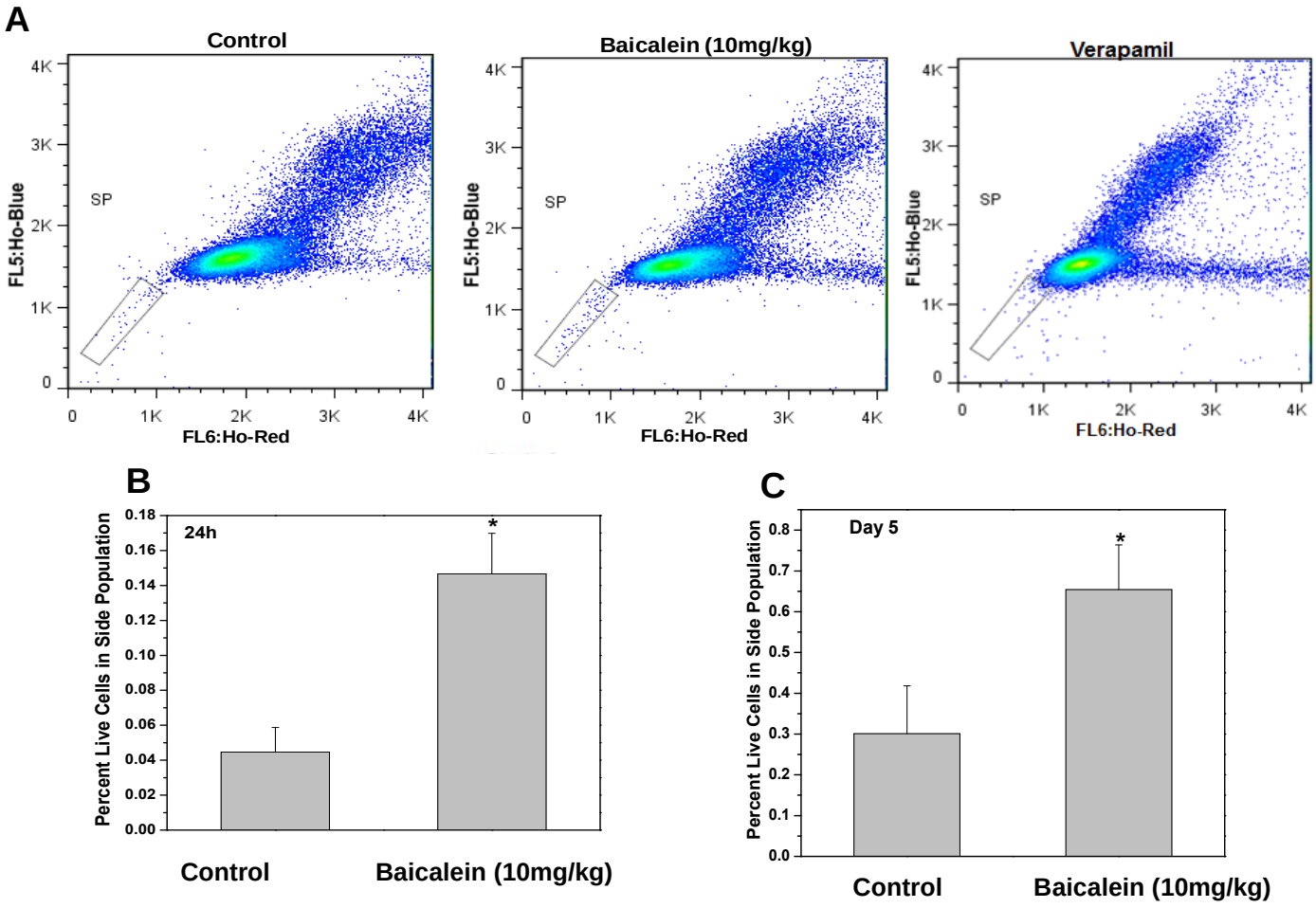


Fig. 2.20 Baicalein enhanced hematopoietic stem cell abundance *BM-MNC were isolated from mice 24h or day 5 after administration of baicalein or vehicle were isolated and were analyzed for side population using Hoechst 33342 dye. Flow cytometric histograms show characteristic side population in live cells (A). Bar graph represents percentage of side population within live cells at 24h (B) and day 5 (C). Bar graph represents percentage of side population cells within live cells (B&C). *p<0.05, as compared to control.*

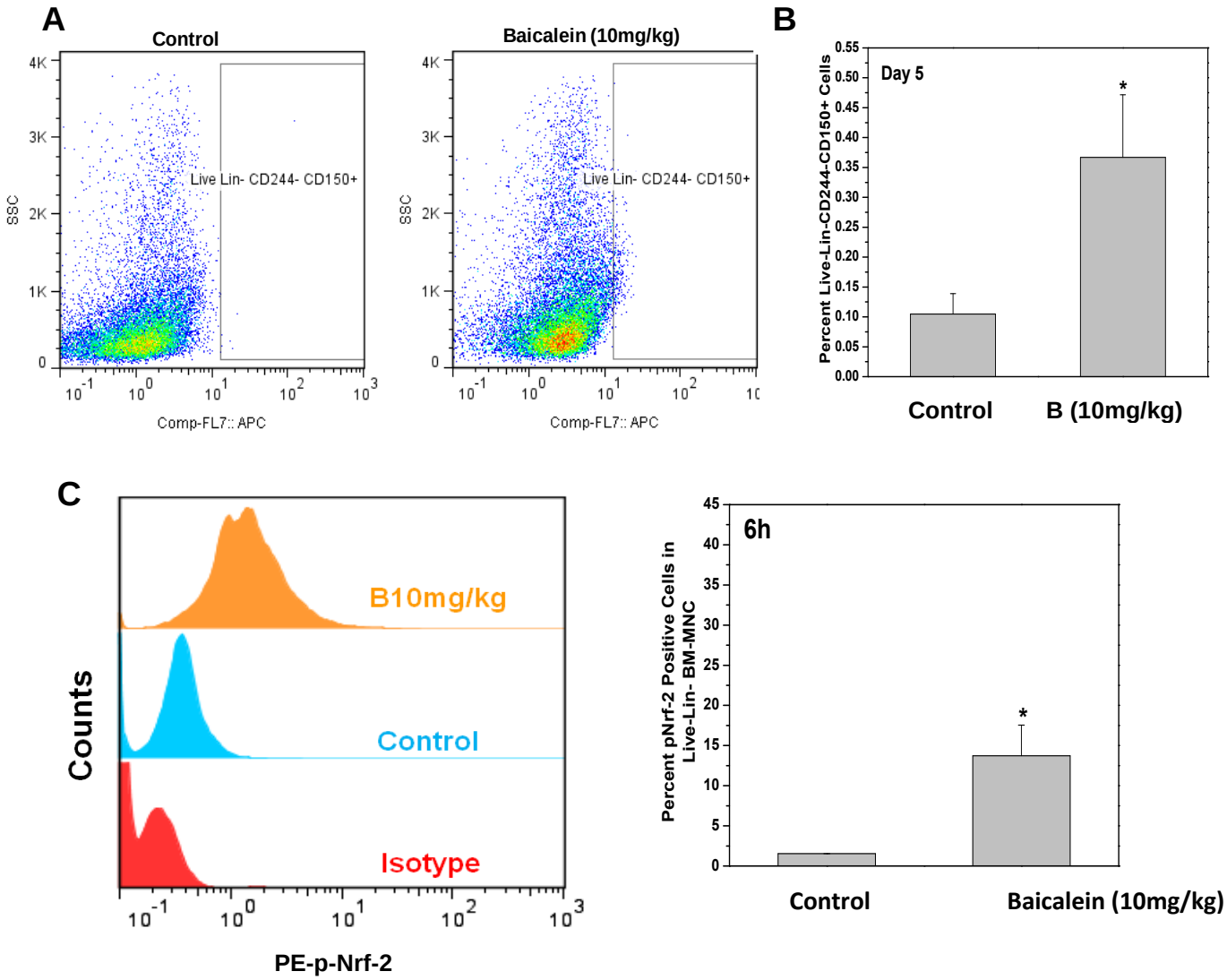


Fig. 2.21 Baicalein enhanced HSC abundance and increased phosphorylation of Nrf-2 in lin-BM-MNC *BM-MNC were isolated 5 days after baicalein administration and were immuno-phenotyped for lin-CD244-CD150+ population by flow cytometry. Bar graph represents per cent live-lin$^-$CD244$^-$ CD150$^+$ cells (A&B). BM-MNC were isolated 6h after baicalein administration and stained with antibodies against FITC labeled lineage markers and pNrf-2-PE. Histograms (C) and bar graph (D) show pNrf-2+ cells within lineage – population of BM-MNC. *p<0.05, as compared to control.*

2.3.13 Baicalein administration induced DNA binding of NF-κB and phosphorylation of ERK in BM-MNC via suppression of MKP3

To investigate the mechanism of action of baicalein for the observed in vivo radioprotection, BM-MNC from baicalein administered mice were isolated at different time intervals and examined for activation of NF-κB & ERK. Baicalein administration induced nuclear translocation of p65 and DNA binding of NF-κB (Fig. 2.22A). It also induced phosphorylation of ERK while decreased MKP3 levels and increased HO-1 levels (Fig. 2.22B).

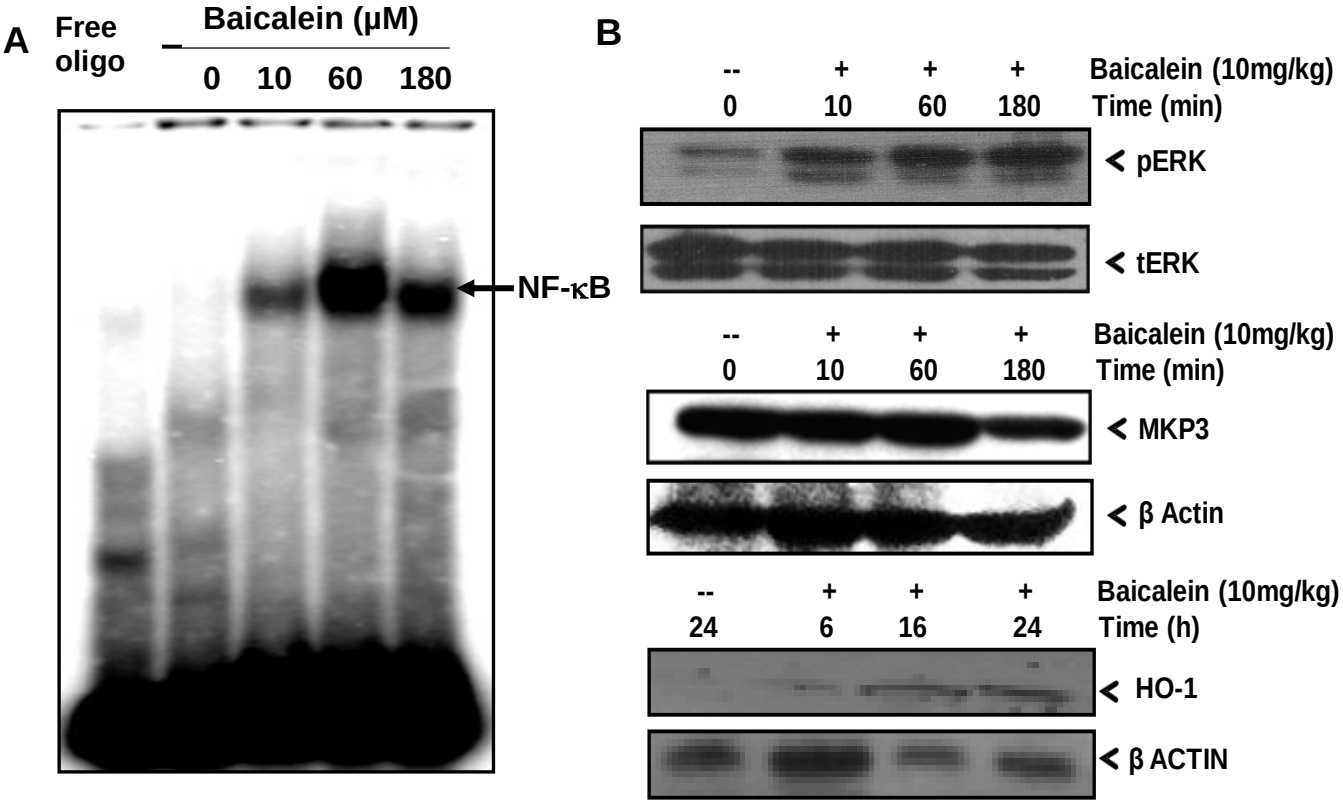


Fig. 2.22 Baicalein administration upregulated levels of pERK and HO-1 in BM-MNC *Nuclear extracts from BM-MNC isolated at different time intervals were probed for NF-κB (A). Whole cell extracts from BM-MNC isolated at different time intervals from mice administered with baicalein or vehicle were probed for pERK, MKP3 and HO-1 by Western blotting (B).*

2.3.14 Baicalein administration significantly enhanced survival of mice exposed to WBI

Mortality of mice exposed to WBI doses between 6Gy to 10Gy is primarily because of hematopoietic syndrome. Since cells of hematopoietic system were rescued from IR induced cell death by baicalein, its ability to reduce WBI induced mortality in mice was investigated. Mice were administered baicalein (10mg/kg bw; i.p.) dissolved in DMSO or DMSO alone for three consecutive days and were exposed to 7.5Gy dose of γ-radiation 10 min after last injection. They were monitored for 30 days for changes in survival and body weight. It was observed that baicalein administration improved the survival of irradiated mice by 60% as compared to WBI (Fig. 2.23A & B). Further, experiment was performed to calculate the DRF for baicalein using survival as an end point. Mice were grouped according to dose of radiation (5.5, 6.5, 7.0, 7.5, 8.5, 9.5 & 10.5 Gy) by fixing the dose of baicalein at 10 mg/kg bw. LD50/30 was calculated after monitoring the survival of mice for 30 days after radiation exposure. It was observed that LD50/30 of mice exposed to radiation alone was 6.5Gy and baicalein administration increased it to 7.5Gy resulting in DRF of 1.153 (Fig. 2.23C & D). To rule out the strain specific differences in radioprotection offered by baicalein, survival studies were carried out in C57BL/6 mice using same dose regimen. It was observed that, baicalein offered 90% protection against WBI induced mortality (Fig. 2.23E & F).

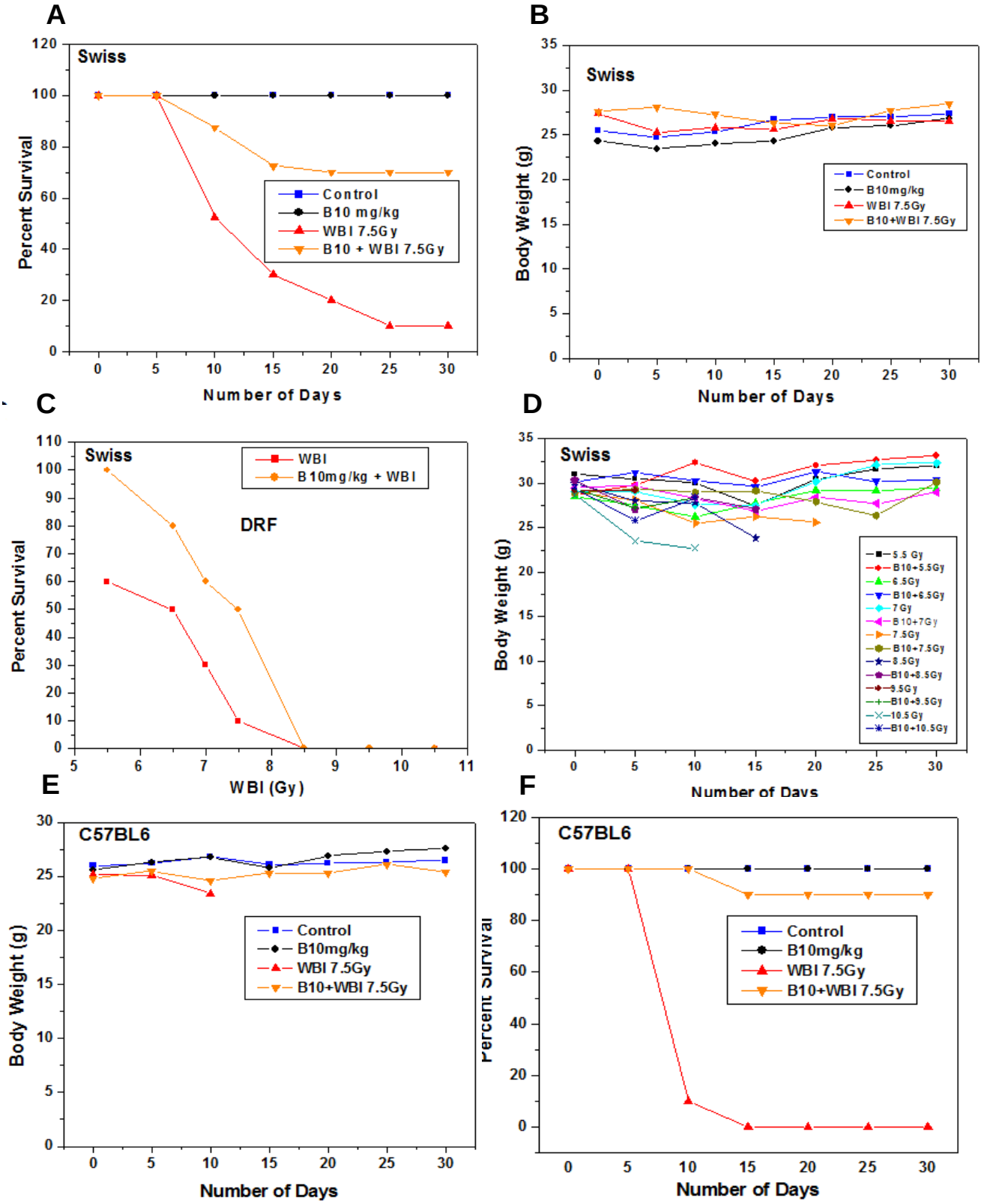


Fig. 2.23 Baicalein significantly reduced radiation induced mortality in mice *Graph represents per cent survival of Swiss mice administered with vehicle or baicalein (10mg/kg b.wt.) for three consecutive days*

and exposed to a dose of 7.5Gy IR 10 min after last injection. Mice were monitored for 30 days survival (A) and change in body weight (B). (C&D) Mice were exposed to different doses of irradiation 5.5, 6.5, 7.0, 7.5, 8.5, 9.5 or 10.5Gy with or without baicalein administration and monitored for 30 days for survival. Changes in the weight were monitored till 30 days in all the groups and representative data of one experiment is shown. This data was used for calculation of DRF. Graph represents per cent survival. (E&F) C57BL6 mice administered with vehicle or baicalein (10mg/kg b.wt.) for three consecutive days and exposed to a dose of 7.5Gy WBI 10 min after last injection. Mice were monitored for 30 days survival and changes in body weight. Significance for survival calculated by Mantel-Cox log rank test using Graphpad prism.

2.3.15 Administration of ATRA abolished baicalein mediated radioprotection

Further, mice were administered with inhibitor of Nrf-2, *all-trans* retinoic acid (ATRA) prior to baicalein treatment bi-weekly till 30 days after WBI (7.5Gy). Survival of mice was monitored for 30 days. Baicalein protected ~70% mice against WBI induced mortality, while ATRA administration reduced survival of baicalein treated mice to ~10% (Fig. 2.24A & B). These results confirmed the role of Nrf-2 in baicalein mediated radioprotection.

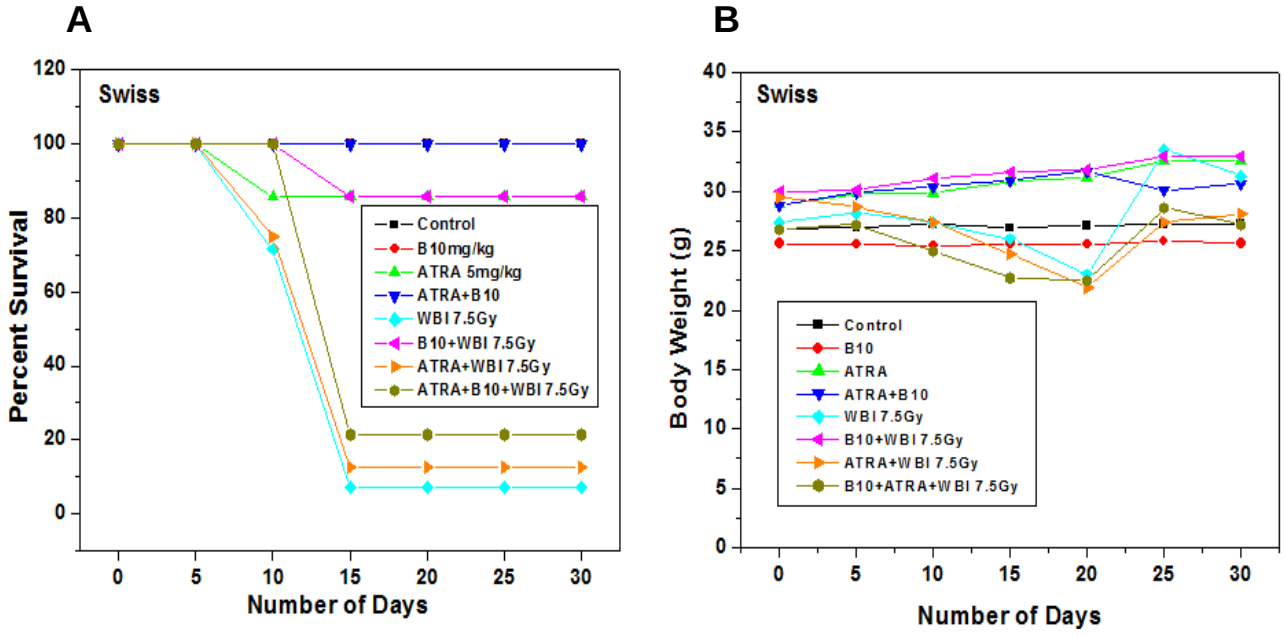


Fig 2.24 Nrf-2 inhibitor abolished baicalein mediated radioprotection in vivo *Mice were administered with baicalein and / or ATRA (5mg/kg i.p. twice in a week till day 30) or vehicle and exposed to 7.5Gy*

WBI. Mice were monitored for 30 days survival and body weight changes. Graph represents per cent survival of mice (A) & body weight changes (B).

2.4. Discussion

IR is known to suppress immune system thereby leading to increased susceptibility to fatal infections resulting in mortality. Exposures above 2 Gy induce hematopoietic syndrome. Lymphocytes serve as a good experimental model to study the effects of radiation and to screen the molecules for protection against hematopoietic injury. Aim of the present study is to investigate radioprotective potential of baicalein in vitro using splenic lymphocytes and in vivo using mouse model.

Exposure to IR 4Gy induces apoptosis in lymphocytes. Pre-treatment of lymphocytes with flavonoid anti-oxidant baicalein could rescue these cells against radiation induced loss in viability. This suggested that baicalein could further be explored for its radio-protective ability.

Before it can be taken further as a radioprotector, it was essential to investigate its effect on tumor cells. One of the most important attributes of radioprotector before it can be employed in clinical settings is that it should not offer survival advantage to tumor cells in presence of radiation. Hence, effect of baicalein on murine T cell lymphoma EL-4 cells which are tumor counterpart of normal lymphocytes was monitored in presence of radiation. Baicalein did not protect EL-4 cells against IR induced cell death, rather radioprotective concentration of baicalein per se induced cell death in EL-4 cells. This revealed anti-tumor potential of baicalein which was further explored and the results are presented in Chapter 4.

Since baicalein has been reported to be a potent antioxidant in different model systems, it was investigated whether the free radical scavenging ability is responsible for radioprotection. Baicalein could scavenge radiation induced ROS at 5µM, but it could not offer any protection to

lymphocytes at this concentration. These findings suggested that radioprotective potential of baicalein may be independent of its ROS scavenging ability. The levels of ROS and GSH determine the intracellular redox environment. Since baicalein scavenged basal ROS levels, the effect on GSH/GSSG ratio was examined and found that it did not perturb the ratio even up to 24h.

Since the possibility of radical scavenging by baicalein as a mechanism for radioprotection was ruled out, experiments were undertaken to unravel the role of some of the well-known pro-survival signaling molecules[304]. Cells are equipped with several key signaling mediators that can regulate cell survival under conditions of oxidative stress. The involvement of MEK, ERK, P38, PI3K, m-TOR and Nrf-2 was investigated by using pharmacological inhibitors. The inhibitors of P38, PI3K and m-TOR failed to abrogate the radioprotection elicited by baicalein (data not shown). Inhibitors of ERK or Nrf-2 could completely inhibit baicalein mediated radioprotection to lymphocytes suggesting their involvement.

ERK is a pro-survival signaling molecule in MAPK pathway [305], whereas Nrf-2 is a redox sensitive transcription factor required for coordinated up-regulation of antioxidant and cytoprotective genes in response to oxidative stress [306]. Baicalein treatment resulted in activation of ERK pathway in terms of increase in phosphorylation of ERK and its upstream kinase MEK in lymphocytes. These results were in agreement with the results obtained using pharmacological inhibitors. Phosphorylation of kinases is finely regulated in cells by specific phosphatases [307]. Therefore, baicalein induced phosphorylation of ERK could be through suppression of phosphatase activity. Lee et al., had shown that baicalein could inhibit a dual specific phosphatase activity in cell free system [308]. Baicalein suppressed total cellular phosphatase activity and also inhibited the levels and activity of dual specificity phosphatase

MKP3. Although there are multiple reports demonstrating the ability of baicalein to activate ERK [249, 309], these results for the first time show the molecular mechanism leading to activation of ERK.

Previous reports have shown that ERK can activate Nrf-2 [219, 310-312]. Therefore, it was investigated that whether baicalein can activate this transcription factor in lymphocytes. Addition of baicalein to lymphocytes indeed increased the nuclear levels of Nrf-2 [310-312]. Nrf-2 can be activated by oxidative stress dependent or oxidative stress independent manner. In later, phosphorylation of serine 40 residue on Nrf-2 signals its dissociation from inhibitory protein Keap1 thereby inducing nuclear translocation. Baicalein induced phosphorylation of Nrf-2 and increased protein levels of HO-1, an important anti-inflammatory molecule [313] in a time dependent manner. Thus, this study for the first time demonstrates that there is a co-ordinated and temporal activation of pro-survival transcription factor Nrf-2 by baicalein in lymphocytes leading to radioprotection.

It is clear from above discussion that baicalein induced ERK phosphorylation is the primary event in lymphocytes which may be regulating activation of other important pro-survival molecules. In agreement with this hypothesis, when cells were incubated with ERK inhibitor prior to baicalein treatment it could abolish activation of redox regulatory transcription factor Nrf-2.

Many phytochemicals show promising results as radioprotectors in cell free and cellular systems but when extended to in vivo models they are ineffective. Viability and functional integrity of hematopoietic cells post radiation exposure to sub-lethal or lethal doses serve as an important indicator for radioprotection. Sustained oxidative stress post radiation exposure causes decline in cell count, viability, survival and proliferation potential of BM-MNC as well as splenic

lymphocytes. Exposure to IR induces apoptosis and senescence in bone marrow cells thereby leading to rapid depletion of cells of hematopoietic system [314-317]. Baicalein protected against loss of functionality of BM-MNC and splenic lymhocytes induced by WBI. It also reduced WBI induced ROS levels in BM-MNC indicating that antioxidant role of baicalein could not be ruled out in radioprotection.

The cytoprotective and anti-apoptotic effects of baicalein were seen systemically as revealed by reduced apoptosis in splenic lymphocytes as compared to vehicle treated mice receiving WBI. Multiple assays for assessing apoptosis further validated the fact that baicalein protected lymphocytes against WBI induced damage. Many lymphocytes that survive oxidative stress become immunologically un-responsive [318]. The lymphocytes recovered from baicalein and WBI treated mice responded better to mitogenic stimulus (Con A) in terms of proliferation and cytokine secretion as compared vehicle treated mice receiving WBI.

Exposure to IR above LD50/30 dose leads to mortality due to hematopoietic syndrome. Since baicalein offered protection to hematopoietic cells, it is imperative to assume that it can protect mice against WBI induced mortality. Administration of baicalein exhibited significant protection against WBI induced mortality over WBI alone group. Better survival results were obtained when baicalein was administered to C57BL6 mice. DRF is the magnitude by which a radioprotector can elevate the dose required to kill 50% population exposed to IR when administered prior to such exposure [156]. It is used to compare the efficacy and potency of radioprotectors across different classes as a standard criterion [156]. Baicalein exhibited a DRF of 1.153 which is very close to genestein (1.16) [189].

In vitro studies revealed the role of MKP3/ERK/Nrf-2/HO-1 axis in baicalein mediated radioprotection. To investigate whether similar mechanism is operative in vivo, BM-MNC

isolated from baicalein administered mice was probed for MKP3, pERK, NF-κB & HO-1 levels. Consistent with the in vitro findings, these cells exhibited decreased levels of MKP3, higher phosphorylation status of ERK, increased DNA binding of NF-κB and increased levels of HO1. Although baicalein administration recovered against IR induced loss in cell count of bonemarrow, it could not offer immediate protection to BM-MNC or lymphocytes (day 0 and day 5; data not shown). Rather, it helped in the process of hematopoiesis thereby generating differentiated cells. Similar to genistein [190], results from colony forming cells assay revealed that baicalein administration offered significant protection against WBI induced loss in the number of hematopoietic progenitors. It could maintain the proliferative potential of hematopoietic progenitors thereby leading to recovery of BM-MNC and lymphocytes (day 9 and day 11).

HSCs play a key role in protecting against IR induced hematopoietic injury. Many studies have shown manipulation of HSC frequency and/or survival as principal mechanism of radioprotection [13, 319, 320]. Survival and recovery of cells of hematopoietic system by baicalein may be the outcome of increased HSC frequency or survival. Hence, HSC frequency was enumerated after baicalein administration. Interestingly, mice administered with baicalein exhibited increased abundance of HSC at 24h as well as 5 days post administration.

There are reports showing the role of Nrf-2 in HSC survival and maintenance under oxidative stress conditions [321-323]. Baicalein treatment lead to increased phospho-Nrf-2 levels in lineage negative BM-MNC. Role of Nrf-2 in baicalein mediated radioprotection was further probed using pharmacological inhibitor of Nrf-2. Mice were administered with Nrf-2 inhibitor [324, 325], ATRA, twice in a week after exposure to WBI 7.5Gy and monitored for 30 days

survival in presence or absence of baicalein. It was observed that, ATRA administration abolished baicalein mediated in vivo radioprotection.

Baicalein, an antioxidant molecule activated ERK by reducing MKP-3 levels thereby triggering Nrf-2 pathway. This resulted in upregulation of cellular pro-survival factors leading protection against IR induced hematopoietic syndrome. The overall mechanism of action of baicalein is summarized in **Fig. 2.25.**

Conclusions

1. Baicalein protected murine splenic lymphocytes against IR induced cell death as assessed by propidium iodide staining, DNA fragmentation, caspase 3 cleavage, homogenous caspase activity, loss in mitochondrial membrane potential and trypan blue viable counting.
2. Baicalein scavenged ROS and did not alter cellular GSH content.
3. Baicalein induced phosphorylation of ERK via suppression of corresponding dual specificity phosphatase MKP3.
4. Baicalein induced nuclear translocation and DNA binding of oxidative stress regulatory pro-survival transcription factor Nrf-2.
5. Baicalein protected BM-MNC, lymphocytes and hematopoietic progenitors against WBI induced loss in count and viability.
6. Baicalein maintained functionality of splenic lymphocytes and retained proliferative potential of progenitors after WBI exposure.
7. Baicalein administration enhanced abundance of HSC in bone marrow.

8. Baicalein offered ~60% protection against WBI induced mortality in mice over radiation control and exhibited a DRF of 1.153.
9. Administration of Nrf-2 inhibitor to mice abolished in vivo radio-protection offered by baicalein.

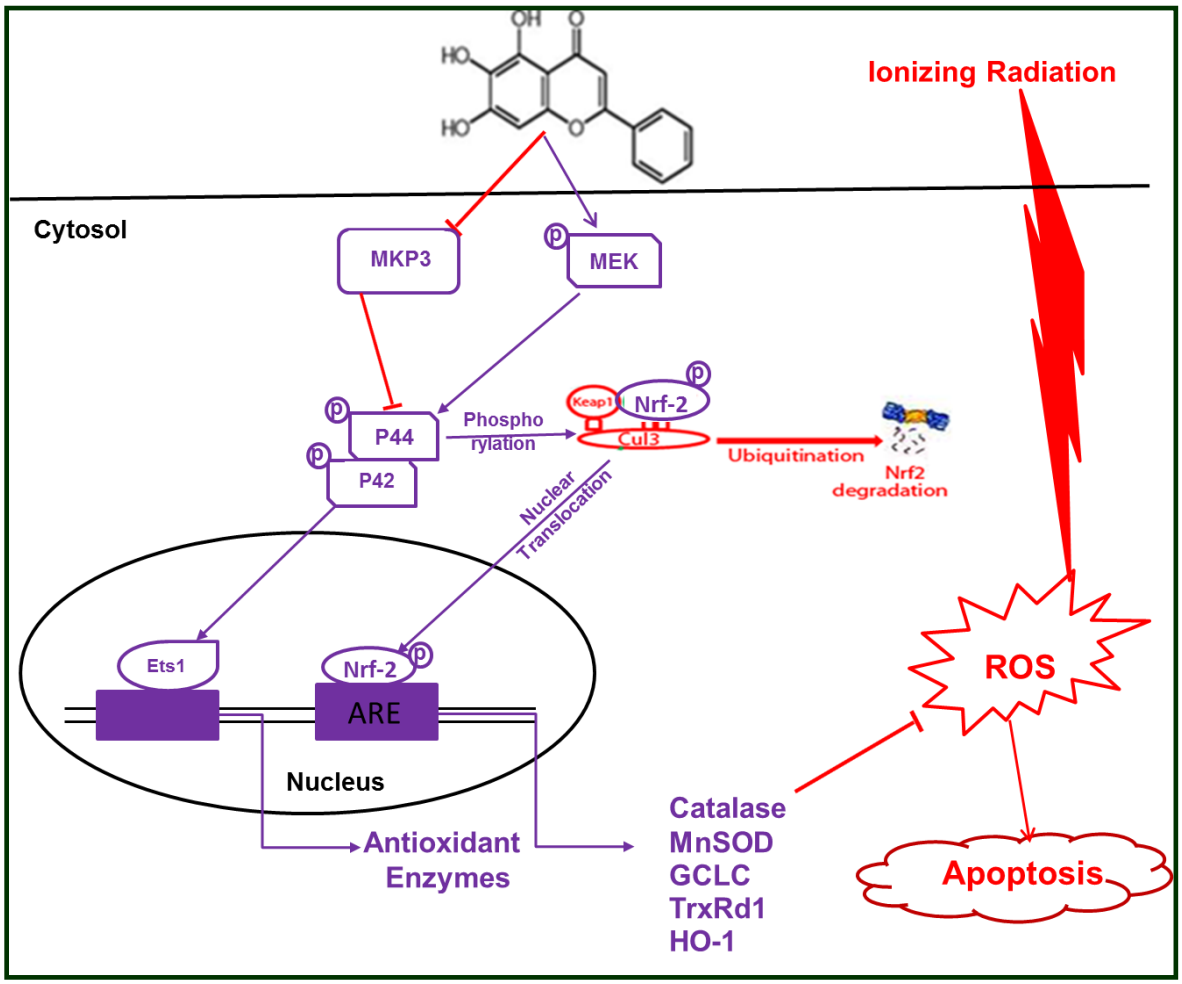


Fig. 2.25 Mechanism of radioprotection of baicalein

CHAPTER 3

IN VITRO AND IN VIVO

ANTI-INFLAMMATORY EFFECTS OF BAICALEIN

3.1. Introduction

3.1.1 Inflammation

Inflammation is a protective immune response characterized by a series of events mounted in response to harmful stimuli like physical, chemical, mechanical injury or invasion of biological agent [326]. It is a complex and dynamic process that involves interplay of cellular and secreted components. The purpose of inflammation is to eliminate the initial cause of cell injury, clear out necrotic cells and initiate tissue repair [327]. Under normal condition a well-regulated inflammation resolves after removal of harmful stimuli and recovery from the tissue injury. In response to the injury, inflammatory mediators recruit cellular components like neutrophils and macrophages at the site of inflammation which engulf damaged cells or infected cells [328]. Inflammation is necessary to recover from injury or infection for removal of pathogenic agent and maintain healthy state of an individual.

Depending on the duration, inflammation is categorized into acute or chronic. Acute inflammation is a short-term process that begins to cease after removal of the injurious stimulus. It is characterized by five cardinal signs heat, pain, redness, swelling and loss of function [329]. Acute inflammation is initiated at site of injury by resident macrophages which recognize generic molecules called pathogen associated molecular patterns (PAMPs) that are broadly shared by pathogens and distinguishable from host cells (Kumar, Collins (1998) leading to release of soluble mediators of inflammation **Table 3.1** [330].

Table 3.1 Mediators of acute inflammation.

Mediator	Vasodilation	Immediate	Sustained	Chemotaxis	Opsonin	Pain
Histamine	+	+++	–	–	–	–
Serotonin (5–HT)	+	+	–	–	–	–
Bradykinin	+	+	–	–	–	++
Complement 3a	–	+	–	–	–	–
Complement 3b	–	–	–	–	+++	–
Complement 5a	–	+	–	+++	–	–
Prostaglandins	+++	+	+?	–	–	
Leukotrienes	–	+++	+?	+++	–	–
Lysosomal proteases	–	–	++	–	–	–
Oxygen radicals	–	–	++	–	–	–

Persistent acute inflammation due to non-degradable antigens, viral infection, persistent foreign bodies or autoimmune reaction leads to chronic inflammation [329]. One of the most important attributes of inflammation is that inflammatory response must be acutely terminated. Failing which unresolved or improperly resolved inflammation can cause bystander damage to surrounding tissue. **Table 3.2** lists the differences between acute and chronic inflammation.

Although, inflammation is tightly regulated, often dysregulated and chronic inflammation is responsible for several disorders including diabetes, arthritis, atherosclerosis, neurodegenerative disorders, cancer, and cardiac problems [329]. A large number of diverse proteins are involved in

the process of inflammation and mutation in any of these may cause malfunctioning or impaired expression. It is considered as an important risk factor in cancer initiation and progression **Fig. 3.1** [224]. During inflammation, NFκB is activated in different immune cells and is responsible for release of these pro-inflammatory mediators and cytokines, activation and migration of cells and induction of oxidative stress [233] [331].

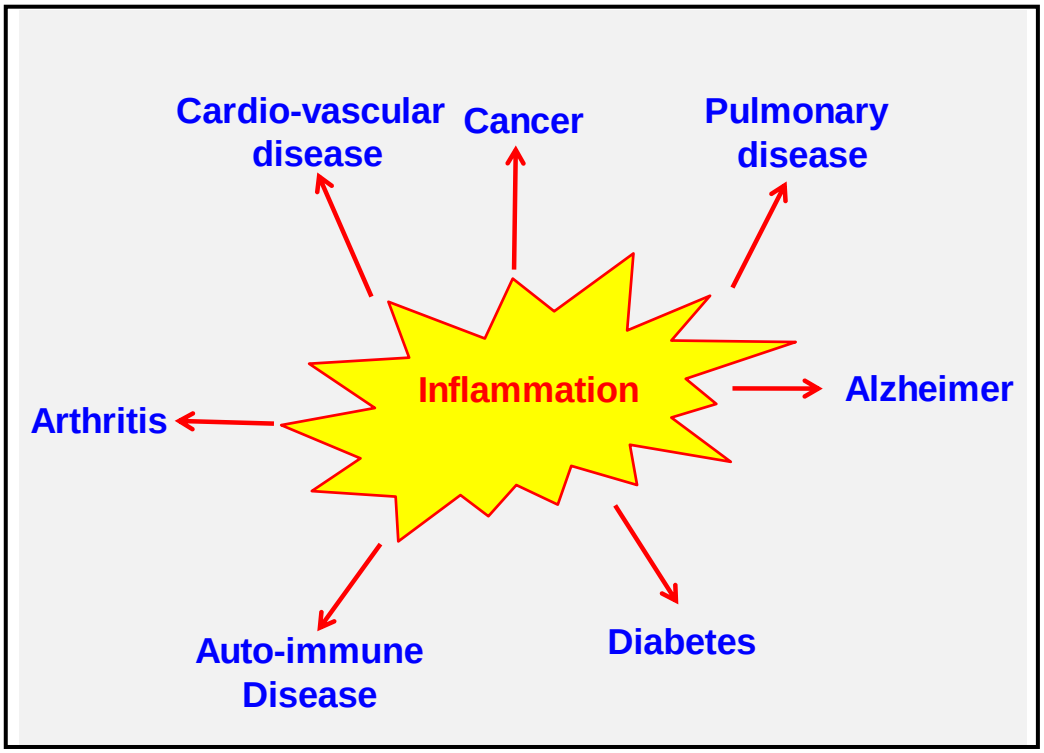


Fig. 3.1 Inflammation

Table 3.2 Differences between Acute and Chronic Inflammation

	Acute	Chronic
Duration	Short (days)	Long (weeks to months)
Onset	Acute	Insidious
Specificity	Nonspecific	Specific (where immune response is activated)
Inflammatory cells	Neutrophils, macrophages	Lymphocytes, plasma cells, macrophages, fibroblasts
Vascular changes	Active vasodilation, increased permeability	New vessel formation (granulation tissue)
Fluid exudation and edema	Present	Absent
Cardinal clinical signs	Present	Absent
Tissue necrosis	Generally absent If present (Suppurative and necrotizing inflammation)	Continuous
Fibrosis (collagen deposition)	Present	Absent
Operative host responses	Plasma factors: complement, immunoglobulins, properdin, etc; neutrophils, nonimmune phagocytosis	Immune response, phagocytosis, repair
Systemic manifestations	Fever, often high	Low–grade fever, weight loss, anemia
Changes in peripheral blood	Neutrophil leukocytosis; lymphocytosis (in viral infections)	Frequently none; variable leukocyte changes, increased plasma immunoglobulin

3.1.2 NF-κB

The transcription factor NF-κB was discovered in 1986 as a nuclear factor that binds to the enhancer element of the immunoglobulin kappa light-chain of activated B cells hence called as NF-κB [332]. In total, five members of this transcription factor family have been identified. All five members of this protein family form homo or heterodimers and share some structural features including a Rel homology domain (RHD), which is essential for dimerization as well as binding to cognate DNA elements designated as p65 (RelA), RelB, c-Rel, NF-κB1 and NF-κB2 [333]. RelA, c-Rel, and RelB each possess a transcriptional activation domain (TAD) **Fig. 3.2.** Out of 15 theoretically possible NF-κB dimers, some function as transcriptional activators (ubiquitous RelA:p50 heterodimer), but others (p50:p50 homodimer) need to recruit specific co-activator proteins, whereas some dimers are not known to bind DNA **Table 3.3**.

These dimers are bound to inhibitory molecules of the IκB family of proteins **Fig. 3.3**. Binding of NF-κB dimers to IκB molecules does not only prevent binding to DNA, but also shifts the steady-state localization of the complex into the cytosol [233]. p105 and p100 are the precursors of p50 and p52 containing ankyrin repeats which are cleaved during maturation [334]. In contrast to the other members of the NF-κB family these two proteins do not contain a trans-activation domain [335]. When p50 or p52 are bound to a member containing a transactivation domain, such as p65 or Rel B, they constitute a transcriptional activator. Different NF-κB dimers have differential preferences for variations of the DNA-binding sequence [336].

Table 3.3 Potential NF-κB dimers [337]

	p50	p52	Rel B	c Rel	Rel A
Rel A	++	++	--	++	++
c Rel	++	++	--	++	
Rel B	++	++	--		
p52	+-	+-			
p50	+-				

++ Binds DNA and activates transcription

+- Binds DNA but do not activate transcription

-- Do not bind DNA

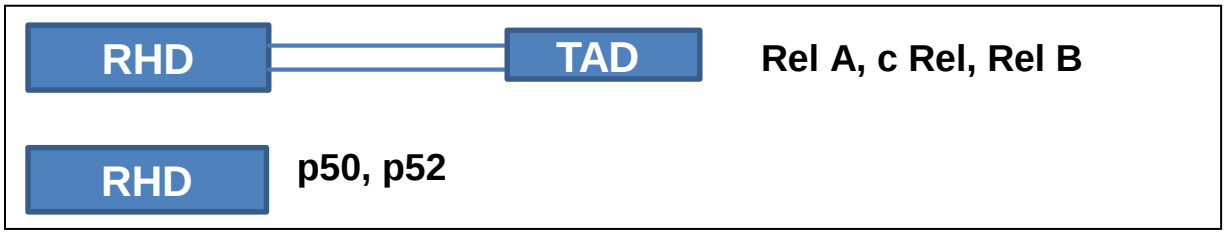


Fig. 3.2 NF-κB family members [337]

Table 3.4 IκB protein family members and signals that induce degradation [337]

Inhibitory Protein	Stimuli that induce degradation
IκBα, IκBβ, IκBε	TNF, LPS, IL-1, TCR, BCR
p105	LPS (B Cells)
p100	LTβ, CD40, BAFF, RANKL, OX40

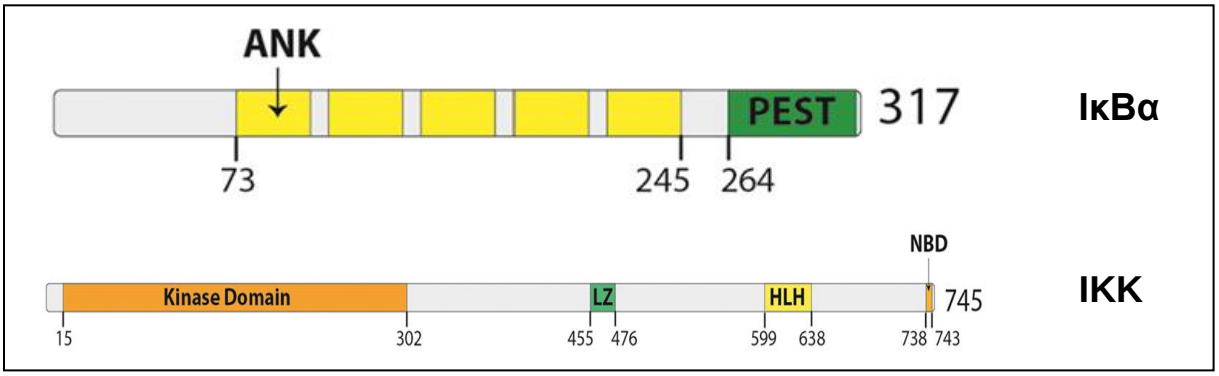


Fig. 3.3 IκB Kinase complex members Adapted from [233]

3.1.3 NF-kB upstream signaling

Activation of NF-κB occurs by release of the inhibitory IκB subunit following its phosphorylation, ubiquitination and degradation or by cleavage of the inhibitory ankyrin repeat domains of p100 and p105 by various stimuli **Table 3.4.** NF-kB can be activated by i) canonical or classical pathway ii) alternative or non-canonical pathway or iii) atypical pathway [338, 339] [340]. Each pathway is triggered by specific stimulus and upstream signaling includes release of NF-kB from its inhibitory protein. Endogenous inflammatory stimuli (e.g., cytokines, TNFα, IL−1β) or pathogen-derived substances (e.g., lipopolysaccharide (LPS) or CpG activate the ubiquitous RelA:p50 dimer through the 'canonical' or 'classical' NF-κB pathway [337]. Engagement of the TNF receptor (TNFR), interleukin−1β receptor (IL−1βR), or TLR causes phosphorylation-dependent activation of the IκB kinase (IKK) complex, composed of the two catalytic subunits, IKKα and IKKβ and the scaffolding protein, IKKγ /NF-κB essential modulator (NEMO). Once activated, the canonical IKK complex phosphorylates IκBα. Degradation of IκBα releases RelA:p50, allowing it to localize to the nucleus to bind DNA and activate gene expression [341] **Fig. 3.4.**

A group of non-inflammatory signals have been shown to activate NF-κB through the (non-canonical) NF-κB signaling pathway [342]. These developmental signals of the TNF-receptor superfamily, such as B-cell activation factor (BAFF) critical for B-cell survival, lymphotoxin β (LTβ) involved in lymph node development, and receptor activator of NF-κB ligand (RANKL) essential for osteoclast differentiation, have been shown to activate NF-κB at a low level for a sustained period of hours to days [340]. The non-canonical pathway is not transduced by a NEMO/IKKβ containing kinase complex, but rather by an IKKα containing kinase complex, whose activation requires NF-κB-inducing kinase (NIK). In addition to the non-canonical IKK-

dependent NF-κB degradation, these signals may also, in certain cellular conditions and contexts, activate the canonical IKK dependent NF-κB activation pathway [340] **Fig. 3.4.**

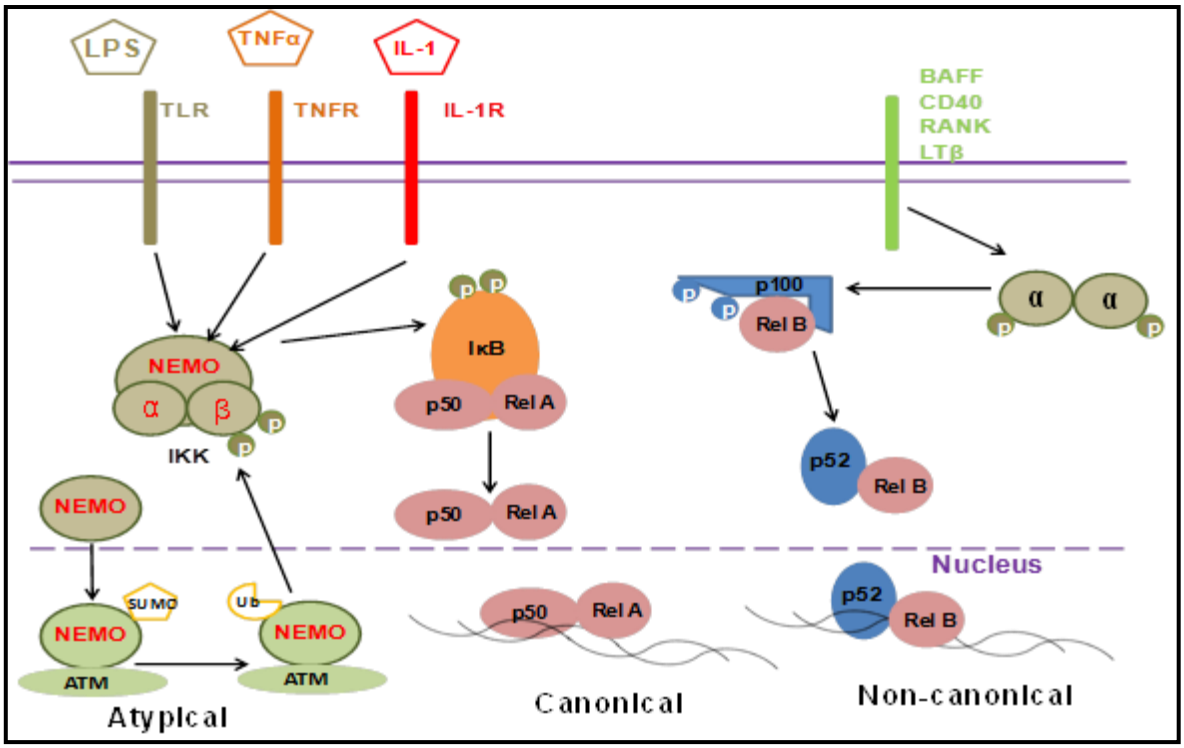


Fig. 3.4 NF-κB signaling pathways [233]

Further, DNA damage induced by irradiation or chemotherapeutic drugs can induce IKK activation. Until recently, it was unclear how a nuclear signal could relay back to the inhibited NF-κB in the cytoplasm to trigger its activation. It was found that DNA damage not only initiates the activation of the nuclear kinase ataxia telangiectasia mutated (ATM), the primary regulator of the tumor suppressor and transcription factor p53, but also initiates the sumoylation of NEMO by the sumo ligase PIASy, promoting the nuclear localization of NEMO [343, 344]. Activated ATM is required for NF-κB activation in response to DNA damage through IKK activation. Wu et al. showed that nuclear sumoylated NEMO associates with and is phosphorylated by the activated ATM, promoting mono-ubiquitination of NEMO, which triggers its export to the cytoplasm. The cytoplasmic ATM–NEMO complex associates with the IKK complex, facilitating ATM-

dependent activation of the canonical IKK complex, leading to IκBα degradation and NF-κB activation **Fig. 3.4** [345].

3.1.4 NF-κB inhibition and inflammation

Persistent and deregulated NF-κB activation with sustained transcriptional activity is implicated in several inflammatory disorders [346]. NF-κB regulates the expression of a large number of genes involved in inflammation. It plays important roles in initiation as well as resolution phase of inflammation.

Chronic inflammatory diseases associated with NF-kB activation [337] [347]

i. Rheumatoid arthritis
ii. Atherosclerosis
iii. Chronic obstructive pulmonary disease (COPD)
iv. Asthma
v. Multiple sclerosis
vi. Inflammatory bowel disease (IBD)
vii. Ulcerative colitis

Given the central role of NF-κB in inflammation, there is a great interest for pharmacological intervention of this pathway using specific inhibitors. Identification of specific and potent inhibitors of NF-κB has been the goal of many researchers and pharmaceutical companies [348, 349]. Finer understanding of the molecular cascade of signaling events has highlighted several steps for specific inhibition of NF-κB activity.

Inhibition of NF-κB can be achieved by one of the following three mechanisms: i) blockage of the stimulating signal resulting in complete abrogation of the signal's effect ii) interference with a cytoplasmic step in the NF-κB activation pathway by blockage of a specific component of the cascade or iii) blockage of the NF-κB nuclear activity [350].

The inhibitors can interfere with one of the following steps: i) signaling upstream of IKK ii) directly at IKK complex iii) ubiquitination or proteasomal degradation of IκBα iv) nuclear translocation of NF-κB v) NF-κB DNA binding or vi) NF-κB gene transactivation [351].

Among these molecules that specifically interfere with DNA binding or trans-activation function are discussed in detail here.

3.1.5 Inhibition of NF-κB DNA binding

Inhibition of NF-κB DNA binding can be achieved by multiple ways including covalent modification of cysteine residues on p65. Many target molecules directly interact with Cys-38 in DNA binding loop of p65 (Rel A). This interaction disrupts ability of p65 to bind to DNA. Another approach includes use of kB-site decoy oligonucleotides that can compete with NF-κB and specifically block its DNA binding [352-354]. Generally these oligonucleotides have modifications to improve their stability and affinity for NF-kB [355, 356]. DNA binding of transcription factors is a redox dependent process and for NF-κB DNA binding Cys-62 in p65 has to be maintained in a reduced state. This reduction is carried out by Trx 1 and Ref 1 proteins inside the nucleus. Inhibition of Trx or TrxR leads to retaining of cysteine residues of p65 in oxidized state thereby inactivating its ability to bind with target DNA [357, 358].

3.1.6 Inhibitors of NF-κB trans-activation

Different NF-κB dimers target different promoters/enhancers in a tissue specific, stimulus-dependent and promoter-specific manner. Covalent modification of NF-κB subunit in terms of serine phosphorylation, acetylation or histone modification in the target locus in the genome can influence gene trans-activation potential. Agents that specifically block RelA phosphorylation have been shown to block tans-activation [359] [360]. LY294002 is a PI3Kinase inhibitor and it does not inhibit IκBα degradation or NF-κB DNA binding, but it blocks IL-1-stimulated phosphorylation of NF-κB, especially the Rel A subunit [361] [362]. .Recently, a relatively new mechanism has been described

wherein NF-κB trans-activation can be blocked through inhibition of TrxR [358]. Earlier, Hirota et. al, have shown that intra-nuclear over-expression of Trx leads to increased NF-κB luciferase activity whereas specific inhibition of Trx by use of shRNA approach blocked NF-κB dependent gene transcription [363].

3.1.7 NF-kB inhibitor drugs as anti-inflammatory agents

Several nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin (sodium salicylate), ibuprofen, sulindac and indomethacin can inhibit activation of NF-κB in cultured cells [364] [365] [366]. Aspirin acts pharmacologically via inhibition of prostaglandin synthesis, however, at higher concentrations it has been shown to block NF-κB by directly inhibiting IKKβ [364]. Glucocorticoids such as dexametasone, prednisone and methylprednisolone are used for their anti-inflammatory properties and also to prevent allograft rejection [367]. Their physiological effects appear to be at least partially mediated through inhibition of NF-κB.

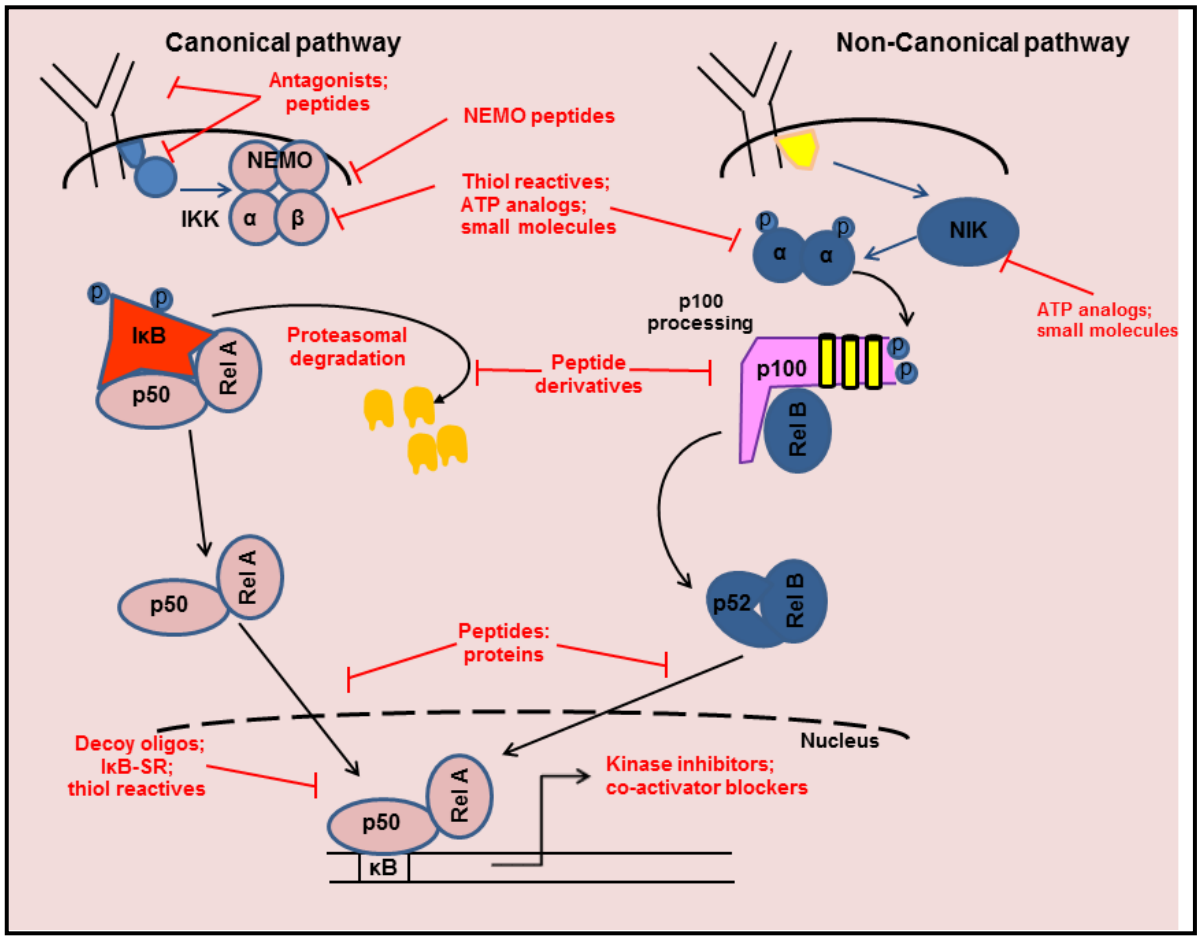


Fig. 3.5 Sites of intervention of NF-kB inhibitors *Adapted from[351]*

Several well-known immuno-suppressants target NF-κB [368]. Cyclosporin A (CsA) inhibits B-cell and T-cell proliferation by blocking the activity of calcineurin, a calcium and calmodulin-dependent serine/threonine phosphatase [369]. Several reports have shown that CsA can also inhibit NF-κB induction by blocking LPS-induced IκB degradation and p105 processing in vivo [370]. PG490 (pure triptolide, a diterpenetriepoxide) is an immunosuppressant molecule that can synergize with CsA to inhibit transcriptional activation by NF-κB [371]. **Table 3.5** lists major targets for developing anti-inflammatory drugs.

Table 3.5 Major targets for developing anti-inflammatory drugs [372]

Enzymes	COX-1, COX-2, IMP dehydrogenase, PGE2, leukotrienes, iNOS, phospholipase, lipoxygenase, matrix metalloproteinase
Cytokines and cytokine receptors	TNF-α, TNF-RII, IL-1β, IL-1RA, IL-2, IL-2R, interferon, G-protein-coupled receptors, histamine 1 and cysteinyl leukotriene 1,
Adhesion molecules	Leukocyte function associated antigen 1 (LFA 1), CD11a, CD2, CTLA4, VLA4
Proteins	NF-kB, MAPKs,

3.1.8 Anti-inflammatory effects of natural products

Long term use of anti-inflammatory drugs by the patients has been shown to cause several side effects. Many of the inflammatory diseases are becoming common throughout the world owing to changing life styles. Corticosteroids have long been used for the management of rheumatoid arthritis, but they suffer from some serious side effects, such as Cushing's habitus (appearance with rounded face, narrow mouth, supraclavicular hump, obesity of the trunk with relatively thin limbs), hypertension, hyperglycemia, muscular weakness, increased susceptibility to infection, osteoporosis, glaucoma, psychiatric disturbances, growth arrest, etc. [373]. Different chemical classes such as alkaloids, steroids, terpenoids, polyphenolics, phenylpropanoids, fatty acids and lipids, and various miscellaneous compounds have been shown to possess anti-inflammatory properties [373]. Among them, flavonoids constitute a class of compounds that are found in human diet and have been shown to possess promising anti-inflammatory effects.

3.1.9 Anti-inflammatory effects of Baicalein

Baicalein is a 5,6,7 trihydroxy flavone isolated from dried roots of *Scutellaria baicalensis* Baicalein belongs to class of polyphenolic compounds called as flavonoids which are ubiquitously present in plants and they also constitute components of functional foods **Table 3.6**.

Table 3.6 Main groups of flavonoids and food sources [374]

Flavonols	Quercetin, Kaempferol, Myricetin, Isorhamnetin etc	Onion, tomato, cherry, apple, green & black tea, grapes
Flavones	Chrysin, Apigenin, Luteolin, Tricetin, Disometin	Parsley, capsicum pepper
Flavonones	Naringenin, Hesperidin, Dihydroquercetin	Orange juice, grape fruit, lemon peel & juice
Flavonols (Catechins)	Silymarin, Silibinin, Taxifolin, (+)- Catechin, (-) Epicatechin	Cocoa, chocolates, cocoa beverages, beans, cherry, grapes, red wine, cider, blackberry
Isoflavonols	Genistein, Glycitein, Daidzein,	Soy cheese, soy flour, soy bean,
Anthocyanins	Cyanidin, Malvidin, Peonidin, Petunidin	Blue berry, black grapes, cherry, rhubarb, strawberry, red wine

It has been reported to possess anti-inflammatory property owing to its ability to inhibit lipoxygenase [246]. Baicalein has also been reported to attenuate endothelium intimal hyperplasia by inhibiting inflammatory signaling molecules including extracellular signal-regulated kinase, protein kinase B or Akt and NF-κB in vascular smooth muscle cells [375]. Baicalein attenuated the radiation-induced inflammatory process in mouse kidney by modulation of NF-κB and Forkhead family of transcription factors [254].

Table 3.7 Anti-inflammatory effects of baicalein in literature

Model	Effect	Mechanism
Mastitis (LPS induced) in mice	Suppressed MPO, TNFα, IL-1β	Blocked degradation of IκBα [376]
Acute lung injury (LPS induced) in mice	Increased lung weight, improved lung histology, reduced pro-inflammatory cytokines	Blocked degradation of IκBα[377]
Renal fibrosis in mice	Decreased expression of TNFα, IL-1β, MCP1	Inhibition of NF-κB and MAPK[378]
Liver ischemia/reperfusion injury in mice	Decreased aminotransferase levels, leukocyte infiltration and histopathologic abnormalities	Inhibition of NF-κB and pro-inflammatory cytokines[379]
Angiotensin II-induced cardiac remodeling	Restored cardiac contractile function, decreased malondialdehyde production	Inhibition of AKT/mTOR pathway[378]
Con A induced hepatitis	Suppressed serum levels of TNFα, IL-1β and abnormalities in liver histopathology	Induction of apoptosis in activated liver mononuclear cells[380]
LPS stimulated RAW 264.7 cells	Inhibited iNOS, COX-2 and TNFα mRNA levels	Suppressed estrogen receptor, JAK/STAT signaling, Inhibition of ROS[381]
6-hydroxydopamine induced neurotoxicity	Inhibited cell death	Activated Nrf-2/ARE pathway[248] [382]
Diabetic Retinopathy	Inhibited microglial activation, reduced VEGF expression	Suppressed IL-18, IL-1β and TNF-α[383]
TNF-induced inflammation inhuman mast cells	Significantly inhibited IL-6, IL-8, and MCP-1 in TNF-alpha-	Inhibition of IκBα phosphorylation[384]
Dextran sodium sulfate induced colitis	Prevention of body weight loss, blood haemoglobin content and rectal bleeding	Suppression of IFN and IL-4[385]

Baicalein inhibited NF-κB mediated inflammatory responses by upregulation of the nuclear factor erythroid 2-related factor-2/heme oxygenase-1 pathway [386]. In the murine macrophage RAW 264.7 cell line, baicalein inhibited LPS-induced inflammation via upregulation of estrogen receptor and inhibited NF-κB-dependent signaling[381]. Baicalein attenuated inflammatory responses by suppressing TLR4 mediated NF-κB and MAPK signaling pathways in LPS-induced mastitis in mice[377]. **Table 3.7** lists the reported anti-inflammatory effects of baicalein in different model systems and its mode of action. However, there were no reports describing anti-inflammatory activity of baicalein on T cell responses.

Although plethora of reports describing anti-inflammatory effects of baicalein are present in the literature, a systematic study on effect of baicalein on T cell responses has not been carried out. Further, in connection with our primary interest of investigating radioprotective properties of baicalein in mouse model, it becomes imperative to study influence of baicalein on inflammatory responses exhibited by T cells. Baicalein is a well-known anti-oxidant and thereby it may be expected to inhibit NF-κB signaling considering that antioxidants are known to suppress this pathway. Contrary to this, our findings from chapter 2 have demonstrated that baicalein treatment induced nuclear translocation and DNA binding of pro-inflammatory transcription factor NF-κB in BM-MNC **Fig. 2.22**. But baicalein suppressed mitogen induced T cell proliferation and cytokine secretion **Fig. 2.18**. This raised an important question that how an agent can activate NF-κB and still suppress its dependent gene expression. Subsequent to nuclear translocation of NF-κB, reducing environment is required for its DNA binding. Critical cysteine residues in p65 need to be reduced before it can bind to DNA [357]. This process is facilitated by Trx with the help of Ref-1. Trx is a small dithiol disulfide oxido-reductase which is reduced to its

native state by TrxR with the help of NADPH. It has been reported that Trx system regulates redox state of different transcription factors [387, 388] [389].

3.1.10 NF-κB and thioredoxin system

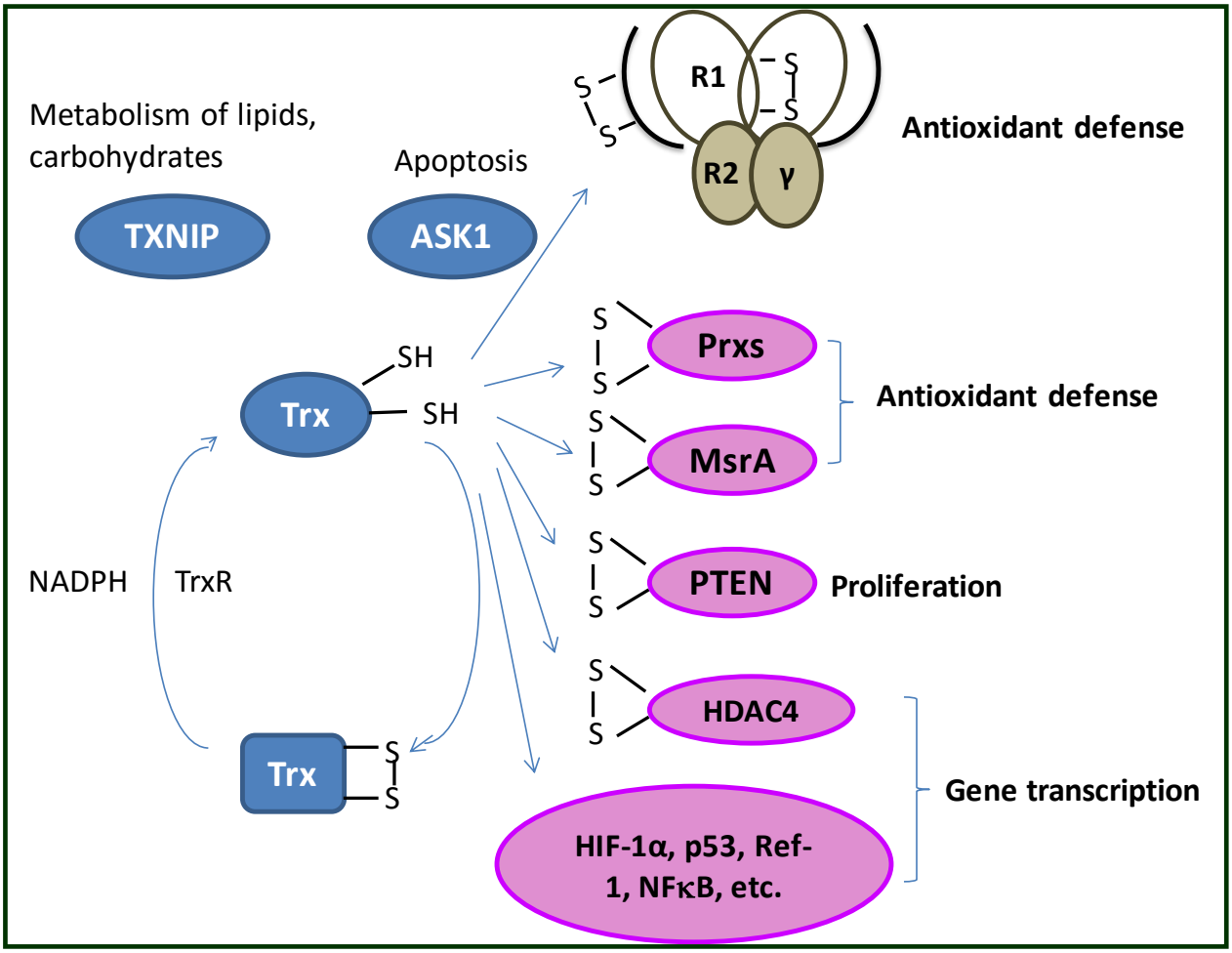


Fig. 3.6 Thioredoxin system as a general disulfide reductase *[389]*

Trx is a 12 kDa dithiol protein and it is conserved from bacteria to humans as it maintains the protein dithiol/disulfide homeostasis [389] [389]. Along with Trx reductase (TrxR), Trx can provide electrons from nicotinamide adenine dinucleotide phosphate (NADPH) to a very large number of critical cellular proteins during a wide range of cellular responses **Fig. 3.6.**

Both cytosolic Trx1 and mitochondrial Trx2 in mammalian cells contain an active site Trp-Cys-Gly-Pro-Cys- in a Trx fold structure. Human Trx1 with 105 amino acid residues

has three structural Cys residues at positions 62, 69 and 73, apart from Cys32 and Cys35 in the active site. Cys62 and Cys69 in Trx-S2 can form a second disulfide bond under oxidative stress conditions **Fig. 3.7**.

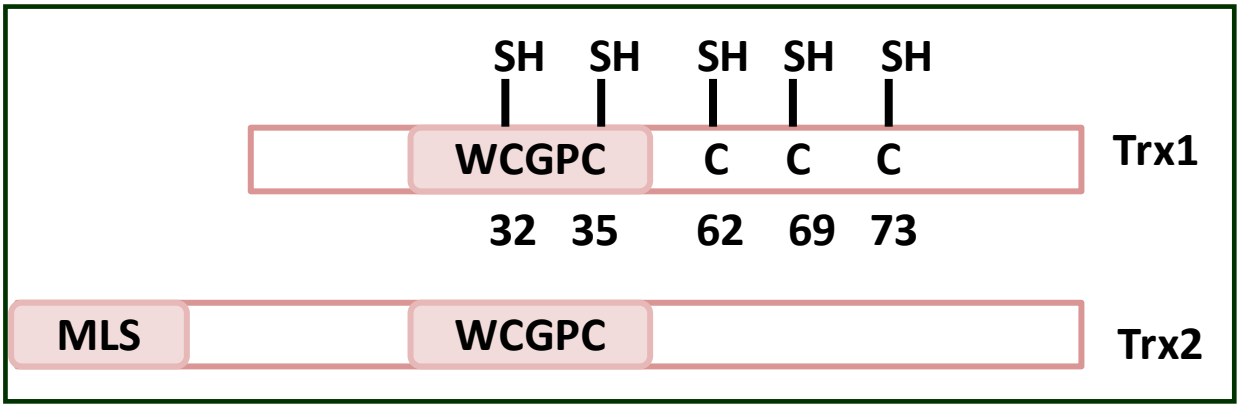


Fig. 3.7 Structure of thioredoxin *[389]*

Trx1 is a central redox regulator and facilitates the activation of many transcription factors involved in cell growth, apoptosis, and inflammation such as NF-κB, activator protein 1 (AP-1), p53, hypoxia-inducible factor 1, and redox factor 1 (Ref-1) [390]. The redox status of some Cys residues in the DNA binding site of the transcription factors, for example, Cys62 of NF-κB, is critical for the DNA binding. Under oxidative stress conditions, Trx1 is translocated from the cytosol to the nucleus [391]. The presence of Trx can maintain the cysteine in its reduced state and hence promote the DNA binding activity of NF-κB. Moreover, Ref-1 can also translocate from the cytosol to the nucleus and it interacts with Trx1 physically. The association of Ref-1 with Trx1 can increase the DNA binding activity of transcription factors [392].

Apart from this, it has also been shown that, Cys-246 and Cys-266 residues of MKK4 are reduced by Trx 1 and MKK4 activates NF-κB for its binding to Mn-SOD promoter [393]. Interestingly, Heilman et al. have shown that NF-κB-mediated gene expression was markedly inhibited in cells lacking TrxR activity suggesting that the trans-activation potential of NF-κB may depend on TrxR activity [358]. Further, selective oxidative stress in the nucleus was shown

to hamper NF-κB reporter activity [394] whereas targeted nuclear over-expression of Trx1 resulted in increased NF-κB luciferase activity [363].

3.1.11 Baicalein and thioredoxin system

Recent study carried out using flavonoids of different class has identified their potential to inhibit TrxR in cell free system [395]. Since our findings revealed that baicalein did not interfere with any of the upstream events in NF-κB signaling but still suppressed its dependent gene expression, the possibility of Trx inhibition as a probable mechanism for its anti-inflammatory effects via suppression of trans-activation functions of NF-κB by baicalein was examined.

3.1.12 Scope of the present study

In the present study, murine splenic lymphocytes are used as model system to investigate anti-inflammatory effects of baicalein. Effect of baicalein on Con A as well as anti-CD3/CD28 mAb induced T cell activation, proliferation and cytokine secretion is studied and the effect of baicalein on mitogen induced NF-κB activation in T cells was investigated. Effect on NF-κB dependent gene expression is evaluated. Molecular mechanism of action of baicalein in suppressing NF-κB dependent signaling is demonstrated. Finally, in vivo and ex vivo anti-inflammatory potential of baicalein is evaluated using graft versus host disease and homeostasis driven proliferation models.

3.2. Materials and methods

3.2.1 Chemicals

Baicalein, sodium selenite (Na_2SeO_3 abbreviated as NaSe), insulin, NADPH, 1,4-dithiobisnitrobenzoic acid (DTNB) and 1-chloro, 2,4-dinitrobenzene (CDNB) were purchased from Sigma Chemical Co. (MO, USA). RPMI 1640, fetal bovine serum (FBS) was obtained

from HiMedia (Mumbai, India). Hoechst 33342, Carboxy fluoresceindiacetate succinimidyl ester (CFSE) was procured from Molecular Probes (NY, USA). ELISA sets for detection of cytokines (IL-2, IL-4, IL-6, and IFN-γ), fluorochrome labelled antibodies against CD25, CD69 and CD54 were procured from BD Pharmingen (CA, USA). HiPure RNA isolation kit, cDNA synthesis kit and Light Cycler SYBR green RT-PCR kit was purchased from Roche (Basel, Switzerland). Oligonucleotide probe of NF-κB and Trx inhibitor PX12 were purchased from Santacruz Biotechnology (Dallas, Texas, USA). T4 polynucleotide kinase and kinase buffers were purchased from New England Biolabs (Ipswich, MA). Over-expression plasmid for Trx1 was purchased from Origene (Rockville MD, USA). All other chemicals used in our studies were obtained from reputed manufacturers and were of analytical grade.

3.2.2 Animal maintenance

Eight to ten weeks old BALB/C or C57BL6 male mice weighing approximately 20-25g, reared in the animal house of the Bhabha Atomic Research Centre were used. Guidelines issued by the Institutional Animal Ethics Committee of Bhabha Atomic Research Centre, Government of India, regarding maintenance and dissection of animals were strictly followed.

3.2.3 Cell line and culture

EL-4 (murine T cell lymphoma) was purchased from Health Protection Agency Culture Collections (Salisbury, UK) and cultured in RPMI containing 10% fetal bovine serum and antibiotics (100U/ml penicillin and 100μg/ml streptomycin) at 37°C in an atmosphere of 5% CO_2. Cells were maintained in exponentially growing conditions with doubling time little less than 24h and sub-cultured thrice in a week.

3.3.4 Proliferation assay

Spleen was aseptically removed from the mice, placed in a sterile petri dish containing RPMI 1640 medium. Single cell suspension of lymphocytes was prepared and stained with CFSE (5μM) as described earlier [396]. CFSE labelled lymphocytes (2 x 10^6) were treated with baicalein (5 to 25μM, 2h) or CDNB (0.5 to 2.5μM) or PX12 (0.5 to 5μM) with or without the pre-treatment with sodium selenite for 24h wherever mentioned and stimulated with Con A (5μg/ml) for 72h at 37°C with 10% FBS in a 95% air/5% CO_2 atmosphere. Vehicle treated cells served as a control. Cell proliferation was measured by dye dilution in a flow cytometer (Partec CyFlow). Cells that showed a decrease in CFSE fluorescence intensity were calculated using FlowJo software and were expressed as percent daughter cells.

3.2.5 Surface staining

Analysis of surface markers on lymphocytes was carried out by surface staining with PE or FITC labelled mAbs as described earlier [302]. Briefly, lymphocytes (3 x 10^6) were treated with baicalein (25μM, 2h) and were stimulated with Con A (5μg/ml) for 24h at 37°C in a 95% air/5% CO_2 atmosphere. Lymphocytes were stained with FITC conjugated CD25 or PE conjugated CD69 or CD54 antibodies. Cells were acquired using Partec Cyflowspace flow cytometer and analyzed using FlowJo software (TreestarInc, Ashland, USA).

3.2.6 Measurement of cytokine secretion

Lymphocytes (2 x 10^6) were treated with baicalein (5 to 25μM, 2h) with or without pre-treatment of Na_2SeO_3 (2μM, 24h) and stimulated with Con A (5μg/ml) for 24h at 37^0C. EL4 cells transfected with Trx over-expression plasmid were treated with baicalein (25μM, 2h) and stimulated with Con A (5μg/ml) for 16h. Cytokine concentration (IL-2) was measured in the culture supernatant using cytokine ELISA sets (BD Pharmingen, USA).

3.2.7 Electrophoretic Mobility Shift Assay

Murine splenic lymphocytes (10 x 10^6/ml) were treated with indicated concentrations of baicalein or PX12 or CDNB or Con A (5µg/ml) for 1h or 4h respectively. Cells were harvested and nuclear extracts were probed for EMSA by incubating with ^{32}P-end-labeled 45-mer double stranded NF-κB oligonucleotides from the human immunodeficiency virus long terminal repeat (5′-TTGTTACAA**GGGACTTTCCGCTGGGGACTTTCC**AGGGAGGCGTGG-3′; bold face indicates NF-κB binding sites. Identity of NF-κB band was confirmed by performing super-shift assay and excess mutant oligonucleotide. The dried gel was exposed to phosphorimage plate and the radioactive bands were visualized using a PhosphorImage plate scanner (Amersham Biosciences, USA).

3.2.8 Estimation of enzyme activities

Trx activity was determined by the micromethod of insulin reduction. All assay tubes contained 0.26M HEPES, pH 7.6, 10mM EDTA, 2mM NADPH, 1mM insulin and 100nM purified E. coli TrxR and cell extract in final volume of 100µl. After incubating at 37^{0}C for 20min reaction was stopped by adding 500µl stopping solution containing 0.2M Tris-HCl, 6M guanidine-HCl, 1mM DTNB and absorbance was measured at 412nm against reagent blank. Reagent blanks were included to determine the background content of SH groups in samples.

3.2.9 Induction of lymphopenia and adoptive transfer for homeostatic proliferation in vivo

Balb/c mice were exposed to 6 Gy whole-body γ-radiation at a dose of 1Gy/min in a Blood irradiator (BRIT, Vashi, India). Lymphopenia induction was confirmed by counting the number of total splenic lymphocytes 48h post-irradiation. Purified CD4+ T cells from Balb/c mice were stained with CFSE and treated with baicalein (25µM) for 2h at 37°C in 5% CO2. These cells were washed and five million cells were injected intravenously into lymphopenic syngeneic

Balb/c mice. Four mice were used in each group. Splenic lymphocytes were isolated from reconstituted mice 72h after injection and analyzed by flow cytometry to enumerate the frequency of donor cells and to quantify cell proliferation by CFSE dye dilution.

3.2.10 Graft versus host disease (GVHD)

Balb/c mice were exposed to 6Gy dose of WBI. To induce GVHD in immune-compromised Balb/c mice, 8 x 10^6 splenic lymphocytes from C57BL/6 donors were injected i.v. 48 h after irradiation. Each mice in control group received vehicle treated splenic lymphocytes, whereas each mice in the treated group received splenic lymphocytes treated with 25 µM baicalein for 2h. The recipient mice were monitored daily to assess the signs of GVHD. A total of 13 mice were used per group. GVHD became evident from rapid and sustained weight loss as well as from features such as hunchback, diarrhoea, hair loss and death. Serum was separated from the blood collected on day 5 from recipient mice (Balb/c) injected with vehicle treated lymphocytes or baicalein treated lymphocytes taken from C57BL/6 mice and levels of different cytokines were estimated using sandwich ELISA.

3.2.11 ELISA & RT-PCR

Lymphocytes (10 x 10^6/ml) were treated with baicalein (25µM, 2h), stimulated with Con A (5µg/ml) and nuclear extracts were probed for levels of Trx1 by ELISA. In another experiment, cells given same treatment were harvested and processed for RNA isolation, cDNA preparation and RT-PCR for IL-2, IL-6 and β actin as described previously [295, 397]. **Table 3.8** shows primer sequences of IL-2, IL-6 and β actin.

Table 3.8 Primer sequence

Gene	Sequence
IL-2	Forward: 5'TGAGCAGGATGGAGAATTACAGG3' Reverse: 5'GTCCAAGTTCATCTTCTAGGCAC 3'
IL-6	Forward: 5'CTGCAAGAGACTTCCATCCAG 3' Reverse: 5'AGTGGTATAGACAGGTCTGTTGG3'
β actin	Forward: 5'GTGACGTTGACATCCGTAAAGA3' Reverse: 5'GCCGGACTCATCGTACTCC3'

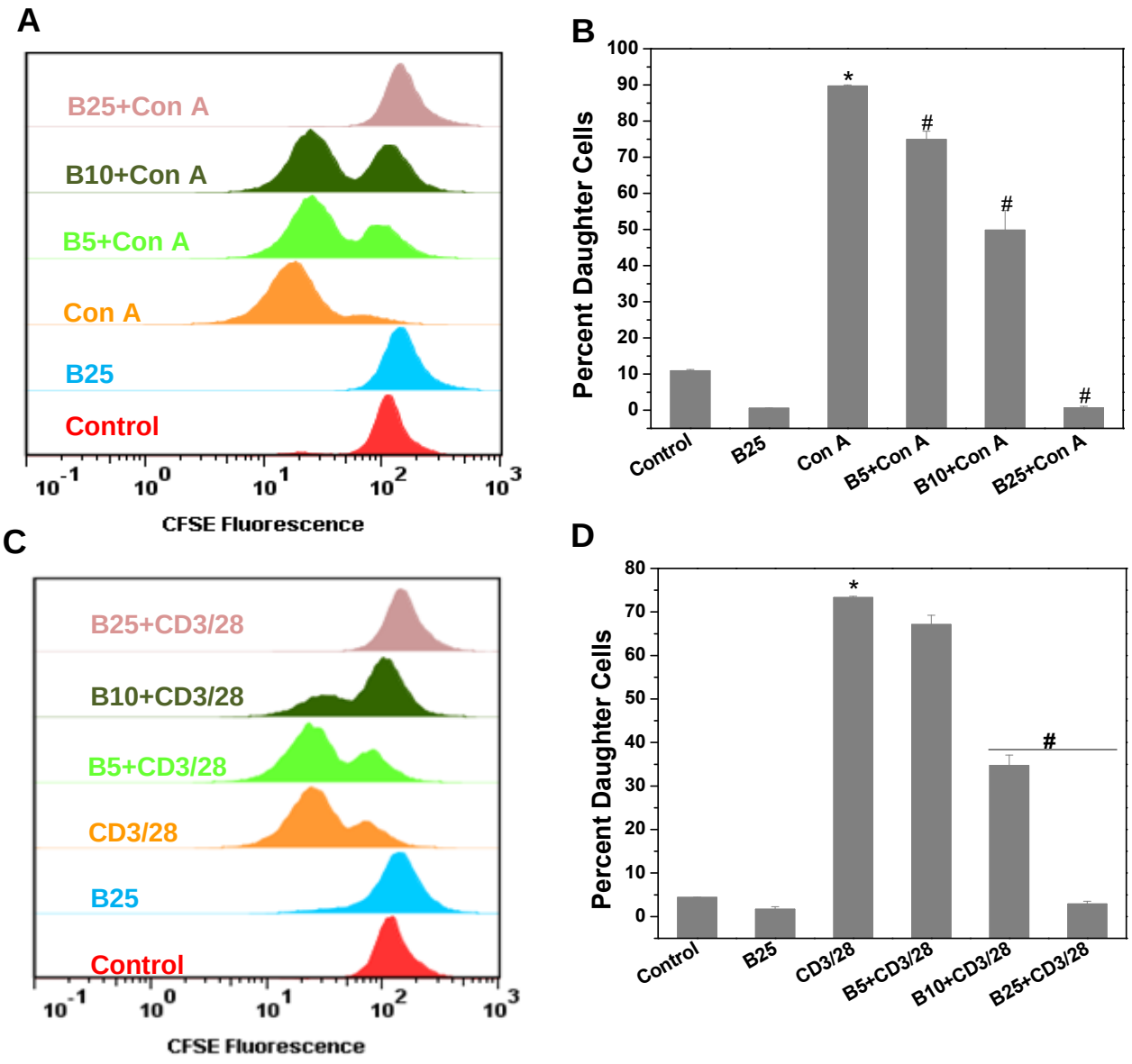
3.2.12 Statistical analysis

The statistical analysis was done using ANOVA by Microcal OriginPro 8.0 software. Data are presented as mean ± SEM. *refers to $p<0.05$, as compared to control, # refers to $p<0.05$, as compared to stimulated cells or irradiated group and $ refers to $p<0.05$, as compared to baicalein treated cells stimulated with mitogen.

3.3. Results

3.3.1 Baicalein suppressed mitogen induced proliferation and cytokine secretion in splenic lymphocytes in vitro

To investigate the anti-inflammatory potential of baicalein, its effect on proliferation of T cells was tested. CFSE stained lymphocytes were pre-treated with different concentrations of baicalein for 2h and stimulated with Con A or anti-CD3/CD28 mAb and proliferation was assessed by CFSE dye dilution using a flow cytometer. Culture supernatants were used for ELISA.



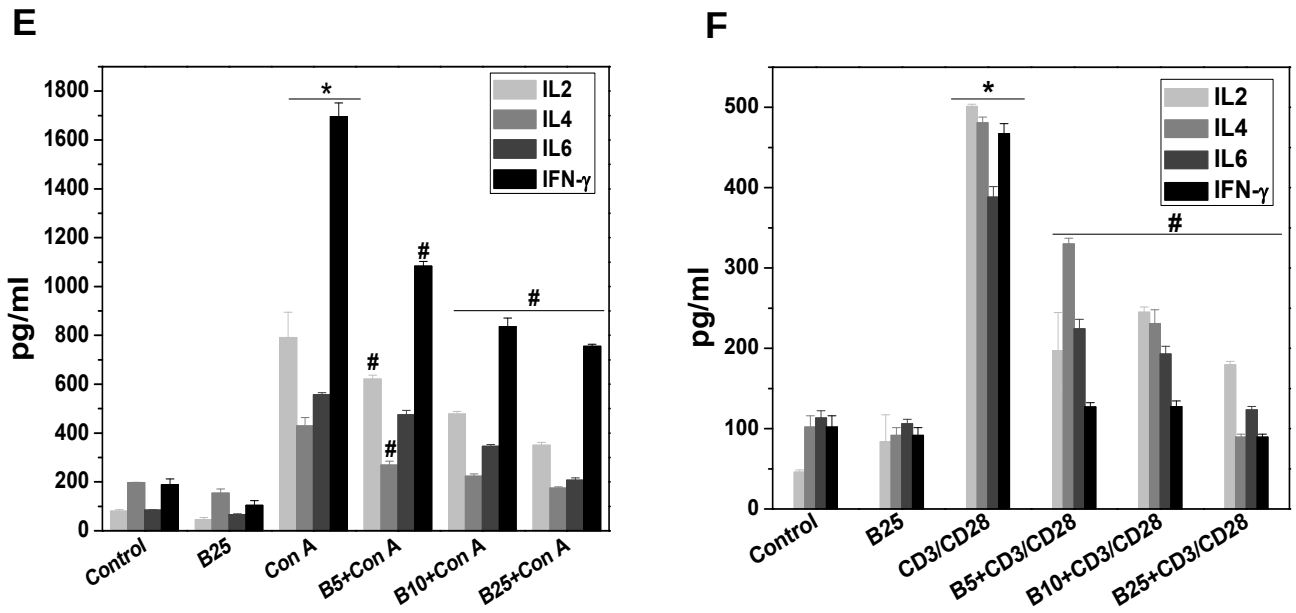


Fig. 3.8 Baicalein suppressed Con A and anti-CD3/CD28 mAb induced proliferation and cytokine secretion in a dose dependent manner *Murine splenic lymphocytes were stained with CFSE, treated with different concentrations of baicalein (5, 10 or 25μM) for 2h, simulated with Con A (5μg/ml) or anti-CD3/CD28 mAb and cultured for 72h or 24h at 37^0C. Cells were acquired on a partec Cyflow flow cytometer and representative histograms are shown (A & C). Bar graph represents percentage of daughter cells calculated by CFSE dye dilution using Flowjo software (B & D). Culture supernatants from cells cultured for 24h were harvested and analyzed for IL-2, IL-4, IL-6 and IFN-γ. Bar graph represents cytokine concentration pg/ml (E & F). *p<0.05, as compared* to control and #p<0.05 as compared to Con A treated group.

stimulatory molecules. Hence, the effect of baicalein on Con A induced T-cell activation markers (CD54, CD69 and CD25) was studied. Baicalein treatment significantly suppressed Con A

Baicalein treatment suppressed Con A as well as CD3/CD28 mAb induced proliferation (Fig. 3.8 A-D) and cytokine secretion at 25μM (Fig. 3.8E & F)

3.3.2 Baicalein treatment suppressed up-regulation of T-cell activation markers

Mitogenic stimulus leads to upregulation of membrane proteins on T cells that serve as co-stimulatory molecules. Hence, the effect of baicalein on Con A induced T-cell activation markers (CD54, CD69 and CD25) was studied. Baicalein treatment significantly suppressed Con A

induced up-regulation of CD69 (Fig. 3.9A & B) and CD25 (Fig. 3.9C & D) (IL2Rα) but not CD54 (ICAM-1) expression (Fig. 3.9E & F).

3.3.3 Baicalein suppressed NF-κB dependent gene expression via inhibition of thioredoxin

Baicalein treatment led to activation of NF-κB (Fig. 3.10A) and it did not suppress Con A induced DNA binding of NF-κB (Fig. 3.10B). Identity of NF-κB band on EMSA gel was confirmed by performing super-shift and cold competition assay (Fig. 3.10C). But, baicalein treatment led to suppression of NF-κB dependent T cell responses. DNA binding and trans-activation of transcription factor NF-κB is regulated by Trx system. Hence, effect of baicalein on thioredoxin system in terms of levels and activity of Trx in nuclear compartment after mitogenic stimulus was investigated. It was observed that baicalein treatment suppressed mitogen induced increase in Trx activity in the nuclear compartment (Fig. 3.10D) but did not affect its nuclear levels (Fig. 3.10E). Treatment with low concentration of sodium selenite is known to increase the levels and activity of TrxR [398]. Activated TrxR can help to trans-activate NF-κB dependent genes after stimulation with Con A. Hence, NF-κB dependent gene expression was studied after treating cells with sodium selenite prior to baicalein treatment followed by mitogenic stimulus. It was observed that, pre-treatment with sodium selenite significantly abrogated suppressive effects of baicalein on NF-κB dependent gene expression (Fig. 3.10F). Since, observed anti-inflammatory effect of baicalein is thought to be mediated via inhibition of Trx, EL4 (murine T cell lymphoma) cells over-expressing Trx were employed and levels of IL2 post Con A stimulation were monitored in presence and absence of baicalein. It was observed that, baicalein treatment significantly suppressed Con A induced IL2 secretion in EL4 cells. However, in Trx over-expressing EL4 cells baicalein did not inhibit Con A induced IL2 production (Fig. 3.10G).

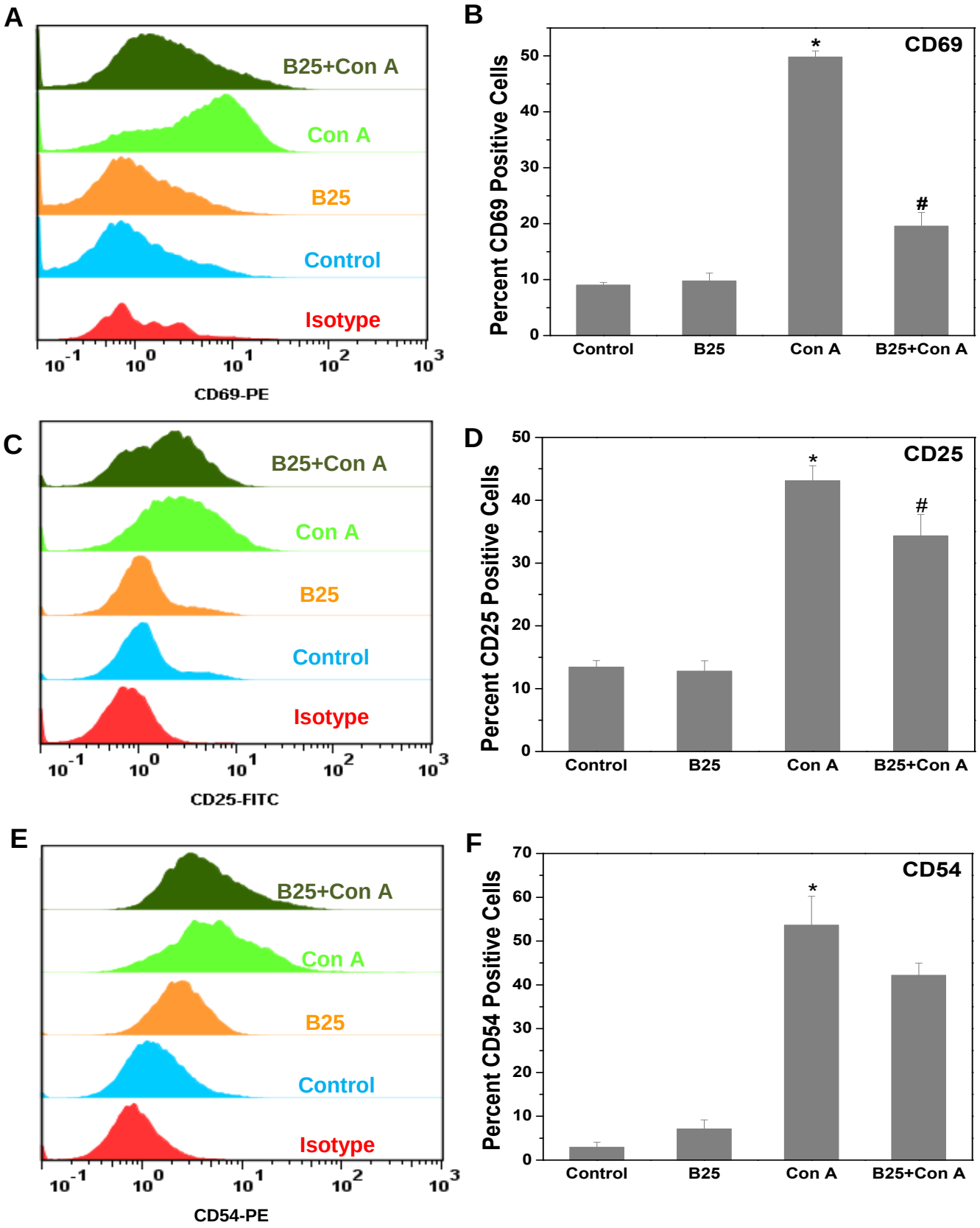
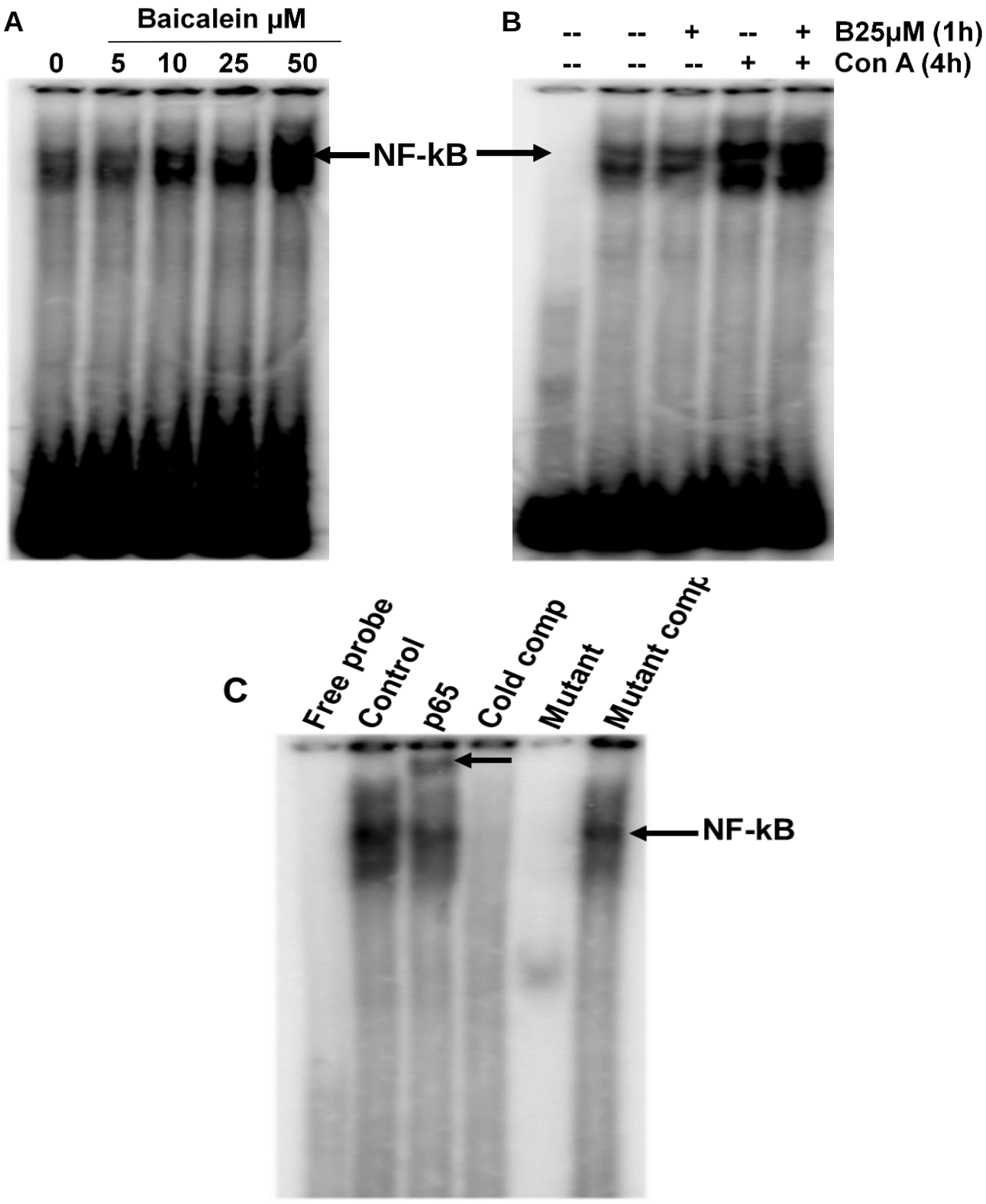


Fig. 3.9 Baicalein suppressed expression of T cell activation markers. *Lymphocytes ($2X10^6$) were treated with baicalein (25µM) and were stimulated with Con A (5µg/ml) and cultured for 24h at 37^0C. Cells were harvested, stained with anti-CD69-PE (A & B), anti-CD25-FITC (C & D) or anti-CD54-PE (E & F) antibodies. Cells were acquired on a flow cytometer and representative histograms are shown (A, C & E). Bar graph represents percent positive cells (B, D & F). *p<0.05 as compared to control.*


A
Baicalein μM
0
5
10
25
50
NF-kB
B
-- -- + -- + B25μM (1h)
-- -- -- + + Con A (4h)
C
Free probe
Control
p65
Cold comp
Mutant
Mutant comp
NF-kB

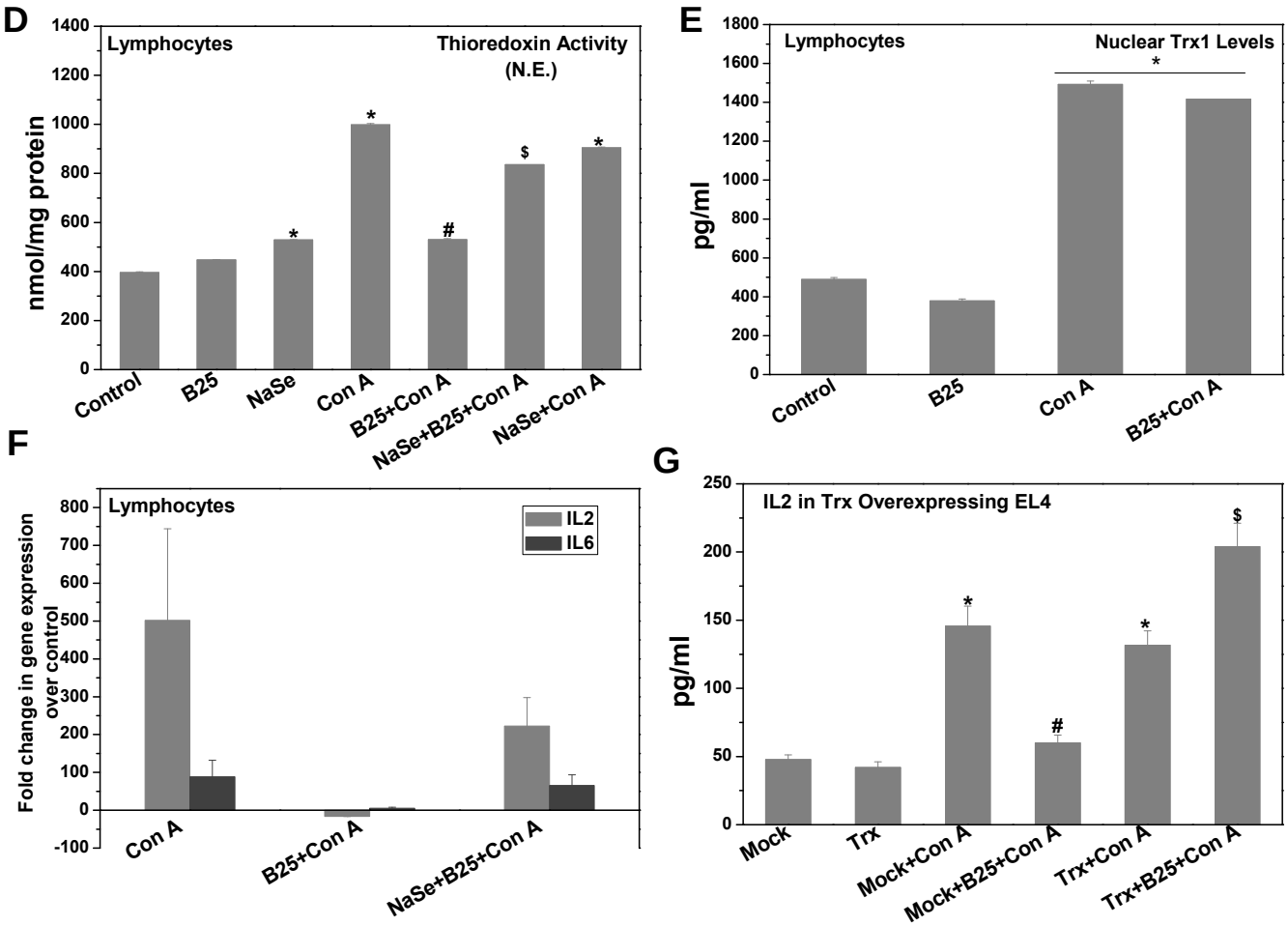


Fig. 3.10 Baicalein did not suppress Con A induced DNA binding of NF-κB but suppressed its dependent gene expression via Trx inhibition: *Nuclear translocation and DNA binding activity of NF-κB was assessed by electrophoretic mobility shift assay. Nuclear extracts from cells treated with baicalein (A) or Con A (5μg/ml, 4h) or both (baicalein 25μM, 2h and Con A 5μg/ml, 4h) (B) were analyzed for presence of NF-κB by EMSA. (C) Band was confirmed by super-shift assay. Nuclear extracts from cells treated with Na_2SeO_3 (2μM, 24h) prior to baicalein treatment (25μM, 2h) and stimulated with Con A (5μg/ml, 4h) were analyzed for Trx activity (D) or Trx 1 levels (E). Bar graph represents Trx activity nmol/mg protein (D) and Trx1 levels (pg/ml) (E). Total RNA was extracted from cells treated with Na_2SeO_3 (2μM, 24h) or baicalein (25μM, 2h) or both, stimulated with Con A (5μg/ml) and cultured for 24h at 37^0C. Relative mRNA expression was estimated by quantitative real time PCR for IL-2 and IL-6 genes. Bar graph represents fold change in gene expression over control (F). EL4 cells were transfected with Trx over-expression plasmid and 24h post transfection cells were treated with baicalein or vehicle for 2h, stimulated with Con A (5μg/ml) and cultured for 16h at 37^0C. Culture supernatants were analyzed for IL-2 levels. Bar graph represents IL-2 concentration (pg/ml) (G). *p<0.05, as compared to control, #p<0.05, as compared to Con A and $p<0.05, as compared to baicalein treated and Con A stimulated cells.*

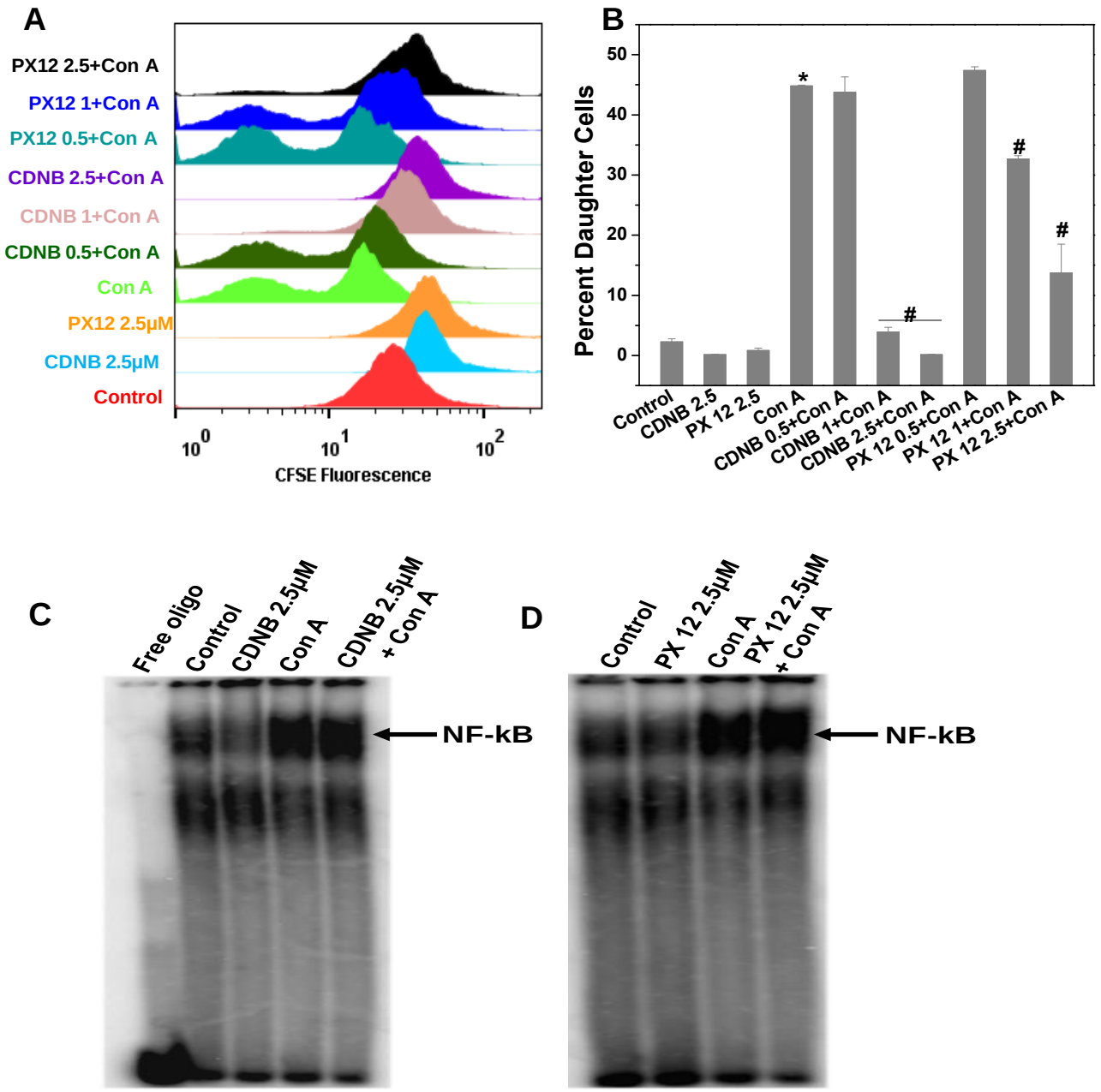


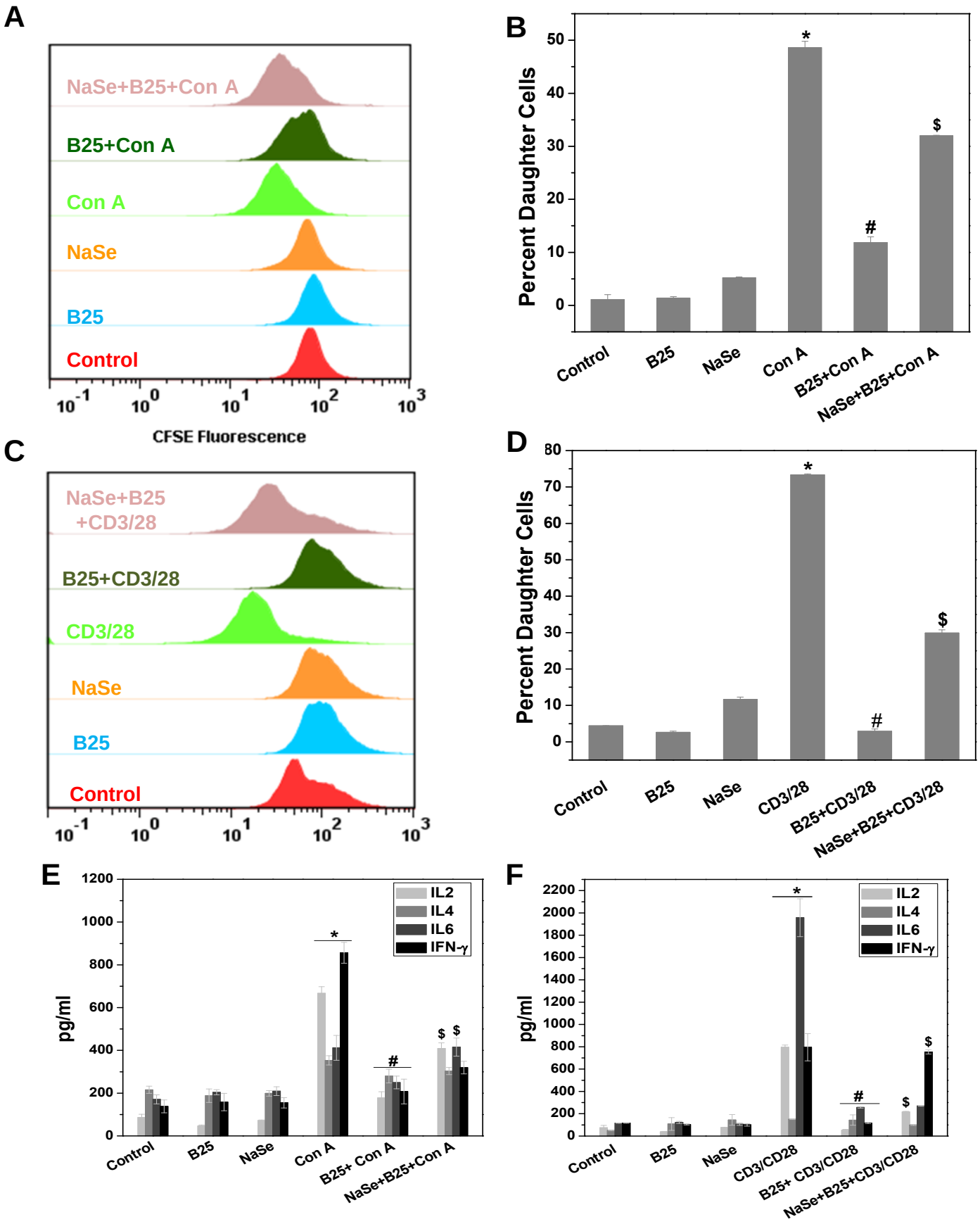
Fig. 3.11 Inhibitors of thioredoxin system inhibited Con A induced T cell proliferation independent of NF-κB suppression. *Murine splenic lymphocytes were stained with CFSE and treated with indicated concentrations of PX12 or CDNB for 4h, stimulated with Con A (5μg/ml) for 72h. Cells were stained with Hoechst 33342 and acquired on partec cyflow flowcytometer. Percent daughter cells are shown. Flow cytometric histograms are shown (A). Bar graph represents percent daughter cells (B). Nuclear extracts from cells treated with anti-inflammatory concentration of PX12 or CDNB for 1h and subsequently exposed to Con A (5μg/ml) for 4h were probed for NF-κB DNA binding by EMSA (C & D). *p<0.05, as compared to control, #p<0.05, as compared to Con A stimulated cells.*

3.3.4 Inhibitors of Trx system suppressed T cell proliferation without suppressing NF-κB

To ascertain whether suppression of NF-κB DNA binding is dispensable for manifestation of anti-inflammatory effects in the absence of Trx system, pharmacological inhibitors of Trx (PX12) and TrxR (CDNB) were used. It was observed that pre-treatment of cells with either of these inhibitors almost completely suppressed Con A induced T cell proliferation (Fig. 3.11A & B). To examine the effect of anti-inflammatory concentration of these compounds on NF-kB DNA binding EMSA was and it was observed that inhibitors of both Trx as well as TrxR could not abrogate Con A induced NF-κB DNA binding in murine splenic lymphocytes (Fig. 3.11C & D). These experiments suggested a role of Trx system in regulating inflammatory responses without affecting NF-kB DNA binding.

3.3.5 Sodium selenite treatment partially abrogated anti-inflammatory effects of baicalein

It was observed that baicalein mediated inhibition of Trx lead to suppression of NF-κB dependent gene expression. Treatment of lymphocytes with sodium selenite prior to baicalein treatment significantly abrogated baicalein mediated suppression of NF-κB dependent gene expression. Hence, effect of sodium selenite on baicalein mediated suppression of mitogen induced T cell proliferation and cytokine secretion was monitored. It was observed that sodium selenite pre-treatment led to significant abrogation in baicalein mediated suppression of Con A (Fig. 3.12A & B) as well as CD3/28 (Fig. 3.12C & D) induced T cell proliferation and cytokine secretion (Fig. 3.12E & F). Sodium selenite pre-treatment partially abrogated baicalein mediated suppression in Con A induced secretion of IL-2 and IL-6 (Fig. 3.12E), whereas it could moderately abrogate suppression in anti-CD3/28 induced secretion of IL-2 and IFN-γ (Fig. 3.12F). These results indicate involvement of Trx system in regulation of T cell responses


A
NaSe+B25+Con A
B25+Con A
Con A
NaSe
B25
Control
CFSE Fluorescence
B
Percent Daughter Cells
Control
B25
NaSe
Con A
B25+Con A
NaSe+B25+Con A
C
NaSe+B25 +CD3/28
B25+CD3/28
CD3/28
NaSe
B25
Control
D
Percent Daughter Cells
Control
B25
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CD3/28
B25+CD3/28
NaSe+B25+CD3/28
E
pg/ml
IL2
IL4
IL6
IFN-γ
Control
B25
NaSe
Con A
B25+ Con A
NaSe+B25+Con A
F
pg/ml
IL2
IL4
IL6
IFN-γ
Control
B25
NaSe
CD3/CD28
B25+ CD3/CD28
NaSe+B25+CD3/28

Fig. 3.12 Baicalein mediated suppression of Con A or anti-CD3/CD28 mAb induced proliferation and cytokine secretion was partially abrogated by sodium selenite pre-treatment. *Lymphocytes were stained with CFSE and were treated with sodium selenite (2μM, 24h) prior to baicalein treatment (25μM, 2h), stimulated with Con A (2.5μg/ml) or anti-CD3/CD28 mAb and cultured for 72h or 24h at 37^{0}C. Cells were acquired on flow cytometer and representative histograms are shown (A & C). Bar graph represents percentage of daughter cells calculated by CFSE dye dilution using Flowjo software (B & D). Culture supernatants from cells stimulated with Con A (5μg/ml) or anti-CD3/CD28 mAb, cultured for 24h were harvested and analyzed for IL-2, IL-4, IL-6 and IFN-γ. Bar graph represents cytokine concentration (pg/ml) (E & F). *p<0.05, as compared to control, #p<0.05, as compared to Con A & $p<0.05 as compared to baicalein treated and Con A stimulated cells.*

3.3.6 Baicalein did not inhibit homeostasis driven proliferation of T-cells in mice but suppressed graft versus host disease associated morbidity and mortality

To study the effect of baicalein on homeostasis driven proliferation (HDP), purified CD4+ T cells were treated with baicalein and transferred to syngenic lymphopenic host. Transient exposure of purified CD4+ T cells to baicalein did not inhibit the homeostatic proliferation of these cells in mice (Fig. 3.13A). These findings suggested that baicalein treatment did not interfere with homeostatic proliferation of T cells and its inhibitory effects are limited to only mitogen induced proliferation.

To study the in vivo anti-inflammatory efficacy of baicalein, its ability to inhibit graft-versus-host disease (GVHD) was studied. Splenic lymphocytes from C57BL/6 mice were incubated with baicalein in vitro (25 μM, 4 h) and adoptively transferred to immunocompromised Balb/c mice. The MHC mismatched recipient mice which received untreated control cells developed GVHD that led to ~80% mortality within 10 days (Fig. 3.13B) and demonstrated typical symptoms of GVHD, including alopecia, scleroderma, hunched posture, diarrhea, and progressive weight loss. However, the mice which received baicalein treated cells showed ~40%

mortality and the survivors exhibited better health for up to 30 days. Further, it was observed that mice receiving baicalein treated lymphocytes experienced inconspicuous weight loss as compared to control group (Fig. 3.13C). It was observed that on day 5 post allo-transplantation, the levels of proinflammatory cytokines (IL-6, IFN-γ and IL-2) were significantly higher in the serum collected from mice receiving vehicle treated allogenic lymphocytes as compared to those in mice which received baicalein treated allogenic lymphocytes (Fig. 3.13D). This observation clearly shows potent anti-inflammatory activity of baicalein in vivo.

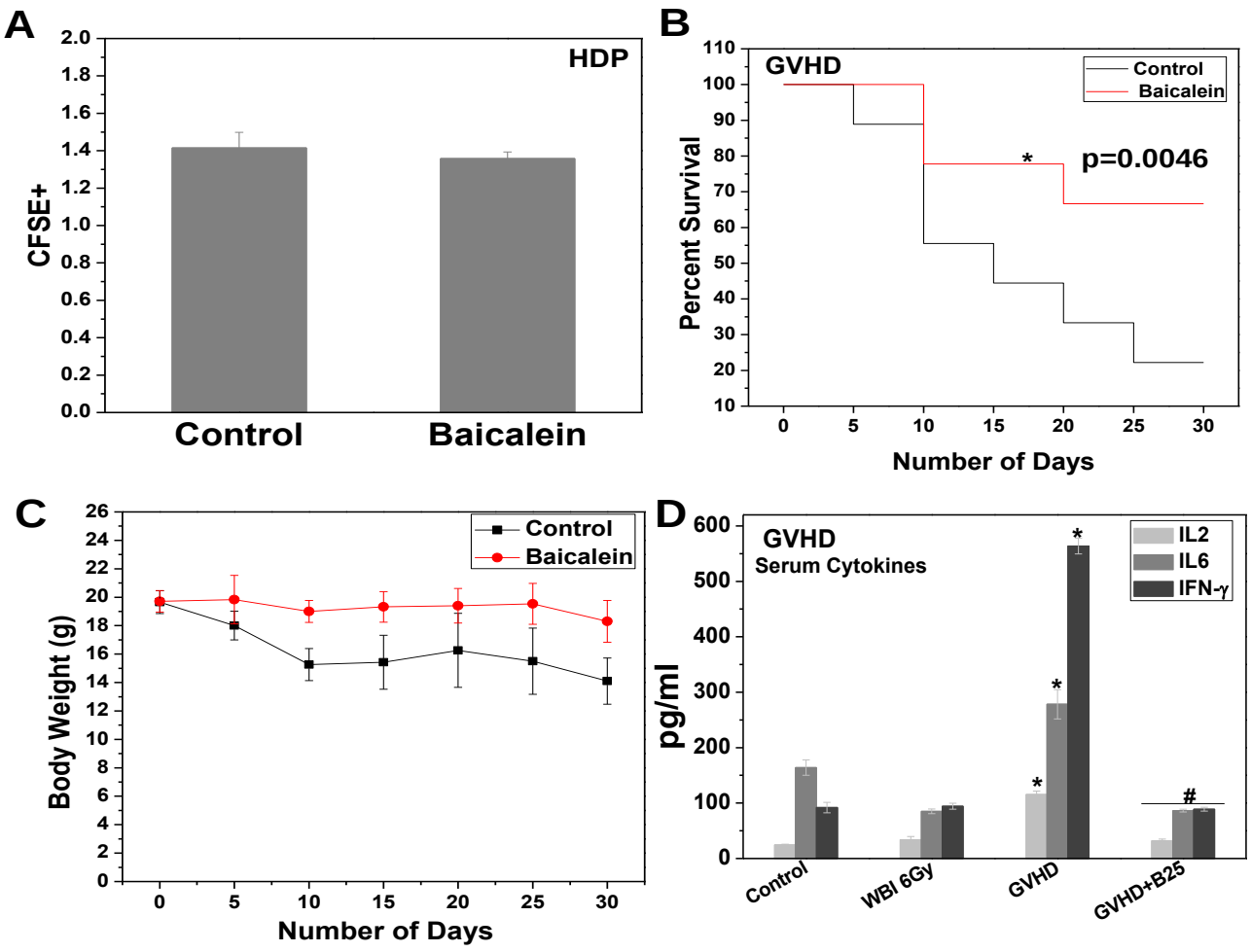


Fig. 3.13 Baicalein treatment did not inhibit homeostasis driven proliferation but suppressed GVHD associated mortality and morbidity: *CFSE stained CD4+ T cells isolated from donor Balb/c mice were treated with vehicle or baicalein (25μM, 2h) and $5x10^6$ cells were injected intravenously into syngenic lymphopenic recipient Balb/c mice. Lymphopenia was induced in Balb/c mice by exposure to 6Gy dose of whole body irradiation. Splenic lymphocytes from lymphopenic host were isolated 48h post injection, acquired on a flow cytometer. Frequency of donor cells was calculated by CFSE dye dilution using flowjo software (A). Graft versus host disease was induced by transplanting $8x10^6$ splenic lymphocytes isolated from C57BL/6 mice into allogenic lymphopenic recipient Balb/c mice. Recipient mice were monitored for weight loss, serum cytokine analysis and 30 days survival. (B) Line graph represents percent survival of recipient mice when monitored for 30 days. (C) Line graph represents change in body weight of mice. (D) Bar graph represents cytokine concentration in serum expressed in pg/ml. *p<0.05, as compared to control and #p<0.05, as compared to GVHD.*

3.4. Discussion

Sustained and dysregulated NF-κB activation leads to uncontrolled inflammation [399]. Systematic and regulated inflammatory response is central to body's defense system. Several anti-inflammatory molecules used in clinic act through modulation of NF-κB signaling [400]. Many molecules that can inhibit other mediators of inflammation such as cyclooxygenase, lipoxygenase, prostaglandins, mTOR etc. also possess promising anti-inflammatory potential [401]. But, suppression of NF-κB remains by far one of the most attractive and preferred strategy for development of anti-inflammatory molecules [402] [351].

It is contradictory to believe that an activator of NF-κB can function as an anti-inflammatory molecule. However, in the present study it is demonstrated that baicalein treatment per se activated NF-κB while still suppressed its dependent gene expression and functions in murine T lymphocytes.

T cells are central players of the adaptive immune response, which help protect the host against different pathogens ranging from bacteria to fungi and viruses [403]. In order to perform their

function, T cells need to be activated, a process that could lead to a variety of responses including proliferation, migration, cytokine production and even apoptosis. The "decision" by T cells to become activated or not is crucial: an inappropriate or exaggerated response could lead to inflammatory diseases while a failure to respond could lead to infection and death [404]. To perform such a complex and sensitive task, T cells must respond to environmental cues that stimulate a complex signaling cascade. Process of T cell activation involves actin polymerization, cytoskeletal rearrangements, integrin expression for increased adhesion and activation of transcription factors that are required for T cell proliferation and effector functions [405, 406] **Fig. 3.14.**

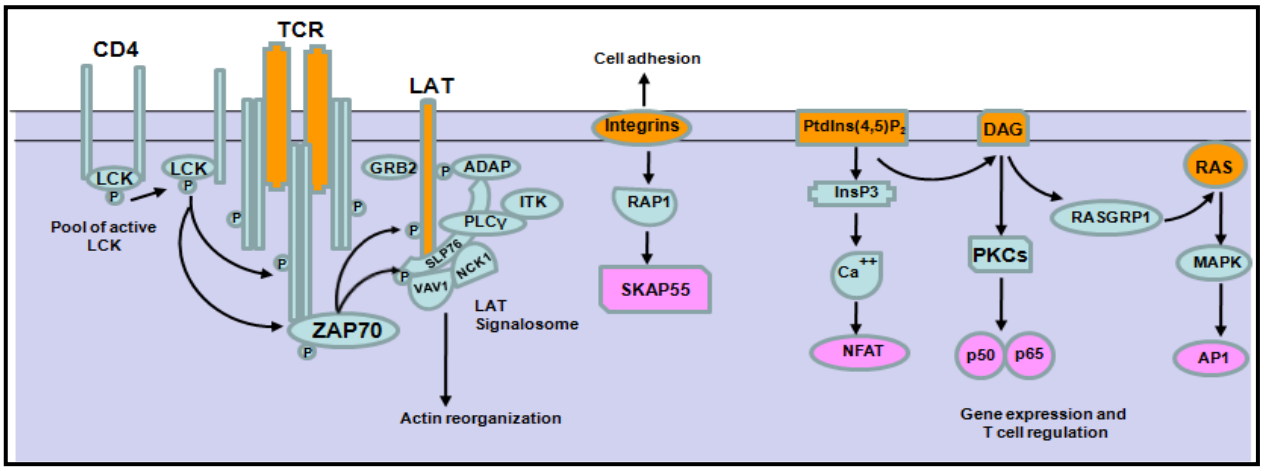


Fig. 3.14 T cell activation cascade *adapted from [407]*

T cell activation is regulated by two distinct signals, signal 1 emanating from T cell receptor and signal 2 coming from the co-stimulatory receptors [408]. Activation of T cells in response to non-pathogenic antigens and self-antigens is controlled by feedback mediated inhibition of activation signals in the absence of signal 2 by induction of a state of specific non-responsiveness (anergy) (Alberts B, Johnson A, Lewis J, et al. New York: Garland Science; 2002). This feedback loop is mediated by a group of E-3 ubiquitin ligases (ITCH, GRAIL and

CBL-B) and transcription factors EGR2 and EGR3 [409]. If the feedback loops are not activated to limit the aberrant effector response, tolerance will be breached, which would lead to immune pathology [410]. To study the effector responses of T cells in vitro, polyclonal mitogens like concanavalin A, phytohemagglutinin (PHA), phorbolmyristic acetate (PMA) and ionomycin or a combination of CD3 + CD28 antibodies are used for T cell activation [411] [412].

Concanavalin A (ConA) is a lectin (carbohydrate-binding protein) originally extracted from the jack-bean, *Canavalia ensiformis [413]*. It is a member of the legume lectin family. It binds specifically to certain structures found in various sugars, glycoproteins, and glycolipids, mainly internal and non-reducing terminal α-D-mannosyl and α-D-glucosyl groups [413]. Concanavalin A (Con A) is an antigen-independent mitogen and functions as signal one inducer, leading T cells to polyclonal proliferation. CD28 is known to be one of major co-stimulatory receptors and to provide signal two in the Con A-induced T cell proliferation [414]. Con A binds specifically α-D-mannosyl and α-D-glucosyl residues in terminal position of ramified structures from [415].

To study anti-inflammatory properties, effect of compounds on T cell activation and effector functions serve as a model system in vitro. Results revealed that baicalein treatment significantly suppressed mitogen (Con A as well as anti-CD3/CD28 mAb) induced proliferation, cytokine secretion and expression of T cell activation markers in a dose dependent manner. Baicalein treatment suppressed expression of surface markers. These results established that baicalein interferes with T cell activation signaling and blocks mitogen induced response. However, this suppression is not absolute as evinced by surface marker expression analysis. Although, it suppressed early (CD69) as well as late (CD25 i.e. IL2Rα) T cell activation markers, this inhibition was not complete. Further, baicalein treatment did not suppress expression of ICAM-1 i.e .CD54 indicating that baicalein has specific targets on T cells.

Activation of NF-κB is a crucial step in T cell proliferation and inhibition of NF-κB and its dependent gene expression can suppress T cell mediated immune responses. Stimulation with Con A leads to increased nuclear translocation and DNA binding of NF-κB. As discussed in detail in the introduction section of this chapter, suppressors of NF-κB function as promising anti-inflammatory modality. Interestingly, baicalein treatment per se led to increased DNA binding of NF-κB and consistent with the literature findings [416], it did not suppress Con A induced DNA binding of NF-κB. It was interesting to observe anti-inflammatory effects of baicalein without affecting its binding to DNA. These findings revealed that baicalein did not interfere with any of the upstream events in NF-κB activation but still suppressed its dependent responses.

Expression of NF-κB dependent genes is regulated at yet another level by controlling transactivation events. Transactivation of NF-κB is regulated by phosphorylation or acetylation of p65 [417]. Phosphorylation of RelA at serine 276 enhances the recruitment of coactivator p300/CBP leading to increased transcriptional activation [418] [419] [420] Serine 536 in the RelA transactivation domain is phosphorylated by IKKs [222] [359] or by ribosomal subunit kinase 1[421] **Fig. 3.15**.

This modification also enhances the transcriptional activity of NF-κB [422] [423]. Apart from this, Trx and TrxR are also shown to regulate NF-κB trans-activation potential. This redox regulatory circuit provides reducing environment for DNA binding of NF-κB and intra-nuclear over-expression of Trx leads to increased NF-κB luciferase activity

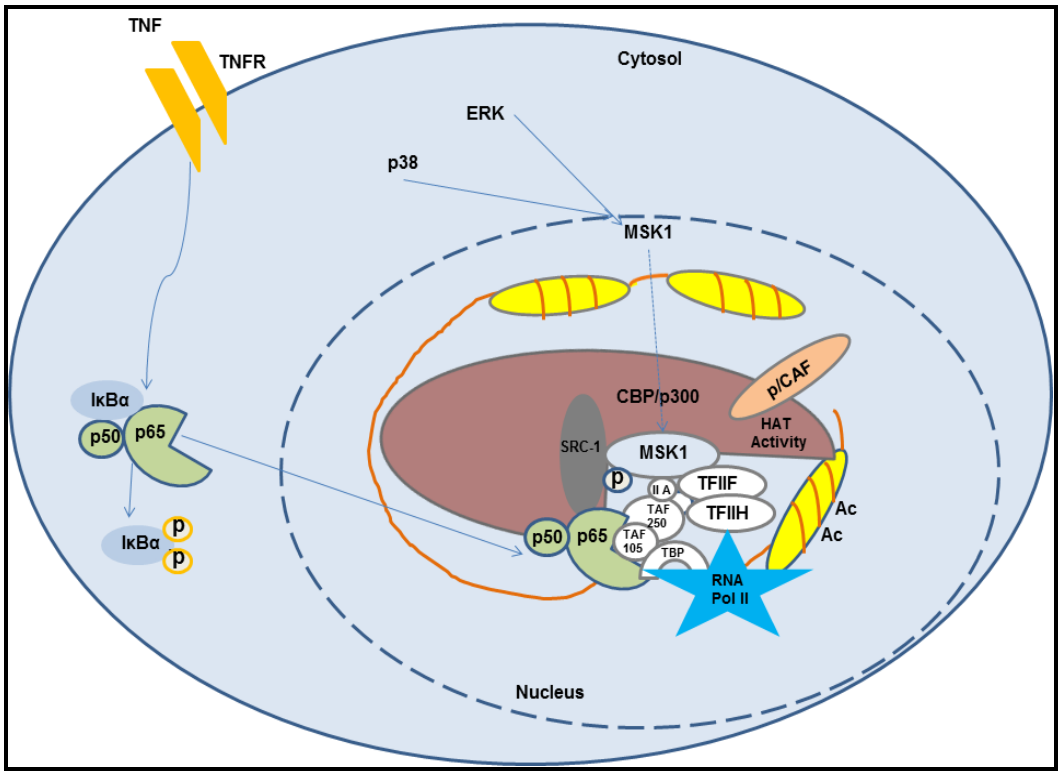


Fig. 3.15 Phosphorylation, acetylation and co-activators regulate NF-κB trans-activation [424]

Further, inhibition of TrxR could suppress NF-κB trans-activation functions in terms of luciferase (reporter gene) activity [358]. Baicalein belongs to class of flavonoid compounds which were recently shown to physically interact with TrxR in cell free system and inhibit its activity [395]. Based on literature reports indicating that flavonoids inhibit TrxR activity, our results showing inhibition of T cell mediated immune responses independent of NF-κB DNA binding by baicalein and literature reports showing NF-κB trans-activation as a druggable target, it was hypothesized that anti-inflammatory activity of baicalein could be through modulation of Trx system.

Activity of Trx in nuclear compartment after treatment of cells with baicalein was monitored in the presence and absence of mitogen. It was observed that baicalein per se had little or no effect on nuclear Trx activity. But, it significantly suppressed mitogen induced increase in nuclear Trx

activity. This can be explained by the fact that, IC50 of baicalein for inhibition of TrxR is in the range of 200μM which is 8 time higher as compared to its anti-inflammatory concentration 25μM. At this low concentration it may not per se affect nuclear Trx activity, but, after mitogen treatment there is an increased nuclear shuttling of Trx1 thereby enhancing probability of interaction with baicalein. In light of these results showing that baicalein treatment per se activates DNA binding of NF-κB, modulation of Trx seems a possible target for baicalein mediated inhibition of NF-κB transactivation.

Trx is maintained in the reduced state by TrxR which has selenocysteine (Sec) residues in the catalytic centers [425]. Selenocysteine is a variant of amino acid cysteine which contains an essential trace element Selenium (Se) in place of Sulphur (S) [425]. Treatment of cells with sub-micromolar concentration of selenium has been shown to activate TrxR [426] [315]. Hence, T cells were treated with sodium selenite prior to baicalein treatment followed by mitogenic stimulus. Rational behind this experiment was to investigate involvement of Trx system in mediating anti-inflammatory effects of baicalein. If the observed effects of baicalein on T cells are to be attributed to Trx system, under conditions of enhanced TrxR activity baicalein mediated immune-suppression would be abrogated. It was observed that sodium selenite treatment partially abolished baicalein mediated suppression in Trx activity after mitogenic stimulus. This finding suggested that stimulation of TrxR in part can neutralize anti-inflammatory effects of baicalein.

Next important question to be addressed remained whether baicalein inhibited nuclear transport of Trx1 after mitogen stimulation. Baicalein mediated suppression in mitogen induced nuclear Trx activity can be attributed to i) interference in nuclear import of Trx1 or ii) inhibition of nuclear Trx activity. Results revealed that baicalein did not inhibit nuclear import of Trx

suggesting it indeed suppressed activity of Trx inside nucleus. Thus, although Con A stimulated increased nuclear accumulation of Trx1, it was not physiologically active in baicalein treated group.

To investigate whether NF-κB dependent gene expression is blocked by baicalein treatment, mRNA levels of IL-2 and IL-6 in lymphocytes pre-treated with baicalein and stimulated with Con A were monitored. Baicalein treatment almost completely suppressed NF-κB dependent gene expression. To investigate role of Trx inhibition in baicalein mediated suppression of NF-κB transactivation, cells were pre-treated with sodium selenite prior to baicalein treatment. It was observed that sodium selenite pre-treatment significantly abolished baicalein mediated suppression in expression of IL-2 and IL-6. To obtain proof of principle, EL4 cells were transfected with Trx over-expression plasmid. Con A treatment induced IL-2 secretion in culture supernatant of EL4 cells. Baicalein treatment suppressed Con A induced IL-2 secretion in EL4 cells but it could not do so in cells over-expressing Trx. These findings pointed out that baicalein mediated inhibition of Trx activity may be the mechanism of its anti-inflammatory activity.

These results established an association between observed anti-inflammatory activity of baicalein and inhibition of Trx system. To further investigate role of Trx in NF-κB trans-activation, employed known pharmacological inhibitors of Trx and TrxR were employed. Anti-inflammatory concentration of Trx inhibitor PX12 and TrxR inhibitor CDNB were determined by performing proliferation assay of CFSE stained T lymphocytes stimulated with Con A in the presence or absence of these inhibitors. Concentration at which there was complete suppression in Con A induced T cell proliferation was chosen for further experiments as it indicated suppression of NF-κB dependent responses. Cells were treated with anti-inflammatory concentration of either of these inhibitors and nuclear extracts from these cells were probed for

NF-κB DNA binding activity. Results revealed inhibition of Trx system did not suppress NF-κB DNA binding suggesting that it did not interfere with any of the upstream signaling events in NF-κB activation. Despite not suppressing NF-κB DNA binding, it could suppress its dependent process of T cell proliferation.

These results provided a clearer picture that inhibition of Trx system in nuclear compartment indeed blocks NF-κB transactivation without interfering with its DNA binding which is the case with baicalein. Thus, it could be concluded that baicalein mediated suppression of nuclear Trx is responsible for its anti-inflammatory activity.

It is evident from the above findings that baicalein suppressed activity of dithiol disulfide Trx in nucleus which is responsible for its anti-inflammatory activity. Further, treatment with sodium selenite, a pharmacological activator of TrxR abrogated suppressive effects of baicalein on Trx activity. Since, sodium selenite treatment partially relieved baicalein mediated inhibition of nuclear Trx activity it was interesting to observe its effect on mitogen induced T cell responses in presence of baicalein. Consistent with our hypothesis, sodium selenite treatment significantly abrogated baicalein mediated suppression in Con A as well as anti-CD3/28 mAb induced T cell proliferation. Partial abrogation in baicalein mediated suppression of IL-2 and IL-6 secretion in case of Con A treatment and IL-2 and IFN-γ in case of anti-CD3/28 mAb stimulation after sodium selenite treatment was also observed. These findings focused on the fact that stimulation of Trx system by activating TrxR partially abrogated anti-inflammatory effects of baicalein. All these results put together indicate that baicalein treatment led to inhibition of Trx system and observed anti-inflammatory effects. **Fig. 3.16** shows mechanism of action of baicalein.

It has long been known that mature T cells are regulated at a population level by homeostatic mechanisms that maintain the total size of the T cell pool at a near-constant level [427] [428]

[429]. Normally, expansion of the T cell pool during an immune response is followed by a deletion phase in which most of the newly generated effector cells are eliminated at the end of the response, thereby restoring total T cell numbers to normal levels [430] [431]. On the other hand, it is also well established that T cells have the capacity to spontaneously undergo extensive proliferation after transfer into immunodeficient hosts [432]. Such "homeostatic" proliferation of T cells occurs when small numbers of T cells are adoptively transferred into T cell–depleted (T-depleted) syngeneic nude, SCID, recombination activating gene (RAG)-deficient, or irradiated hosts [433] [434]. Homeostatic proliferation of CD4+ and CD8+ cells requires contact with self-MHC class II and I molecules, respectively [435] [436-438]. Research from several laboratories strongly suggest that homeostatic proliferation applies to naive T cells and is driven by low-affinity interactions with self-MHC molecules loaded with self-peptides [436, 438, 439].

Exposure of mice to myeloablative doses of IR leads to induction of lymhpopenia. T cells attempt to re-establish homeostatic conditions by proliferating in response to lymphopenia. Effect of baicalein treatment on homeostasis driven proliferation (HDP) of purified CD4+ T cells was monitored in vivo. It was observed that baicalein treatment did not inhibit HDP suggesting it selectively suppressed mitogen induced proliferation of T cells and did not disturb their homeostatic division.

Graft-versus-host disease (GvHD) is a common complication following an allogeneic tissue transplant [440] [441]. Immune cells in the graft recognize host as antigenically foreign and attack them. For GvHD to occur minimum three criteria must be met that include i) graft should contain viable and functional immune cells ii) recipient should be histo-incompatible iii) recipient should be immune-compromised [442].

It can occur during bone marrow transplantation, blood transfusion and organ transplant due to MHC mismatch [443]. Graft-versus-host-disease can largely be avoided by performing a T-cell-depleted bone marrow transplant [444]. But, these types of grafts suffer from disadvantage of general immunodeficiency thereby reducing period of disease free survival [445]. The use of umbilical cord stem cells have reduced incidence of graft rejection [446]. Pre-treatment of graft cells with immunosuppressant drugs is one of the strategies to reduce engraftment failure. Currently, methotrexate, cyclosporine and tacrolimus are used to improve chances of successful transplant.

This serves as a useful model to test the immune-suppressive effects of drugs under investigation. GvHD can be induced in mice by transplanting $CD8^+$ T cells from mismatched donor into immune-compromised recipient. Baicalein treated lymphocytes from C57BL/6 mice were transferred intravenously into lymphopenic BALB/C mice. Lymphopenia was induced by exposure to sub-lethal (6Gy) dose of IR. Mice receiving vehicle treated lymphocytes developed typical symptoms of GvHD including hair loss, body weight loss, and alopecia, hunched posture, nausea and vomiting. Mice receiving baicalein treated lymphocytes survived in better health for more than 30 days. Baicalein treatment suppressed GvHD associated mortality and morbidity in mice and also suppressed levels of pro-inflammatory cytokines in serum.

3.5. Conclusions

1. Baicalein suppressed mitogen induced T cell proliferation and cytokine secretion.

2. Baicalein significantly suppressed Con A induced expression of T cell activation markers CD69 and CD25 but did not interfere with expression of ICAM-1.

3. Baicalein treatment induced DNA binding of NF-κB in murine splenic lymphocytes.

4. Baicalein did not suppress Con A induced DNA binding of NF-κB.

5. Baicalein treatment suppressed Con A induced increase in nuclear Trx activity but did not inhibit nuclear import of Trx1.

6. Pharmacological activator of TrxR, sodium selenite, abrogated baicalein mediated suppression in NF-κB dependent gene expression.

7. Baicalein could not suppress Con A induced IL-2 secretion in EL4 cells over-expressing Trx1.

8. Inhibitors of thioredoxin system also suppressed mitogen induced T cell proliferation but did not inhibit DNA binding of NF-κB.

9. Pre-treatment of T cells with sodium selenite partially abrogated immune-suppressive effects of baicalein.

10. Baicalein did not inhibit homeostasis driven proliferation of T cells.

11. Baicalein suppressed GvHD associated mortality and morbidity in mice.

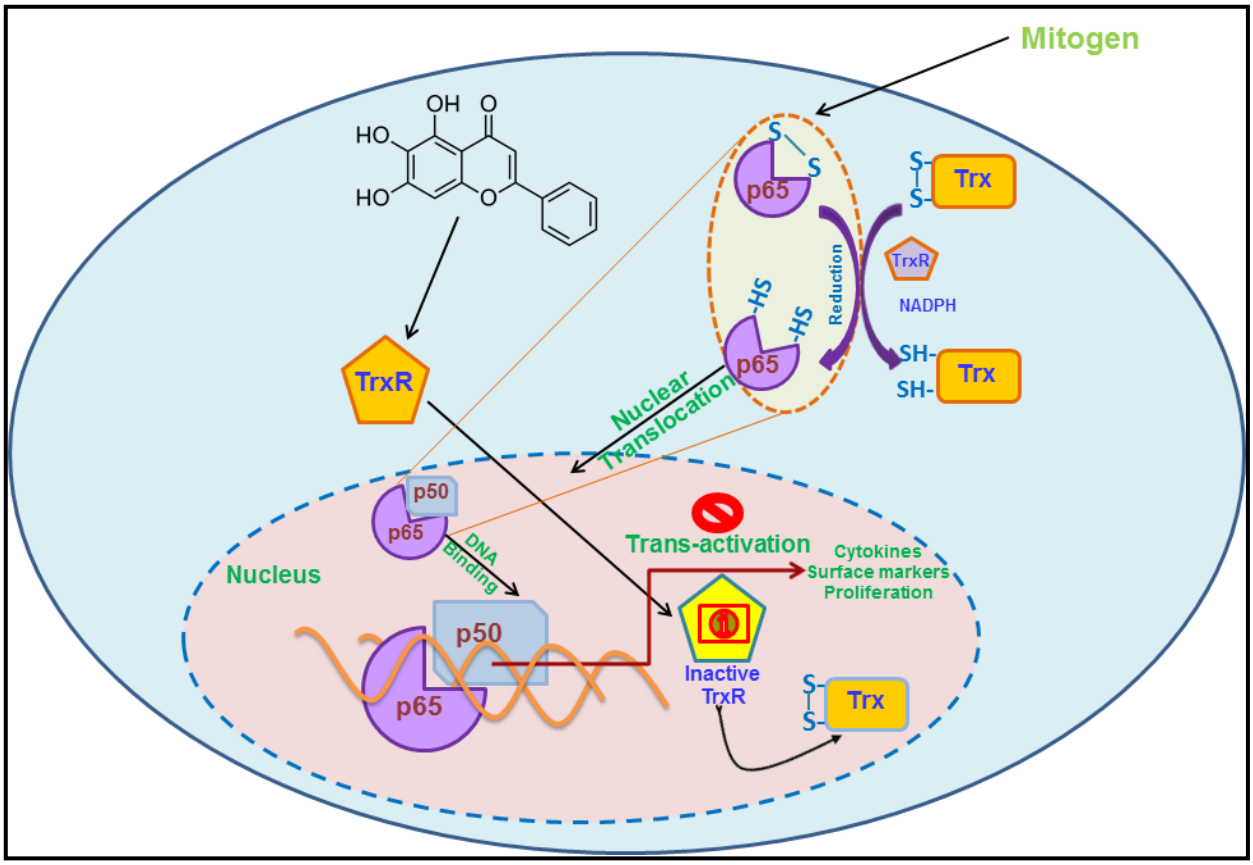


Fig. 3.16 Proposed mechanism of anti-inflammatory action of baicalein

Novelty

This study for the first time demonstrates that an agent that can per se activates NF-κB and still exhibit anti-inflammatory property. The study highlights a novel strategy for development of anti-inflammatory agents.

Future Directions

This study provides platform for designing novel anti-inflammatory molecules that can specifically target trans-activation of NF-κB.

CHAPTER 4

ANTI-TUMOR EFFECTS OF BAICALEIN

4.1. Introduction

Radiation therapy is an integral component of treatment of different types of solid cancers. Tumor cells possess inherent and / or exhibit acquired resistance to radiation induced cytotoxicity. Inherent radioresistance refers to constitutively active oncogenic, proliferative and/or anti-apoptotic signals, whereas acquired radioresistance refers to induction of pro-survival genes/proteins [447]. Exposure to clinically relevant doses of IR induces multilayered signaling response in cancer cells by activating both cytoplasmic and nuclear signaling. Improved understanding of the causes for constitutive and induced radioresistance in tumor cells may pave the way for designing effective treatment modality.

4.1.1 Cancer cells and oxidative stress

Cancer cells being metabolically active live in a highly oxidative stress environment [448, 449]. However, development of radioresistance in cancer cells would suggest that they have acquired the ability to eliminate the ROS and maintain a steady state level. Effective elimination of ROS depends on how efficiently they are neutralized by antioxidants inside cells so that IR induced damage is not permanently fixed. Previous studies from our laboratory have demonstrated that intrinsic radioresistance of lymphoma cells vis-à-vis normal lymphocytes may be due to lower basal and inducible ROS levels [450]. Further, in the same study it is also shown that GSH levels and antioxidant enzyme activities were higher in lymphoma cells as compared to normal lymphocytes.

Generation and persistence of ROS in the mitochondria / nucleus after exposure of cells to IR make them vulnerable to DNA damage, mitochondrial dysfunction and genomic instability [451, 452]. DNA damage induced per cell per Gy post low LET IR exposure is chemically identical to those formed by ROS [453]. Thus, type and severity of DNA damage induced in tumor tissue

depends upon redox status of the cell. A strong and effective antioxidant response against oxidative stress challenge can eliminate ROS which are potential threats to genomic integrity [454].

IR induced oxidative stress in tumor cells is effectively managed by constitutive and inducible antioxidant defense systems. Considering the nature and type of IR mediated damage responsible for tumor cell killing, role of ROS in influencing the outcome of radiotherapy cannot be overlooked. In fact, hyperactive antioxidant machinery is one of the strategies employed by tumor cells to overcome ROS-mediated DNA damage to evade IR-induced cell killing [455, 456].

4.1.2 Antioxidant network in cancer cells

The levels of intracellular antioxidants and antioxidant enzymes are regulated by nuclear factor erythroid-2 related factor-2 (Nrf-2) [455]. Under oxidative stress, Nrf-2 is released from Keap1 and translocates to nucleus where it binds to antioxidant response element (ARE) in DNA and thereby induces transcription of a myriad antioxidant enzymes viz. catalase, Mn-superoxide dismutase, glutathione peroxidase (GPX), glutathione-s-transferase (GST), hemoxygenase I etc. [457].

Cellular defense against oxidative stress and maintenance of redox homeostasis also depends on the regulation of thiol-disulfide exchange [458, 459]. Formation of reversible protein disulfides or protein-SSG mixed sulfides (PSSG) (glutathionylation) act as regulatory switches in response to alteration in cellular redox [460].

Cells harbor two major independent systems of redox buffers in the form of monothiol tripeptide “GSH” and dithiol protein “thioredoxin”. First system contains glutathione (GSH) as reducing agent, which primarily functions in neutralizing ROS either directly or through GPx-catalyzed

reactions and protecting protein-SH groups [457, 461]. Second system comprises of dithiol protein of low molecular mass namely, Trx which is a protein disulfide oxido-reductase and TrxR that reduces Trx [462]. Trx is one of the major determinants of cell fate as it regulates pro-apoptotic protein ASK1 [463, 464]. Besides, it has an important role in regulating several redox reactions vital for cell survival under normal as well as stress conditions [465, 466].

4.1.3 Cellular targets for tumor radio-sensitization

It is important to identify potential targets to sensitize tumors to radiotherapy without significant collateral damage to normal tissues. Thus it is required to identify molecular targets that are differentially expressed between normal and tumor cells.

ERK and Nrf-2

Recent reports indicate that IR activates Nrf-2 pathway and targeting this pathway may impact outcome of radiation therapy [467, 468]. Further, it was reported that upregulation of Nrf-2 in lymphocytes augmented their radioresistance in vitro and also prevented radiation induced morbidity and mortality in mice [120]. **Fig. 4.1** shows role of Nrf-2 in cancer. Based on these reports, it can be hypothesized that activated ERK and Nrf-2-ARE pathway [310] may contribute to the constitutive and inducible radioresistance in tumor cells vis-à-vis normal cells.

GSH and thioredoxin system

Considering oxidative metabolism in tumor cells cellular GSH content plays a vital role in regulating oxidative stress. Further, cancer cells are over-dependent on Trx

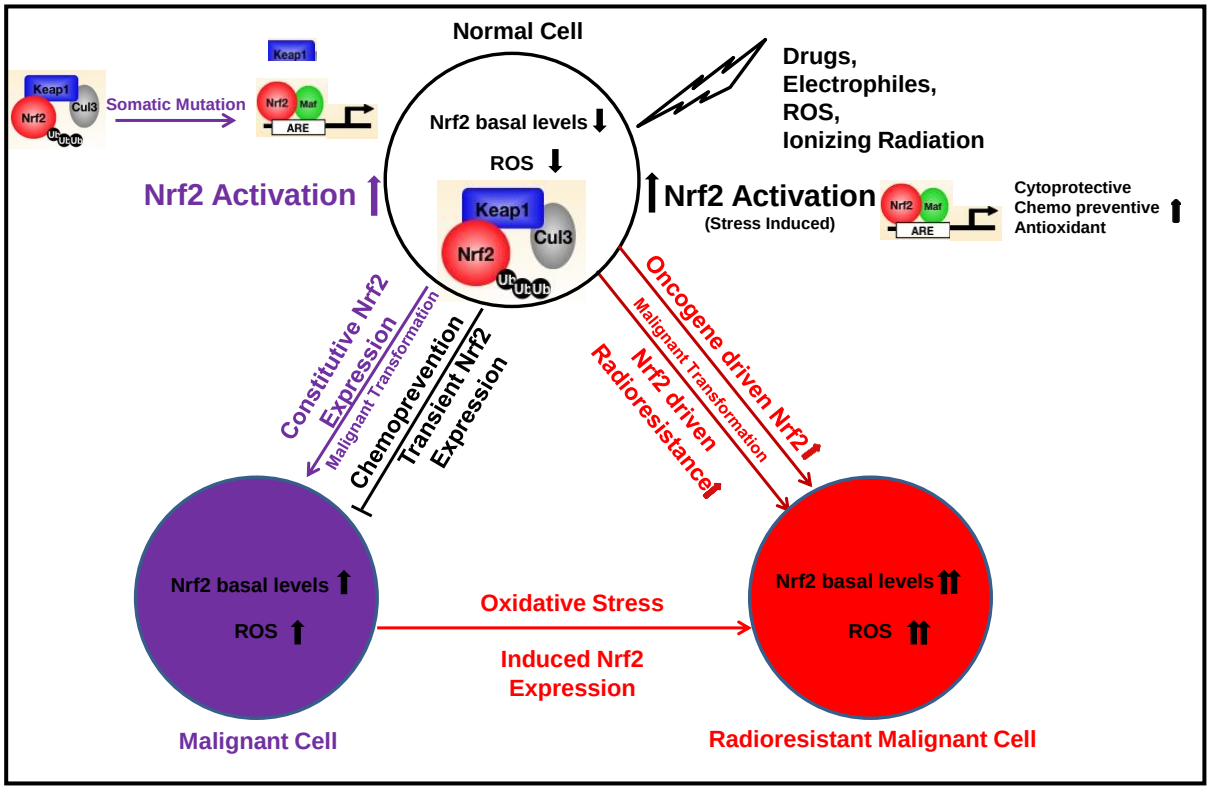


Fig. 4.1 Nrf-2 and cancer

system for constant supply of precursors of DNA and management of oxidative burden. Inhibition of TrxR is already being employed as a strategy for development of novel and effective anti-tumor agents [469, 470]. Considering the role Trx system plays in regulation of tumor growth, its inhibition may prove as a beneficial strategy for sensitizing tumors to radiation. Based on the present literature, it could be perceived that strategies targeting simultaneous disruption of glutathione and Trx metabolism in tumor cells may improve the outcome of radiotherapy treatment.

Effect of radioprotector on tumor cells

One of the most important attributes of a radioprotector is that it should not offer any survival advantage to tumor cells under conditions of radiation exposure. Baicalein is a 5, 6, 7 trihydroxy flavone and its anti-tumor effects are widely reported in the literature. Flavonoids constitute

components of functional food and possess diverse biological properties such as antioxidant, anti-proliferative, anti-inflammatory or antibiotic activity which may contribute to their chemo-preventive action [471, 472]. Recent research finding has identified potential of different flavonoids to inhibit TrxR in cell free system [395]. Several literature reports have described anti-inflammatory and anti-tumor effects of baicalein and various mechanisms have been proposed for this effect [473-477]. In light of the ability of baicalein to protect murine splenic lymphocytes against IR induced damage, it is imperative to investigate its effect on the murine T cell lymphoma EL4 cells which are their tumor counterpart.

Scope of the study

I) To probe the molecular mechanism of differential radiosensitivity between tumor and normal cells i.e. murine splenic lymphocytes from C57BL/6 mice and syngenic murine T cell lymphoma cells (EL-4). The aim of this study is to determine the contribution of ERK/Nrf-2-ARE pathway in tumor radioresistance.

II) In order to appreciate the role of GSH and Trx antioxidant network in determining the intrinsic radio-resistance of tumor cells, experiments were carried out to study the radiation induced spatio-temporal changes in different cellular redox parameters. Further, role of either of these antioxidants in determining tumor radio-sensitivity was evaluated.

III) In order to understand the effect of baicalein on murine T cell lymphoma, study was undertaken to investigate the possible correlation between inhibition of Trx system and its implication on anti-tumor activity by baicalein.

4.2. Materials and Methods

4.2.1 Chemicals

Baicalein, Propidium Iodide (PI), insulin, NADPH and 1,4-dithiobisnitrobenzoic acid (DTNB), HEPES, EDTA, EGTA, PMSF, leupeptin, aprotinin, benzamidine, dithiothreitol (DTT), NP40, propidium Iodide (PI), dimethyl sulfoxide (DMSO), histopaque, dihydrodichlorofluoresceindiacetate (DCFDA), dihydroethidium (DHE) and dihydrorhodamine 123 (DHR123) were purchased from Sigma Chemical Co. (MO, USA). JC-1 and pharmacological inhibitors of Nrf-2, MEK, P38, JNK, TrxR, hemoxygenase I were purchased from Calbiochem (Darmstadt, Germany). Inhibitory peptide for NF-κB was purchased from IMGENEX (San Diego, CA). Plasmid miniprep kit, lipofectamine 2000, mitosox red and alexafluor 633 C5 maleimide were purchased from Invitrogen(Grand Island, NY). shRNA plasmids for TrxR1, Trx1, Trx2, ERK, Nrf-2 and over-expression plasmid for Trx1 were purchased from Origene (Rockville MD USA). Polynucleotide kinase, kinase buffer for EMSA were purchased from New England Biolabs (Ipswich, MA). Oligonucleotide probe for Nrf-2 was purchased from Santacruz Biotechnology (Dallas, Texas, USA). Trx1 and TrxR1 ELISA sets and total thiol detection kit was purchased from Cayman Chemicals (Michigan, USA). RPMI 1640, fetal bovine serum (FBS) was obtained from HiMedia (Mumbai, India). Hoechst 33342 was procured from Molecular Probes (NY, USA). Monoclonal antibodies against CD3 & CD28 and fluorochrome labeled antibodies against HO-1 and annexin V PE-AAD kit were procured from BD Pharmingen (CA, USA). HiPure RNA isolation kit, cDNA synthesis kit, TUNEL-FITC kit and Light Cycler SYBR green RT-PCR kit was purchased from Roche (Basel, Switzerland). Antibody against pASK1 was procured from Cell Signaling Technologies (CA, USA). All other

chemicals used in this study were obtained from reputed manufacturers and were of analytical grade.

4.2.2 Cell culture and isolation of human peripheral blood mononuclear cells (PBMCs)

EL-4 (Murine T cell lymphoma) and Jurkat (Human T cell lymphoma) cells were obtained from Health Protection Agency Culture Collections and cultured in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin and 100µg/ml streptomycin. Cells were incubated at 37°C in a 5% CO_2 humidified atmosphere in a CO_2 incubator. Cultures were maintained in exponentially growing conditions with doubling time little less than 24h and sub-cultured thrice in a week. Human venous blood from healthy volunteers was collected in heparinized tubes. PBMCs were separated using Ficoll histopaque by density gradient centrifugation. For performing experiments using human blood samples permission was obtained from BARC Hospital Medical Ethics Committee (BHMEC) under the project no. BHMEC/DNB/15/10.

4.2.3 Animal maintenance

Eight to ten weeks old C57BL/6 male mice weighing approximately 20-25g, reared in the animal house of the Bhabha Atomic Research Centre were used. Guidelines issued by the Institutional Animal Ethics Committee of Bhabha Atomic Research Centre, Government of India, regarding maintenance and dissection of animals were strictly followed.

4.2.4 Exposure to IR

Cells (EL4/Jurkat/Splenic lymphocytes) were suspended in medium and exposed to IR using a ^{60}Co γ-irradiator at a dose rate of 1.319Gy/min (Blood Irradiator 2000, BRIT, Mumbai). Dose rates and doses delivered were calculated on a regular basis by Radiation Safety & Systems Division (RSSD) of BARC using Fricke dosimetry.

4.2.5 Estimation of apoptosis (Propidium iodide staining and DNA ladder assay)

EL4 cells (1 x 10^6) were treated with different concentrations of baicalein for indicated time intervals at 37^0C in RPMI1640 medium supplemented with 10% FBS and incubated for 48h. EL4/Jurkat/Splenic lymphocytes (1 x 10^6) were exposed to IR 4Gy and incubated for 24h or 48h. Vehicle treated cells served as control. These cells were then harvested, stained with PI and acquired in Partec Cyflow flowcytometer [295]. Analysis was performed using FlowJo software and percent cell death was calculated from pre-G1 population. DNA ladder assay was performed to confirm apoptosis. EL-4 cells (5 x 10^5) were exposed to 4Gy IR and cultured for 24h/48h in RPMI 1640 medium supplemented with 10% FBS in a 5% CO_2 atmosphere. Unirradiated cells served as control. The cells were washed with PBS and processed for DNA fragmentation. Briefly, EL4 cells were lysed in mammalian cell lysis buffer (10mM Tris-Cl, 100mM EDTA, 0.5% SDS & 100µg/ml RNase A). The lysate was centrifuged at 12000g/4˚C/20 min, supernatants were transferred to new tube and incubated with RNase A followed by proteinase K. Phenol/ chloroform/ isoamyl alcohol extraction was performed. The aqueous phase was collected after centrifugation in a new tube. Two volumes of 100% chilled ethanol and 1/10^{th} volume of 3M sodium acetate (pH 5.2) were added and DNA was allowed to precipitate. After centrifugation the pellet was washed with 70% ethanol, air dried and dissolved in de-ionized DNase free water. Samples were run on 1.2% agarose gel at 60V for 2h and DNA ladder was visualized using Gel Documentation System (DNR Biosystem).

4.2.6 Intracellular ROS measurement

To detect intracellular ROS, EL-4, Jurkat and murine splenic lymphocytes were incubated with 20µM oxidation-sensitive dichlorofluoresceindiacetate (DCF-DA) [303] or 5µM dihydroethidium or 5µM dihydrorhodamine 123 or mitosox red for 25min at 37°C [450] before

exposure to 4Gy IR. Increase in fluorescence resulting from oxidation of H_2DCF to DCF(485/535nm) or DHE to hydroethidium (480/610nm) or DHR to rhodamine (500/536nm) or mitosox red (480/610) was measured using a spectrofluorimeter.

4.2.7 Intracellular GSH assay

GSH/GSSG ratio in Jurkat/EL4 cells ($1X10^6$/ml) after IR exposure was measured by conventional enzyme cycling method [478].

4.2.8 Transfection

Two separate shRNA plasmids were tested for knocking down of either ERK or Nrf-2. Effective plasmid (Cat. No. TF515053 for Nrf-2 and TF502598 for ERK Origene) was used in subsequent experiments. The cells ($4x10^5$) were seeded in 800ul medium free of antibiotic and FBS in a 6 well plate. For each transfection, DNA (1µg): lipofectamine 2000 (10µg) complex was prepared separately and incubated for 45-60 min at RT and added to cells. Cells were further cultured for 48 hrs for transgene expression. Jurkat cells (2×10^6/ml) were incubated in R buffer (Invitrogen) containing 5µg shRNA plasmid DNA for TrxR1/Trx1/Trx2/scrambled sequence at RT for 10min. Cells were electroporated using Neon Electroporator (Invitrogen) at pulse voltage of 1325 volts, pulse width 10ms, pulse number 3 at a cell density of 2 x1 0^7cells/ml. Cells were cultured for 48h and observed under fluorescence microscope for transfection. Knockdown of TrxR1 was confirmed by ELISA, whereas knockdown of Trx1/Trx2 was confirmed by RT-PCR.

Trx1 over-expression (OE) plasmid (cat no. TF515053, Origene) was used for over-expressing Trx1 in EL-4 cells. Transfection was performed using Neon® Transfection System (ThermoFisher Scientific, Waltham, Massachusetts, USA) following manufacturer`s protocol. Briefly, 2 million cells were electroporated (pulse voltage 1680V and pulse width 20ms) using

1µg of Trx OE plasmid in antibiotic free medium. Cells were further cultured for 24h for transgene expression. Overexpression was confirmed by RT-PCR for Trx1.

4.2.9 Measurement of change in MMP

EL-4 cells (1 x 10^6) were treated with different concentrations of baicalein for indicated time intervals. Mitochondrial membrane potential (MMP) was assessed using the mitochondrial-specific cationic fluorescent probe JC-1 (5µM) by spectro-flourimetric method as described previously [479].

4.2.10 Surface and intracellular antibody staining

Analysis of surface markers on EL-4 cells was carried out by surface staining with PE or FITC labelled mAbs as described earlier [302]. EL-4 cells (2 x 10^6) were stained with annexin V-PE antibody. Cells were acquired using Partec Cyflow Space flowcytometer and analyzed using FlowJo software (TreestarInc, Ashland, USA). For intracellular antibody staining, EL-4 cells (2 x 10^6) were treated with baicalein 100µM for indicated time intervals and staining was performed using pASK1 antibody followed by PE conjugated secondary antibody. For TUNEL-FITC staining EL4 cells (2x10^6) treated with baicalein 100µM for indicated time intervals were harvested and stained with FITC labelled dUTPs as per manufacturer's protocol. Vehicle treated cells served as loading control. Changes in intracellular protein levels were measured using a flow cytometer (Partec CyFlow) and analysed using FlowJo software.

4.2.11 RNA isolation, cDNA synthesis and quantitative real time PCR

mRNA levels in the samples were quantified by quantitative real-time polymerase chain reaction (qPCR) as described previously [295]. EL4 (1 x 10^6) cells given indicated treatments were processed for RNA isolation by homogenizing in trizol reagent and vortexed after adding chloroform. Cells were incubated for 5min at RT and then centrifuged at 14000 rpm for 15min at

4^0C. Supernatant was collected in new tube and was allowed to precipitate by adding equal volume of isopropanol at RT for 20min. Cells were spun down at 14000 rpm for 15min at 4^0C, supernatant was discarded and pellet was washed by chilled 70% ethanol, air dried and dissolved in RNase free water. Purity and quantity of RNA was estimated in a 96-well quartz plate using Synergy H1 Hybrid reader (Biotek) at 260 and 280nm. 1µg RNA was used to prepare cDNA using cDNA synthesis kit (Sigma) as per manufacturer's instructions. Different dilutions of cDNA were made to calculate the efficiency of real time PCR. Real time PCR for was performed using respective forward and reverse primers (Table 1 & 2) and 2X SYBR mix from Sigma. Melting Temperature was set at 95^0C, cycling conditions were 95^0C-20 sec, 58^0C-20 sec, 72^0C-30 sec (40 cycles) and extension was carried out at72^0C-5min.The expressions of genes were normalized against that of a housekeeping gene β-actin, and plotted as relative change in the expression with respect to control. In another experiment **Table 4.1** shows primer sequences of Trx1, Trx2 and β actin for human and Trx1 and β actin for mouse.

Table 4.1 Primer sequence

Gene	Sequence
Trx1(h)	Forward:5'GTGAAGCAGATCGAGAGCAAG3' Reverse: 5'CGTGGCTGAGAAGTCAACTACTA 3'
Trx2(h)	Forward: 5'CTGGTGGCCTGACTGTAACAC 3' Reverse: 5'TTGTCAAGGAGATCCTCGTGG3'
β-actin(h)	Forward: 5'CATGTACGTTGCTATCCAGGC3' Reverse: 5'CTCCTTAATGTCACGCACGAT3'
Trx1(m)	Forward: 5'CATGCCGACCTTCCAGTTTTA3' Reverse: 5'TTTCCTTGTTAGCACCGGAGA3'
β-actin(m)	Forward: 5'GTGACGTTGACATCCGTAAAGA3' Reverse: 5'GCCGGACTCATCGTACTCC3'

4.2.12 Estimation of enzyme activities

Trx and TrxR activity was determined by the micromethod of insulin reduction as described elsewhere [480]. All assay tubes contained 0.26M HEPES, pH 7.6, 10mM EDTA, 2mM NADPH, 1mM insulin and 100nM purified E. coli TrxR and cell extract in final volume of 100µl. After incubating at 37^0C for 20min reaction was stopped by adding 500µl stopping solution containing 0.2M Tris-HCl, 6M guanidine-HCl, 1mM DTNB and absorbance was measured at 412nm against reagent blank. TrxR activity was also determined by same method except purified human Trx was included in the assay mixture to catalyze insulin reduction. Reagent blanks were included to determine the background content of SH groups in samples.

4.2.13 ELISA

EL4 cells (1 x 10^6/ml) treated with baicalein (100µM, 24h) were harvested and whole cell extracts were probed for levels of Trx1 or TrxR1 by ELISA using antibody coated wells provided by Cayman Chemicals as per manufacturer's protocol.

4.2.14 Electrophoretic mobility-shift assay (EMSA)

Jurkat or EL4 cells (2 x 10^6/ml) exposed to IR 4Gy were harvested at different time intervals and nuclear extracts were probed for EMSA [264] by incubating with ^{32}P-end-labeled (5'-TGGGGAACCTGTGCTGATCACTGGAG-3') Nrf-2 oligonucleotide as described earlier [481]. EMSA was performed by incubating 8 µg of nuclear protein with 16 fmol of ^{32}P-end-labeled, Nrf-2 oligonucleotide in the presence of 2 µg of poly(2′-deoxyinosinic–2′-deoxycytidylic acid) in binding buffer (20mM Hepes, pH 7.9, 0.4mM EDTA, 0.4mM DTT and 5% glycerol) for 30 min at 37°C. The DNA–protein complex formed was separated from free oligonucleotide on 7.6% native polyacrylamide gels using buffer containing 50mM Tris, 400mM glycine, and 2mM EDTA, pH 8.5. The gel was dried and exposed on a PhosphorImager screen and the bands were

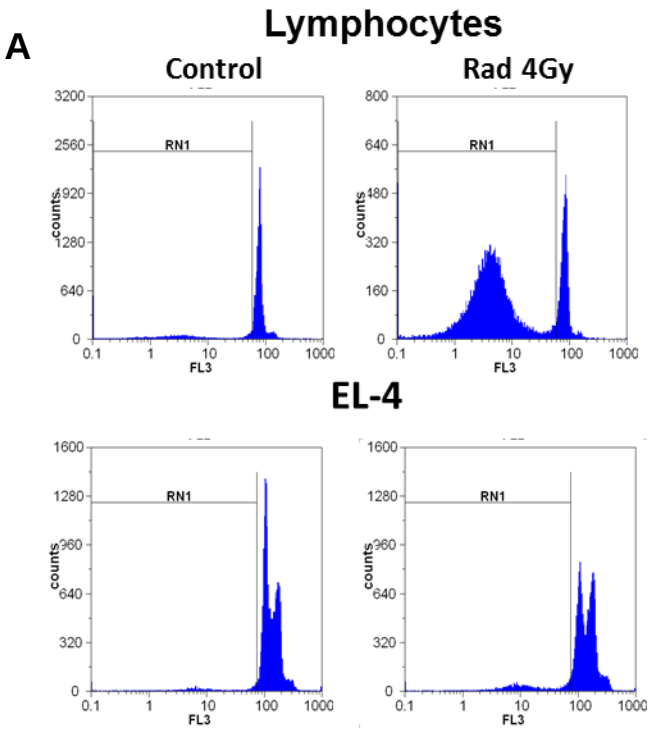
visualized using a phosphorImage scanner (Amersham). Cold oligonucleotide of same sequence in excess and labeled mutant oligonucleotide alone or in combination with wild type labeled oligonucleotide were used to confirm the specificity of Nrf-2 band. The dried gel was exposed to phosphorimage plate and the radioactive bands were visualized using a PhosphorImage plate scanner (Amersham Biosciences, USA).

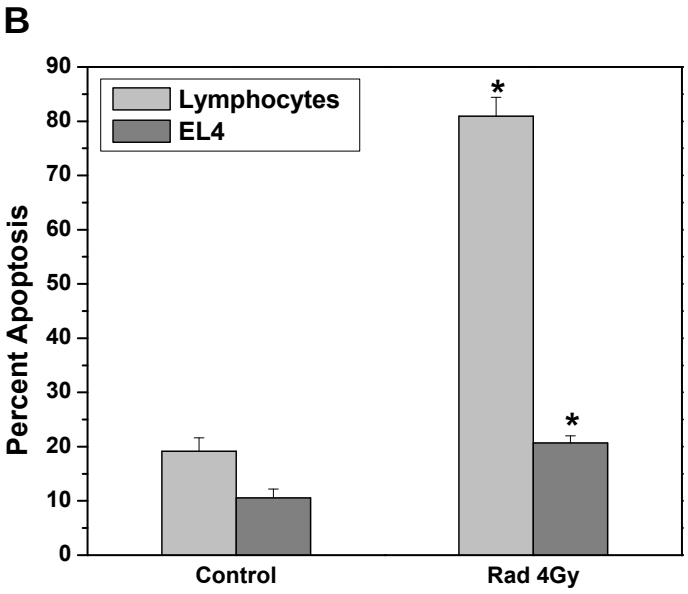
4.2.15 Statistical analysis Data are presented as mean ± SEM from three replicates in each experiment. For each parameter three independent experiments were carried out. The statistical analysis was done using performing ANOVA using Microcal Origin 6.0 software. *refers to $p<0.05$, as compared to control and # refers to $p<0.05$, as compared to irradiated cells.

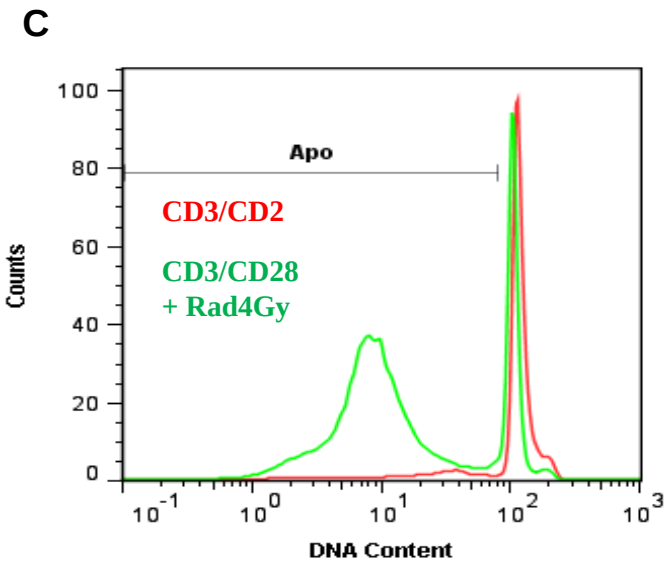
4.3. Results

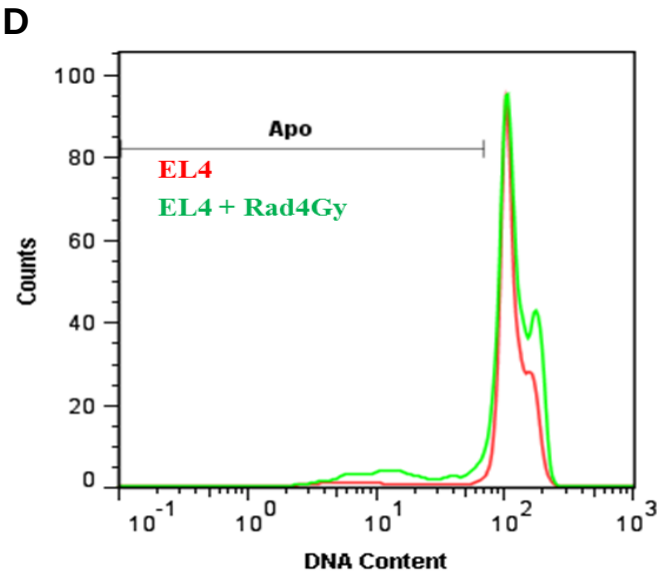
4.3.1 EL-4 cells are more resistant to IR induced cell death as compared to normal as well as activated murine splenic lymphocytes

Fig. 4.2 shows IR induced apoptosis in mouse T lymphoma cell line EL-4 vs. resting and activated mouse splenic lymphocytes (Fig. 4.2A and B). IR induced apoptosis in about 60% of murine splenic lymphocytes. EL-4 lymphoma cells showed significantly lower radiation induced apoptosis (~10%) as compared to murine lymphocytes. Normal lymphocytes are non-proliferating. Hence antibody stimulated lymphocytes were used as additional control. Anti CD3/CD28 stimulated lymphocytes exhibited significantly higher cell death as compared to EL-4 cells in response to radiation (Fig. 4.2C-E).


A
Lymphocytes
Control
Rad 4Gy
RN1
counts
FL3
EL-4


B
Lymphocytes
EL4
Percent Apoptosis
Control
Rad 4Gy
*


C
Apo
CD3/CD2
CD3/CD28
+ Rad4Gy
Counts
DNA Content


D
Apo
EL4
EL4 + Rad4Gy
Counts
DNA Content

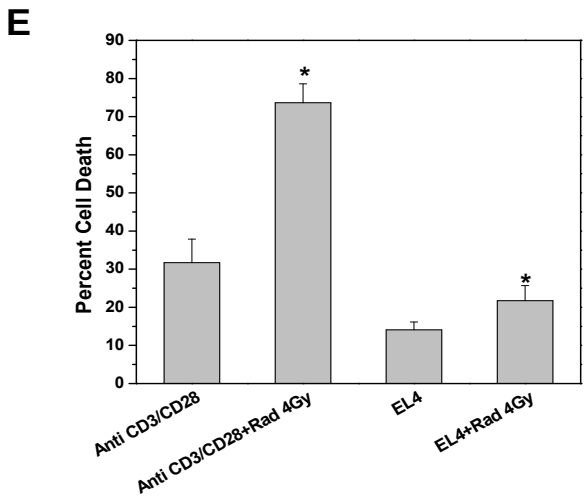


Fig. 4.2. EL-4 cells are more resistant to ionizing radiation induced cell death as compared to normal murine lymphocytes *(A) Flow cytometric profile of propidium iodide stained normal murine splenic lymphocytes cultured for 24h post 4Gy irradiation. Pre-G1 peak (gate RN1) represents apoptotic population. (B) Bar graph represents percent apoptotic cells. (C-E) 1x10^6 splenic lymphocytes were stimulated with anti-CD3/anti-CD28 antibodies for 72h in 24-well plate or EL-4 cells were exposed to IR (4Gy) and cultured for 24h. Cells were stained with propidium iodide and acquired on flowcytometer.*p<0.05, as compared to activated and irradiated lymphocytes.*

4.3.2 Murine T cell lymphoma cells have active redox circuits

Basal and IR induced levels of GSH were estimated in normal lymphocytes and lymphoma cells by conventional enzyme cycling method. There was a significant decrease in GSH/GSSG ratio post-irradiation in normal as well as tumor cells at all the time points (Fig. 4.3A). EL-4 cells showed increase in Trx activity from 2 to 12 h after exposure to 4Gy radiation which correlated with their higher radioresistance (Fig. 4.3B). Basal levels of cellular ROS (hydroxyl, superoxide and H_2O_2) were significantly lower in tumor cells as compared to their normal counterpart (Fig. 4.3C).

4.3.3 Inhibition of ERK or Nrf-2 increased radio-sensitivity of EL4 cells

EL-4 cells were incubated with pharmacological inhibitors of ERK(U0126) or JNK(JNKi) or P38 (P38i)or Nrf-2(ATRA)or HO-1(SnPP) or TrxR(auranofin) or NF-κB inhibitory peptide prior to exposure to 4Gy and cultured for 48h. Inhibitors of ERK, Nrf-2, HO-1 and TrxR significantly

enhanced radiation induced cell death in EL-4 cells suggesting their potential role in cellular radioresistance (Fig. 4.4A). EL-4 cells were transfected with ERK shRNA or Nrf-2 shRNA or (ERK+Nrf-2) shRNA plasmids and cultured for 48h. Then cells were washed with medium and exposed to 4Gy IR. Cells transfected with scrambled shRNA plasmids or vector alone and exposed to radiation served as control. Cell death was measured by PI staining followed by flow cytometry or DNA ladder assay. ERK or Nrf-2 single knockdown cells showed higher radiation induced apoptosis as compared to wild type cells. There was a significant increase in radiation induced apoptosis in ERK and Nrf-2 double knockdown cells as compared to wild type cells or single knock down cells (Fig. 4.4B). DNA fragmentation which is a hallmark of apoptosis was assessed in wild type and knockdown cells post-radiation exposure. These results also confirm that ERK and Nrf-2 are essential for tumor cell survival under normal conditions (Fig. 4.4C).

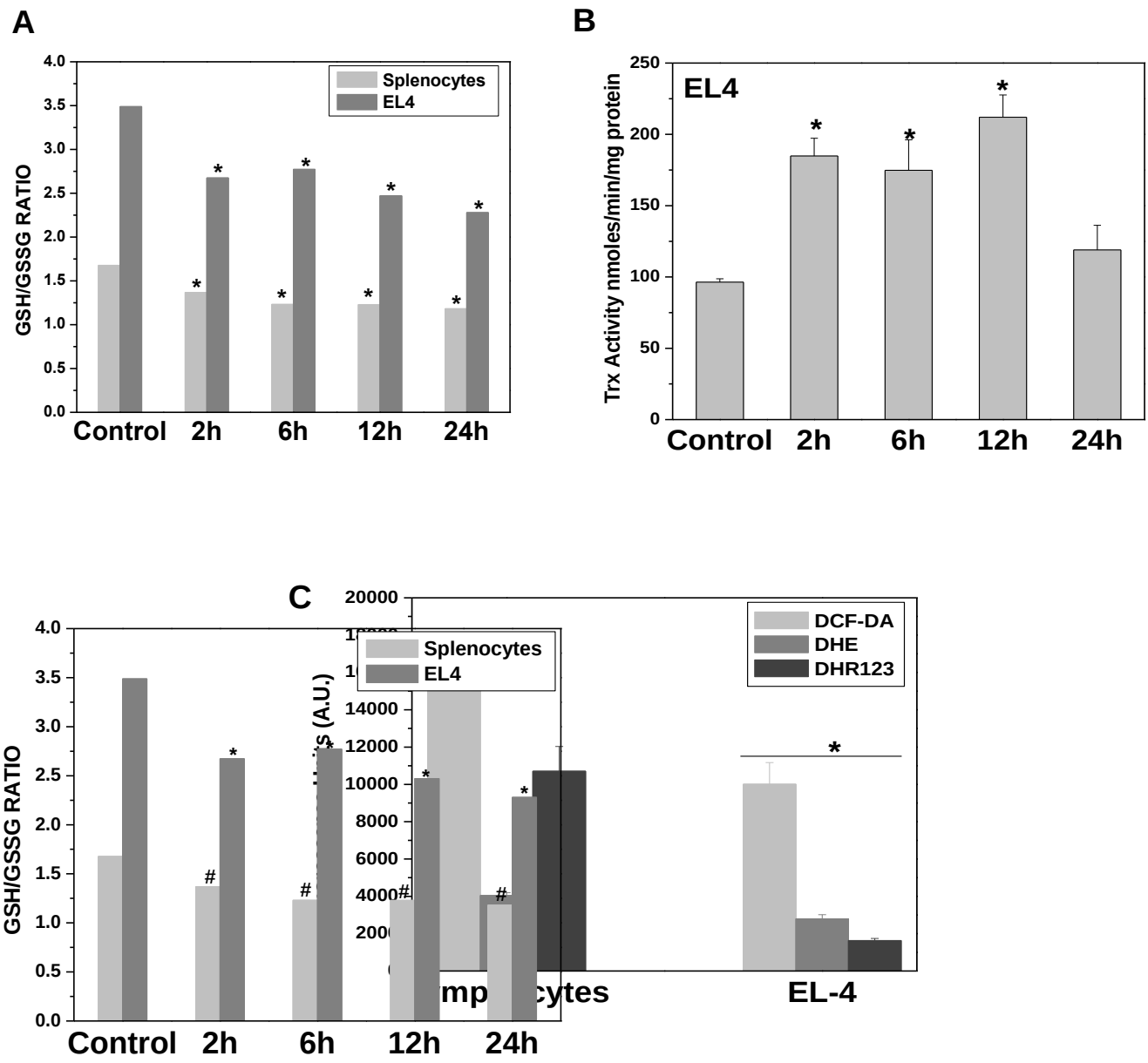


Fig. 4.3 Murine T cell lymphoma have active redox circuit *(A) Intracellular GSH levels were measured at 2, 6, 12 or 24 h post irradiation (4Gy) in normal lymphocytes and EL-4 lymphoma cells. Bar graph represents GSH/GSSG ratio in normal murine splenic lymphocytes and EL-4 cells. (B) Thioredoxin activity in wild type EL-4 cells at 2, 6, 12 & 24 h post IR4Gy was measured. Bar graph shows thioredoxin activity in EL-4 cells. (C) ROS levels in normal splenic lymphocytes and EL-4 cells were estimated by staining with DCF-DA (20μM), DHE or DHR123 (5μM each) 30min at 37°C followed by fluorescence emission measured at their respective wavelength. Bar graph shows relative fluorescence units indicating ROS levels. *p<0.05, as compared to control.*

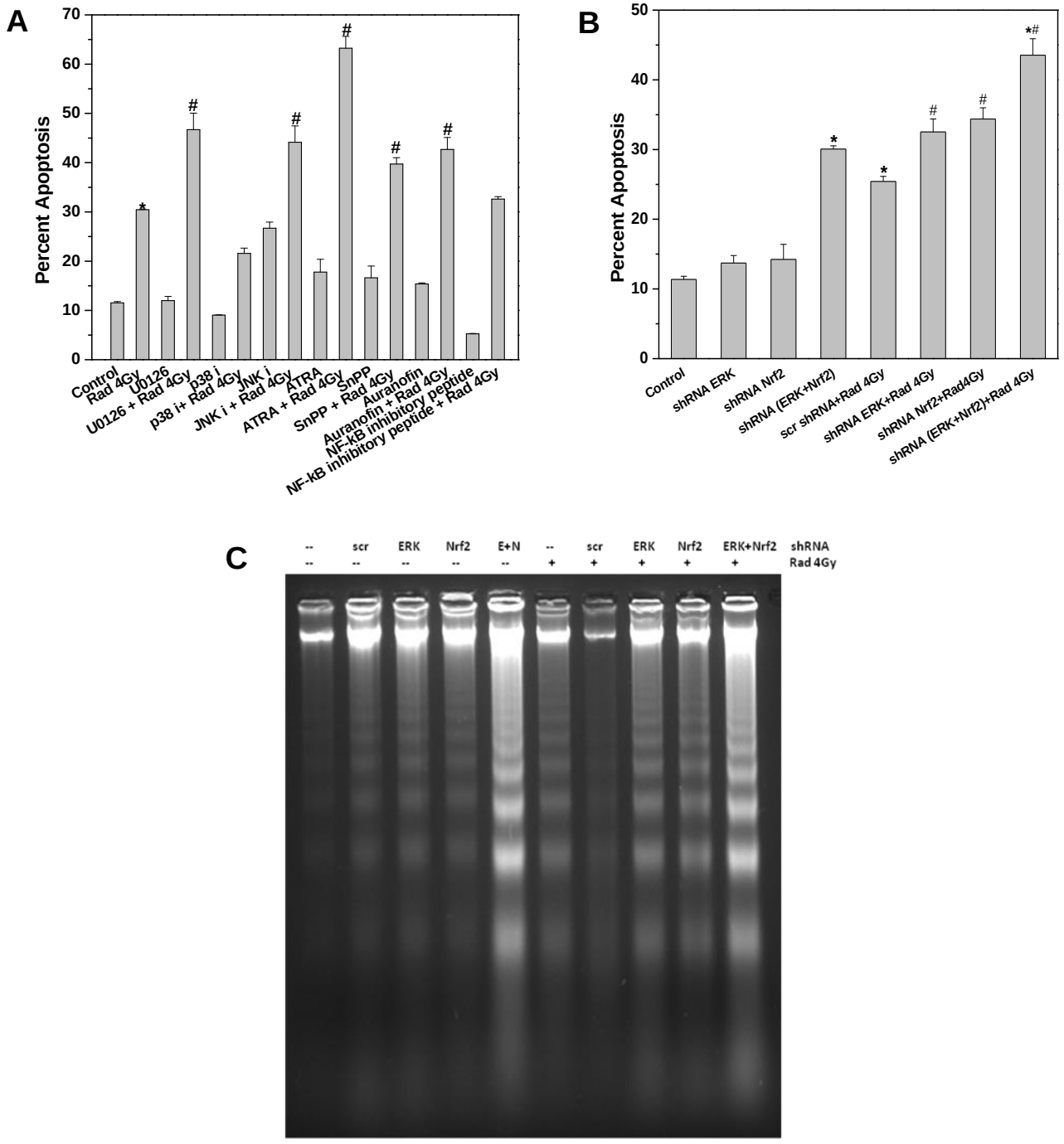


Fig. 4.4 ERK and Nrf-2 inhibitor increased radiosensitivity of EL4 cells *(A) EL-4 cells were incubated with different concentration of pharmacological inhibitors of ERK (10μM for 2h) or JNK (10μM for 2h) or P38 (10μM for 2h) or NF-kB inhibitory peptide (10μM for 2h) or ATRA (5μM for 2h) or SnPP (5μM for 2h) or auranofin (25nM for 2h) or Ras (10μM FTA for 2 h) and were cultured for 48h in 5% CO_2 at 37^0C with or without exposure to IR 4Gy. Cell death*

*was analyzed by propidium iodide staining and flow cytometry. Bar graph represents radiation induced apoptosis in EL-4 cells incubated in the presence of different inhibitors. (B) EL-4 cells transfected with scrambled shRNA or ERK or Nrf-2 shRNA plasmidwere exposed to IR 4Gy and cultured for 48h. Apoptosis was estimated by propidium iodide staining and flow cytometry. The frequency of apoptotic cells (gate RN1) is shown in the bar graph. (C) Genomic DNA from wild type and knock down EL-4 cells cultured for 48h post radiation exposure or alone was resolved on agarose gel and stained with ethidium bromide. DNA ladder indicates cells undergoing apoptosis. *p<0.05, as compared to control and # p<0.05, as compared to IR 4Gy.*

4.3.4 Jurkat Cells but not human PBMC displayed resistance to IR induced apoptosis

Jurkat cells exposed to 4Gy IR were cultured for different time periods (16h, 24h, 36h & 48h) and analyzed for cell death by flow cytometry. It was observed that IR exposure led to 30% increase in cell death over control at 48h (Fig. 4.5A & B), whereas at 16h, cells were arrested in S+G2/M phase. To compare the effect of similar dose of IR on corresponding normal cells, human PBMC were exposed to IR 4Gy and cultured for 24h. Exposure to IR led to ~70% cell death over control in human PBMC at 24h. On the contrary, only ~5% cells underwent cell death at 24h in Jurkat cells (Fig. 4.5C & D).

4.3.5 IR induced Nrf-2 in Jurkat cells

A significant increase in DNA binding of Nrf-2 at 6h post IR exposure (Fig. 4.6A) was observed. Band of Nrf-2 was confirmed with the use of cold and mutant oligonucleotides (Fig. 4.6B).

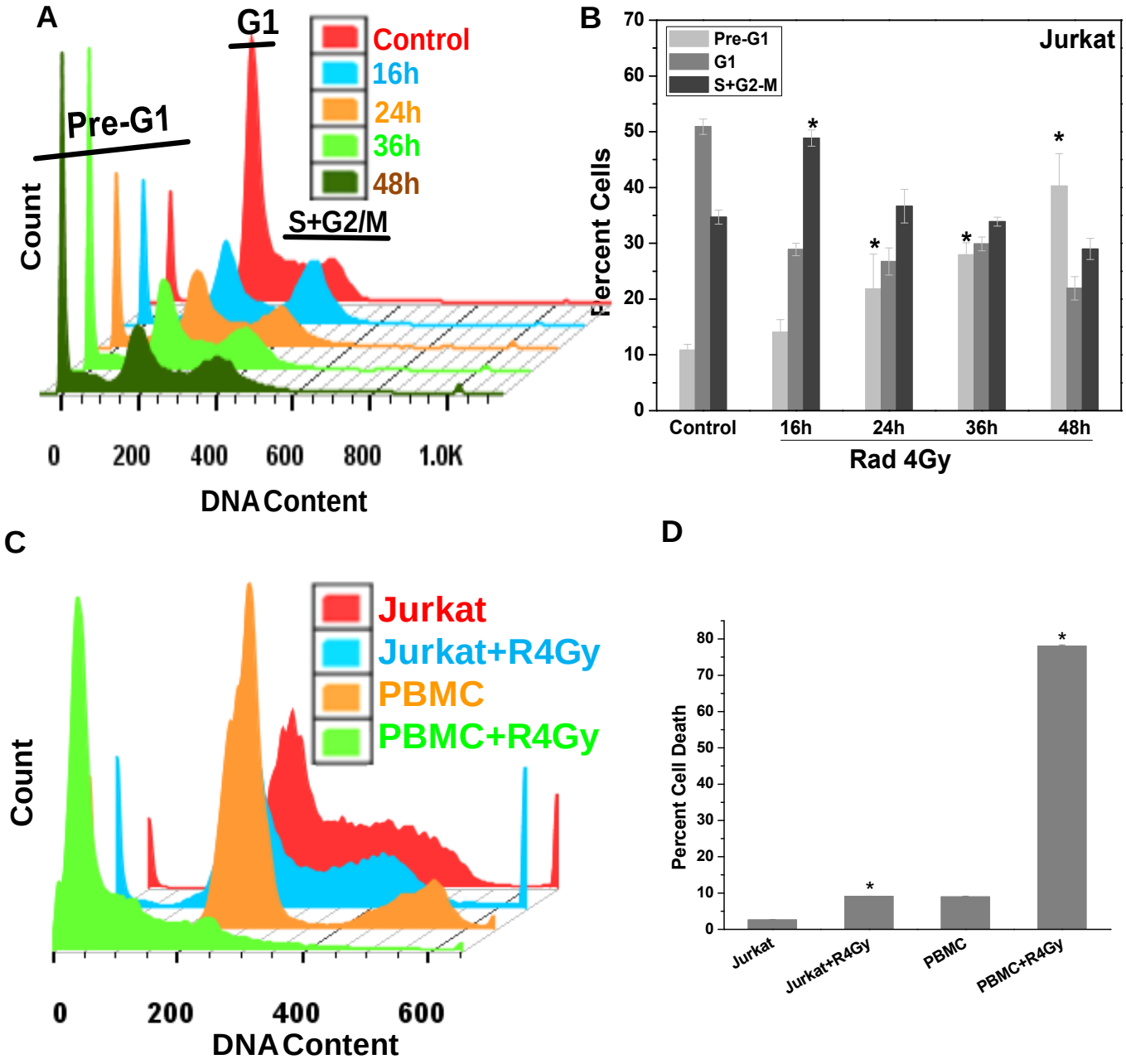


Fig. 4.5 Jurkat cells but not human PBMC are resistant to IR induced apoptosis *(A&B) Jurkat cells (4X10^5) or (C&D)* human PBMC (1X10^6) *were exposed to IR4Gy and cells were harvested at indicated time points. Cells were stained with PI (50µg/ml) and acquired on Partec, Cyflow flowcytometer. Cell cycle and Pre-G1 peak analysis was carried out using FlowJo Software. Representative flow cytometric histograms are shown. Bar graph represents percent cell death.*p<0.05, as compared to control.*

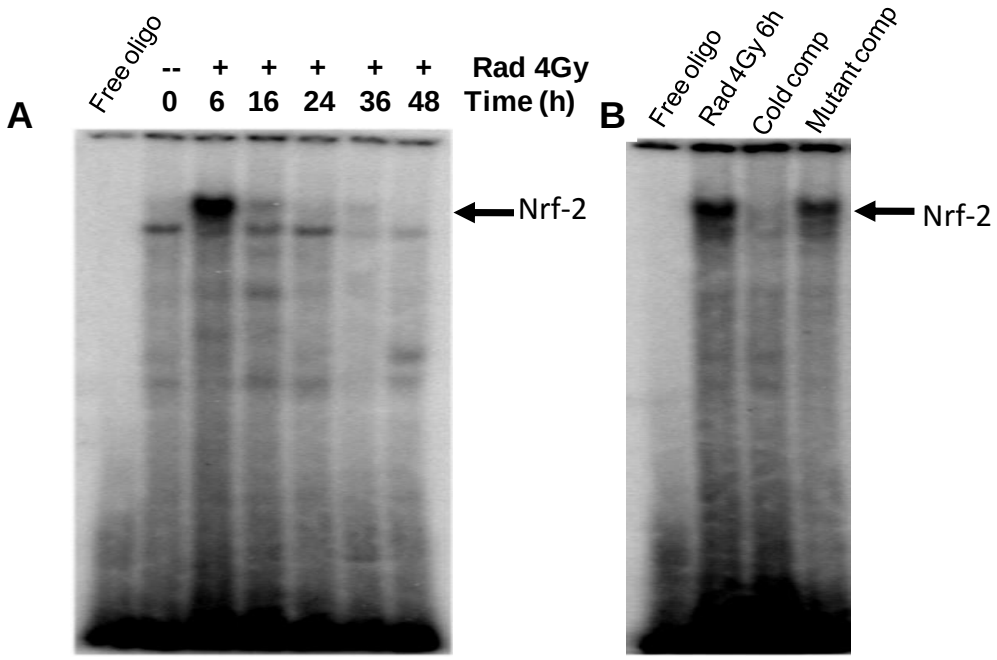
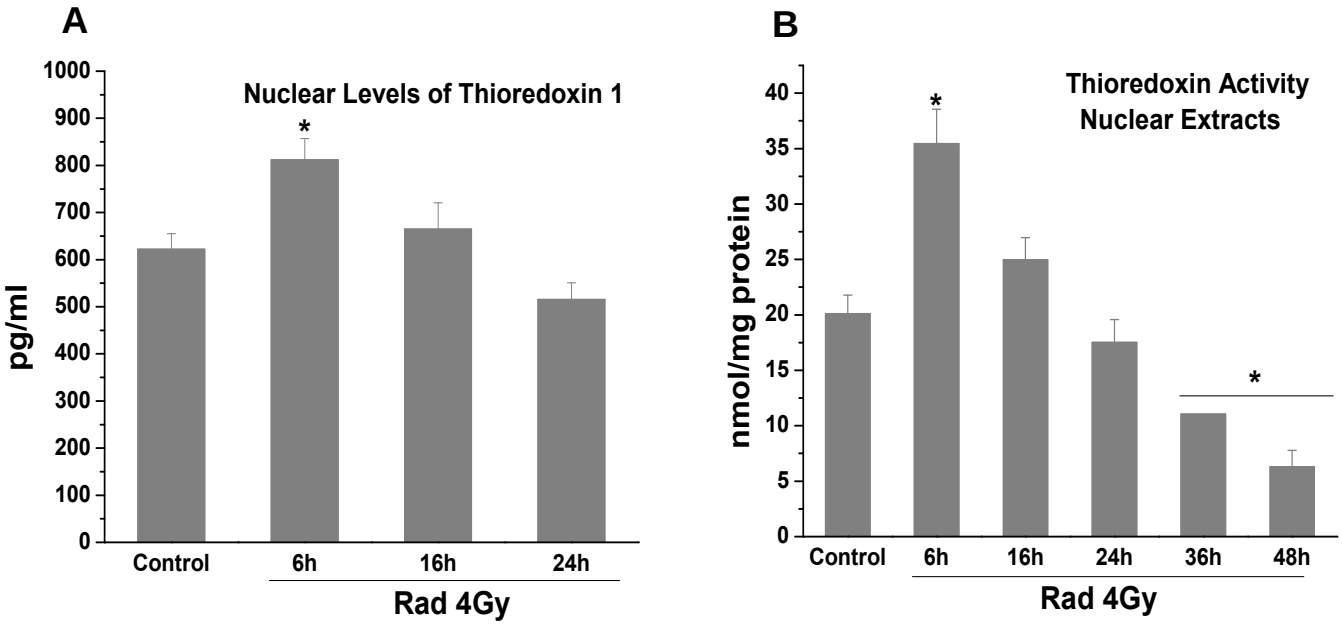


Fig. 4.6 Ionizing radiation induced Nrf-2 in Jurkat cells: *(A & B) Jurkat cells ($2x10^6$) exposed to IR 4Gy were harvested at indicated time points. Nuclear extracts were prepared and probed for Nrf-2. Gel shift assay was performed with Nrf-2 wild type and mutant consensus sequences. Representative image is shown.*



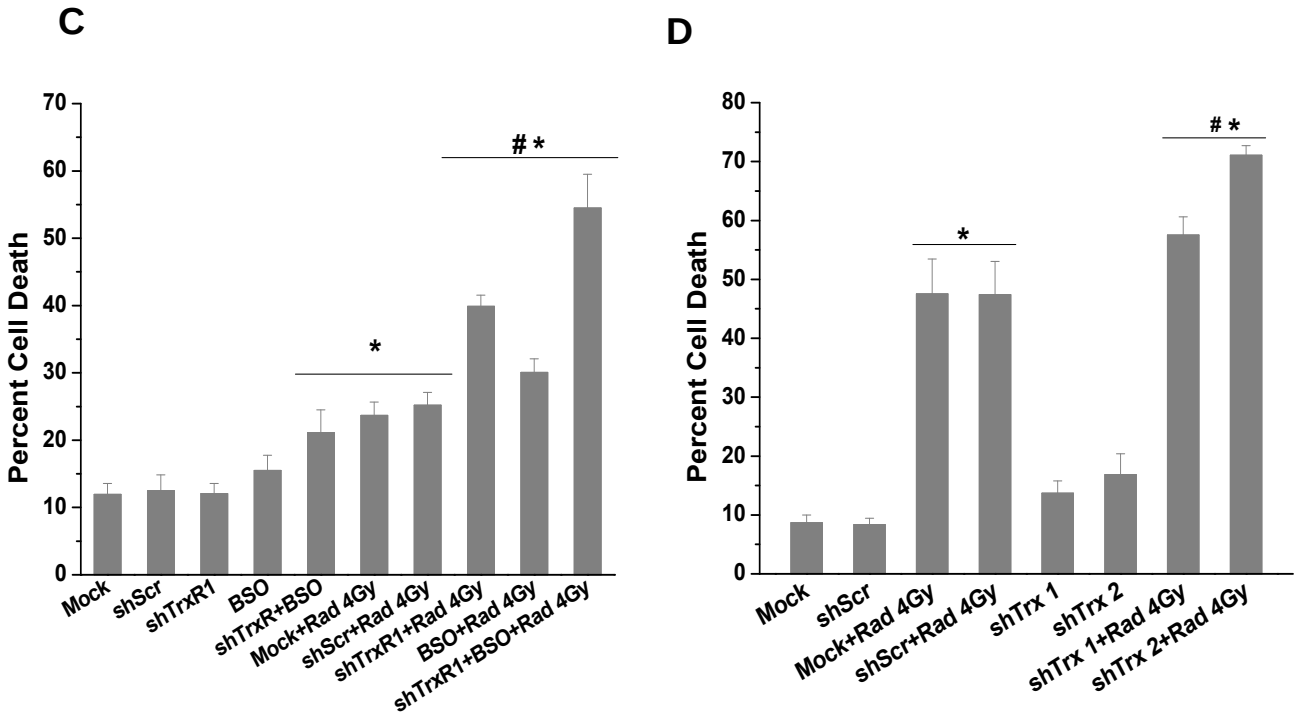


Fig. 4.7 GSH and thioredoxin system is involved in determining radioresistance in Jurkat cells *(A) Nuclear extracts from Jurkat cells exposed to IR 4Gy were probed to detect levels of Trx1 by ELISA. Bar graph represents pg/ml Trx1. (B) Trx activity was determined in the nuclear extracts of Jurkat cells exposed to IR 4Gy. Bar graph represents Trx activity expressed as nmol/mg protein. (C) Jurkat cells ($1x10^6$) were electroporated using shRNA plasmids of TrxR1/scrambled sequence and cultured for 48h or treated with BSO for 24h. Cells were subsequently exposed to 4Gy IR and cultured for 48h. Cells were harvested, stained with PI and acquired on flow cytometer for Pre-G1 peak analysis. Bar graph represents percent cell death. (D) Jurkat cells ($1x10^6$) were electroporated using shRNA plasmids of Trx1/Trx2/scrambled sequence and cultured for 48h. Cells were exposed to IR 4Gy and analyzed for Pre-G1 population by PI staining followed by flow cytometry. Bar graph represents percent cell death.* $p<0.05$, as compared to control and # $p<0.05$, as compared to IR 4Gy.*

4.3.6 GSH and thioredoxin system is involved in determining radioresistance in Jurkat cells

Binding of transcription factors to DNA is a Trx dependent process [482] [388] [357]. It was observed that, IR exposure significantly increased levels (Fig. 4.7A) and activity of Trx1 (Fig. 4.7B) in nucleus at 6h which may be responsible for the DNA binding of Nrf-2. These results revealed that time point at which there was enhanced DNA binding of Nrf-2, coincided with

increased activity of Trx in the nuclear extracts. This indicated the association of Trx system in assisting activation of oxidative stress induced DNA binding of Nrf-2. Glutathione synthesis was inhibited using BSO, an inhibitor of glutamate cysteine ligase catalytic subunit (GCLC), which is an enzyme catalyzing the rate limiting step in GSH synthesis. TrxR was inhibited using pharmacological inhibitor, auranofin. Depletion of GSH or inhibition of TrxR led to significant increase in ROS levels in the absence of radiation which was further enhanced after radiation exposure (Fig. 4.7C). When cells were exposed to IR, there was a significant increase in cell death as compared to IR alone (Fig. 4.7C). When GSH synthesis was blocked along with knockdown of TrxR1, it displayed a synergistic effect in cell death. Interestingly, when Trx1/2 knockdown cells were exposed to IR they also displayed increased cell killing. Especially, when Trx2 knockdown cells were exposed to IR, cells displayed significant increase in cell death as compared to Trx1 (Fig. 4.7D).

4.3.7 Baicalein induced cell death in EL4 cells

To investigate the effect of baicalein treatment on tumor counterpart of normal murine splenic lymphocytes i.e. murine T cell lymphoma EL4 cells were used. Baicalein induced apoptosis in EL4 cells in a dose dependent manner as assessed by propidium iodide staining followed flow cytometry and Pre-G1 peak analysis (Fig. 4.8A). Baicalein treatment lead to significant loss of mitochondrial membrane potential (Fig. 4.8B), time dependent increase in TUNEL positive cells (Fig. 4.8C & D) and progressive increase in annexin V positive cells (Fig. 4.8E & F) at indicated time points.

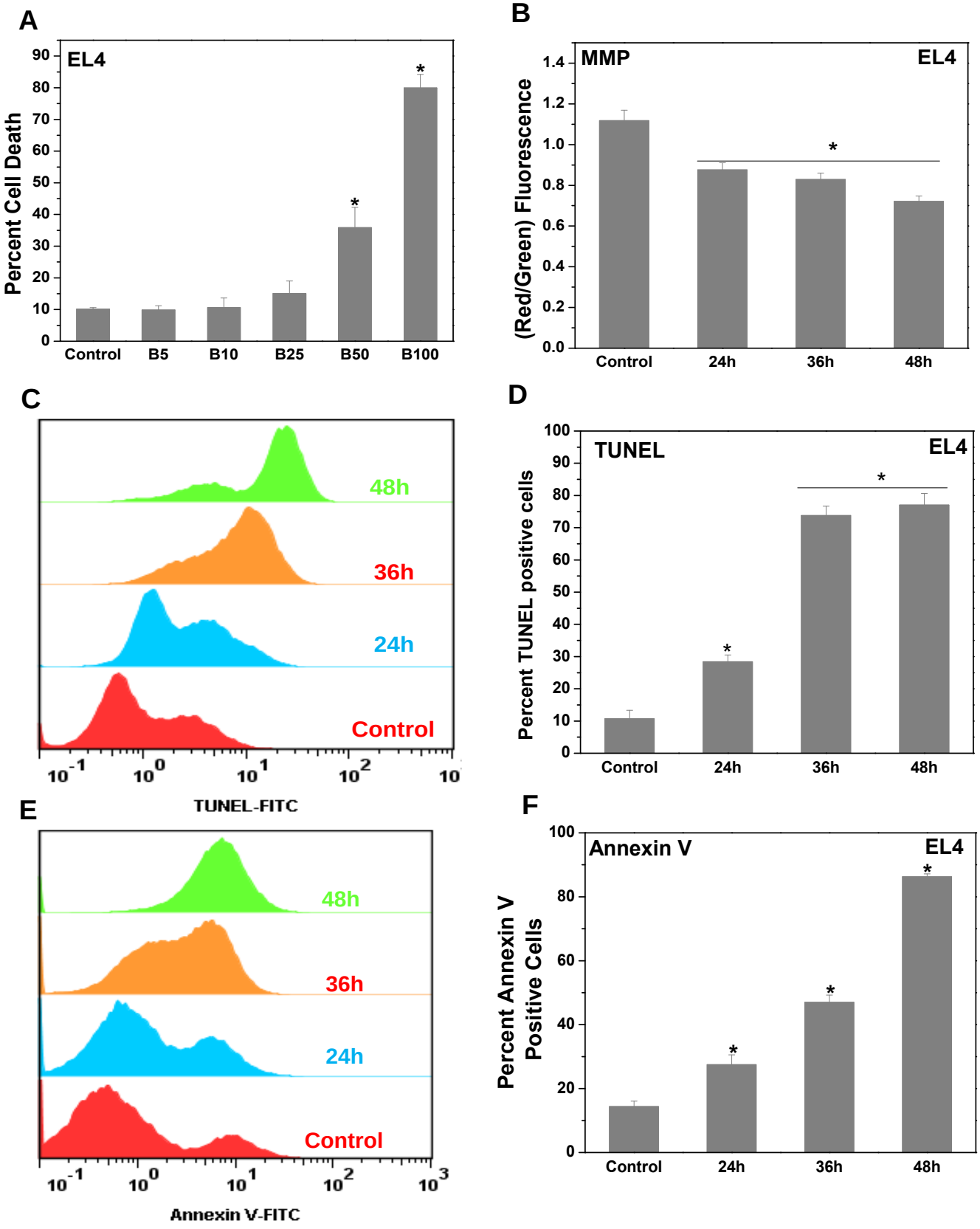

A
EL4
Percent Cell Death
Control
B5
B10
B25
B50
B100
B
MMP
EL4
(Red/Green) Fluorescence
Control
24h
36h
48h
C
48h
36h
24h
Control
TUNEL-FITC
D
TUNEL
EL4
Percent TUNEL positive cells
Control
24h
36h
48h
E
48h
36h
24h
Control
Annexin V-FITC
F
Annexin V
EL4
Percent Annexin V Positive Cells
Control
24h
36h
48h

Fig. 4.8 Baicalein induced cell death in EL4 cells *(A) EL4 cells were treated with baicalein (5, 10, 25, 50 & 100μM) and cultured for 48h at 37^{0}C. Cells were harvested, stained with propidium iodide and acquired on a flow cytometer. Percent cell death was calculated by sub-G1 peak analysis. Bar graph represents percent cell death. (B) EL4 cells treated with baicalein for indicated time points were harvested, stained with JC-1 dye, incubated at 37^{0}C for 30 min and fluorescence was measured using spectrofluorimeter. Bar graph represents ratio of red/green fluorescence. EL4 cells were treated with baicalein (100μM) for indicated time points. Cells were harvested, stained with TUNEL-FITC and acquired on flow cytometer. Flow cytometric histograms are shown (C). Bar graph represents percent TUNEL-FITC positive cells (D).EL4 cells treated with baicalein for indicated time points were harvested, stained with Annexin V-PE and acquired on flow cytometer. Flow cytometric histograms are shown (E). Bar graph represents percent annexin V positive cells (F).* p<0.05, as compared to control.*

4.3.8 Baicalein induced cell death is via inhibition of Trx system

The effect of baicalein on the levels and activity of the components of Trx system namely Trx and TrxR was examined. Baicalein treatment led to inhibition of TrxR activity in cell free system (Fig. 4.9A). Incubation of EL4 cells with baicalein lead to inhibition of TrxR activity (Fig. 4.9B & C) and suppressed their levels (Fig. 4.9D). It is known that inhibition of Trx leads to increase in levels of pro-apoptotic protein ASK1. Hence the levels of pASK1 in baicalein treated EL4 cells were investigated. Baicalein treatment significantly increased phosphorylation of ASK1at 48h (Fig. 4.9E). Over expression of Trx1 abolished anti-tumor effects of baicalein (Fig. 4.9F).

4.4. Discussion

Differential radio-sensitivity of normal and tumor cells depend on the inherent capacity to prevent or repair DNA damage [483, 484]. Since, ROS are the principal mediators of IR induced DNA damage, prevention of this damage and manifestation of radio-resistance depends on efficiency of antioxidant defense mechanism inside the cell.

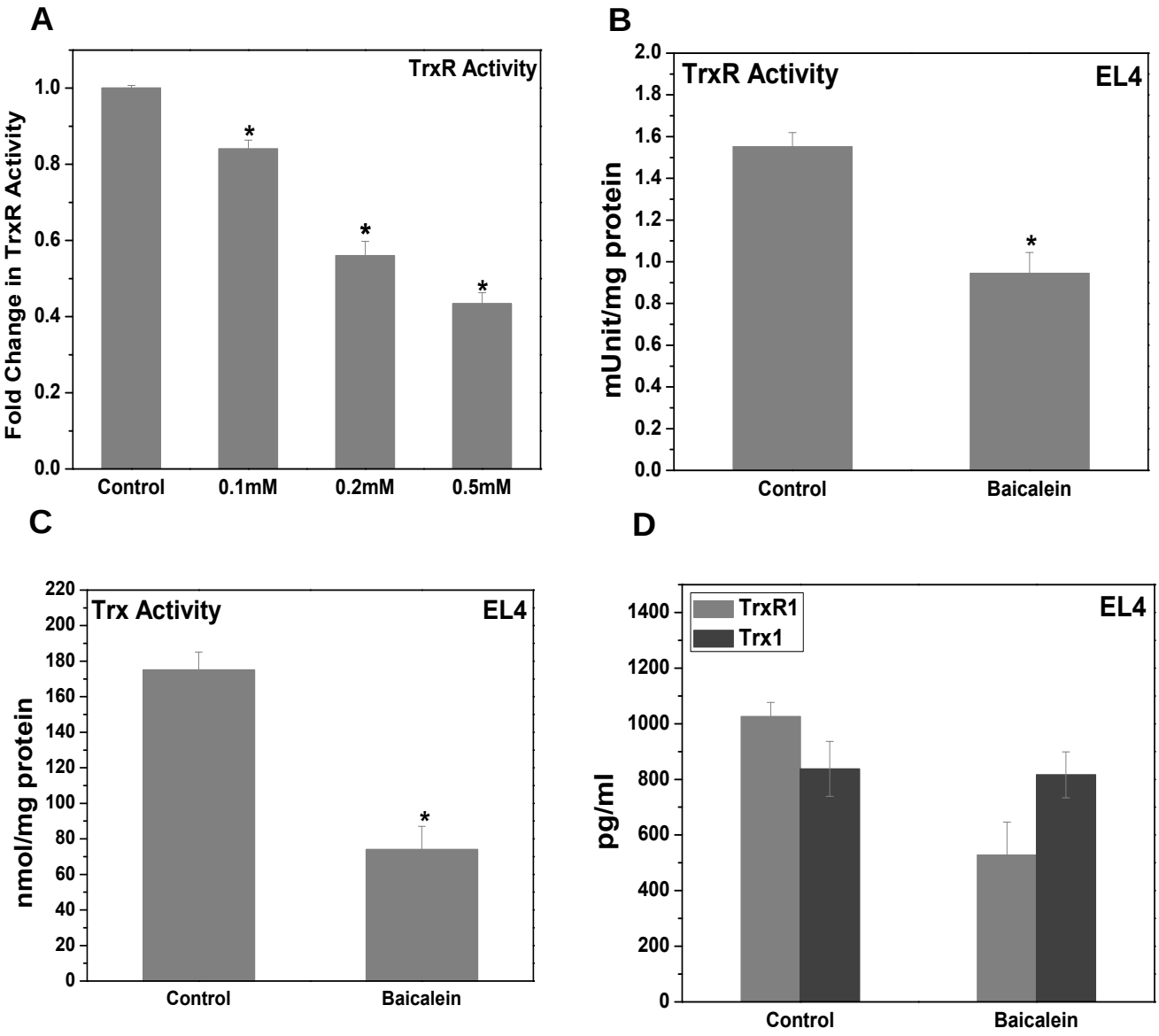


Fig. 4.9 Baicalein induced cell death is via inhibition of Trx system *TrxR was incubated with different concentrations of baicalein (0.1, 0.2 & 0.5mM) for 30 min at 37^0C. Bar graph represents fold change in TrxR activity (A). EL4 cells treated with baicalein (100μM, 16h) were harvested, extracts were probed for estimation of thioredoxin and thioredoxin reductase activity. Bar graph represents thioredoxin reductase activity expressed in mUnit/mg protein (B) and thioredoxin activity expressed in nmol/min/mg protein (C).Baicalein treated EL4 cells were harvested and extracts were probed for detection of levels of Trx1 or TrxR1 by ELISA. Bar graph represents Trx1 or TrxR1 levels expressed in pg/ml (D). *p<0.05, as compared to control.*

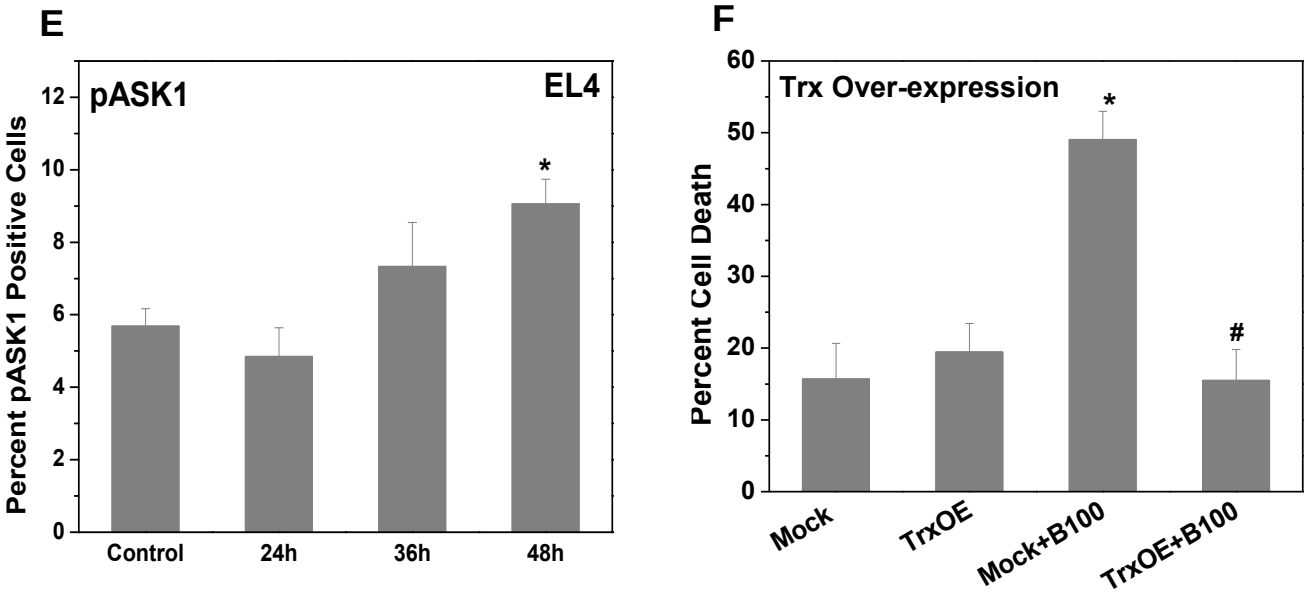


Fig. 4.9 Baicalein induced cell death is via inhibition of Trx system EL4 cells were treated with baicalein (100μM) for indicated time points. Cells were harvested, stained with pASK1 antibody and acquired on flow cytometer. Bar graph represents percent pASK1 positive cells (E). EL4 cells were transfected with Trx over-expression plasmidand were treated with baicalein (100μM, 48h), harvested at the end of the incubation, stained with propidium iodide and acquired on a flow cytometer. Percent cell death was calculated by sub-G1 peak analysis using FlowJo software. Bar graph represents percent cell death in EL4 cells (F). *p<0.05, as compared to control, #p<0.05, as compared to baicalein treated group.

Survival of cells exposed to IR depends on how efficiently cellular redox imbalance is restored to homeostatic conditions. Restoration of redox homeostasis depends on efficiency of constitutive antioxidant defense systems and their interplay with inducible oxidative metabolism [389] [485]. Shankar et al., has shown that decreased generation of radiation induced ROS in mouse lymphoma cells was associated with reduced extent of radiation induced apoptosis compared to that in normal lymphocytes [450]. In the present study role of cellular redox signaling in murine lymphocytes and EL-4 lymphoma cells after exposure to IR was further investigated.

Further, the ratio of GSH to GSSG was significantly higher in EL-4 lymphoma cells as compared to normal lymphocytes under basal conditions. However, there was a significant decrease in

GSH/GSSG ratio in both lymphocytes and EL-4 cells after exposure to IR suggesting their involvement in restoration of cellular reduction potential. On the contrary, activity of Trx increased significantly in EL-4 cells harvested up to 12 h after exposure to radiation. Increased Trx activity may restore cellular redox equilibrium in EL-4 cells and thus contribute to radioresistance.

Cellular redox balance is sensitive to disruption of intracellular thiol systems. These are sensitive to two electron oxidants and are controlled by the Trx and GSH. Cellular redox state can influence the pro-survival/pro-apoptotic signaling targets and thereby decide the fate of a cell [486]. Oxidative stress activates pro-survival signaling molecules such as MAPKs, NF-kB, and Nrf-2 etc. [487]. Pharmacological inhibitors of MAPK, Nrf-2, HO-1 and TrxR significantly enhanced radiosensitivity of EL-4 cells. It was observed that knockdown of ERK or Nrf-2 enhanced radiation induced apoptosis. Further, the double knock down cells were more sensitive to IR induced cell death as compared to single knockdown or wild type cells. **Fig. 4.10** summarizes these findings.

Similarly, Jurkat cells exhibited resistance to IR induced apoptotic death. Nrf-2 constitutes a unique “redox switch” that can be turned on in response to redox imbalance caused by oxidative and electrophilic stresses [456, 488] [458]. However, such adaptive response to external stress is normally transient and prone to be readily saturated [488].

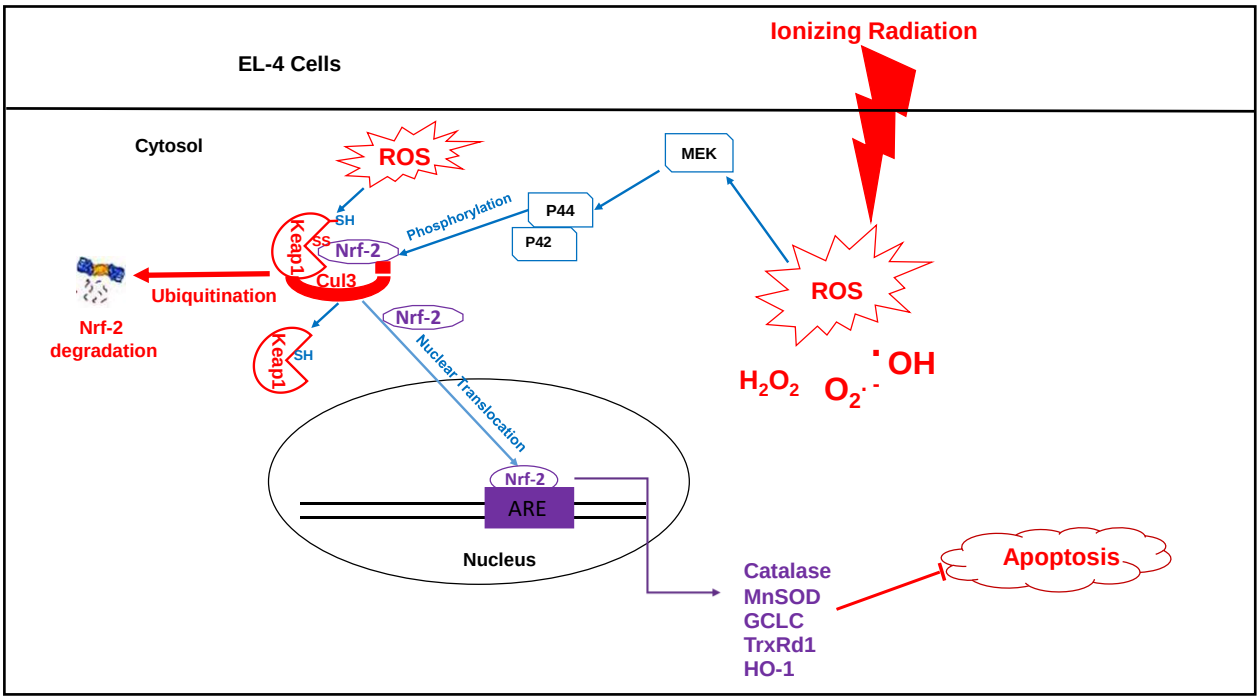


Fig. 4.10. Moleular players regulating radioresistance of EL4 cells

The dysregulation of Nrf-2 in cancer has been suggested to protect and offer growth advantages to various cancers and may offer resistance to chemotherapy [452]. Jurkat cells post radiation exposure showed significant increase in DNA binding of Nrf-2.

Nuclear accumulation and DNA binding of Nrf-2 requires phosphorylation at Ser-40 residue. DNA binding of Nrf-2 is a Trx dependent process. It was observed that, levels and activity of components of Trx system increased significantly post IR exposure at the time point when there was enhanced DNA binding of Nrf-2. These results and literature reports suggest that, Trx system might have provided assistance in DNA binding of Nrf-2 after IR exposure in Jurkat cells. Nrf-2 executes transcription of a myriad genes involved in mediating antioxidant response against oxidative stress.

Glutathione and Trx systems are the two major constitutive antioxidant regulatory systems inside cells that compensate and complement with each other in maintenance of redox homeostasis [489] [490]. The Trx/TrxR system regulates protein thiol content in cell [491]. Oxidative

modification of protein thiols and subsequent loss of function is one of the contributing factors for cell death after exposure to IR. Trx1 is associated with ASK1, once it is oxidized ASK1 is released from it and downstream signaling for apoptosis gets activated [482]. Trx is involved in reduction of protein disulfides of proteins critical for cell survival that involve regulating DNA synthesis (ribonucleotide reductase) [492] and repair, antioxidant defense (peroxiredoxin), cell proliferation (PTEN), transcription factors (Nrf-2, NF-κB, AP1, P53, Hif1αetc.) [493] [390]. Trx provides reducing conditions inside nucleus for transcription factors to bind to DNA after oxidative stress [466]. Knockdown of TrxR1 along with blocking GSH synthesis significantly enhanced radiosensitivity of Jurkat cells. Similar results were observed when either Trx1 or Trx2 were knock down. **Fig. 4.11** summarizes these findings.

Further, kinetics of tumor growth and progression has direct correlation with activity of Trx system [489]. Many tumors exhibit elevated Trx and TrxR activity. Thus, Trx inhibition provides promise to limit tumor growth. Therefore, the anti-tumor activity of baicalein was also studied using lymphoma EL4 cells. Murine T cell lymphoma (EL4) cells are tumor counterpart of normal T lymphocytes.

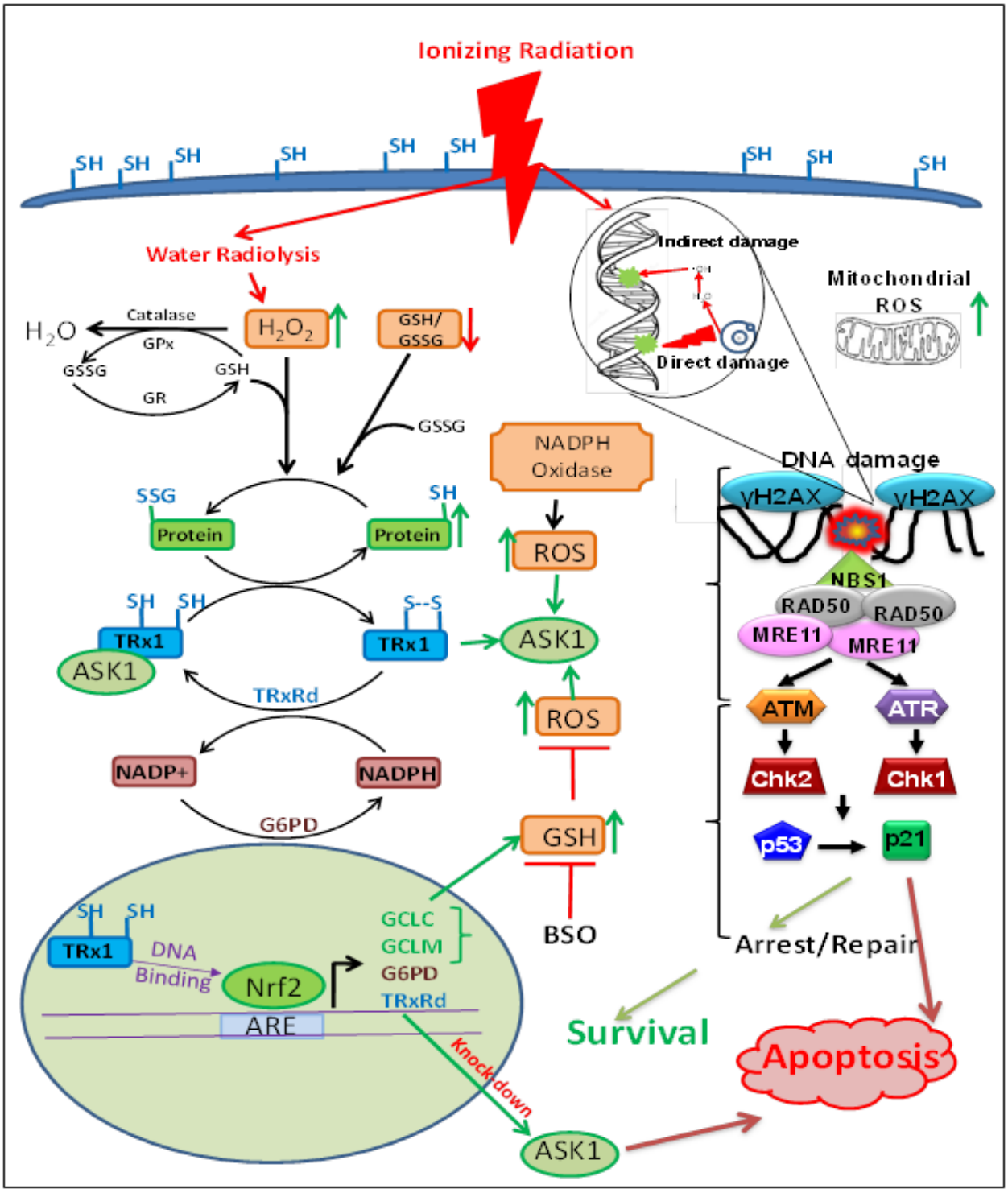


Fig. 4.11. Radioresistance in Jurkat cells

Baicalein induced cell death in EL4 cells in a dose dependent manner at 48h. This was confirmed by increase in percentage of TUNEL and annexin V positive cells and loss of mitochondrial membrane potential. Although, numerous reports describing anti-tumor activity of baicalein using different tumor cell types are present, studies pertaining to implication of inhibition of Trx activity for the observed anti-tumor effects of baicalein have not yet been reported in EL4 cells. Baicalein suppressed activity of Trx system without reducing their levels in EL4 cells which lead to increase in phosphorylation of pro-apoptotic protein ASK1at 48h. Further, EL4 cells transiently over-expressing Trx were refractive to baicalein mediated cell killing. Mechanism of anti-tumor action of baicalein is shown in **Fig. 4.12**.

Conclusions

1. Murine as well as human T cell lymphoma cells were resistant to IR 4Gy induced apoptosis as compared to their normal counterpart murine splenic lymphocytes or human PBMC respectively.
2. Both EL4 and Jurkat cells showed hyperactive anti-oxidant machinery in terms of GSH / GSSG ratio, nuclear levels of Nrf-2 and Trx activity.
3. Inhibition of Nrf-2 enhanced radiosensitivity of EL4 cells whereas blocking GSH and Trx resulted in significant cell death in Jurkat cells.
4. Radioprotective concentration of baicalein induced cell death in EL4 cells.
5. Baicalein mediated cell death in EL4 cells is via inhibition of Trx system.

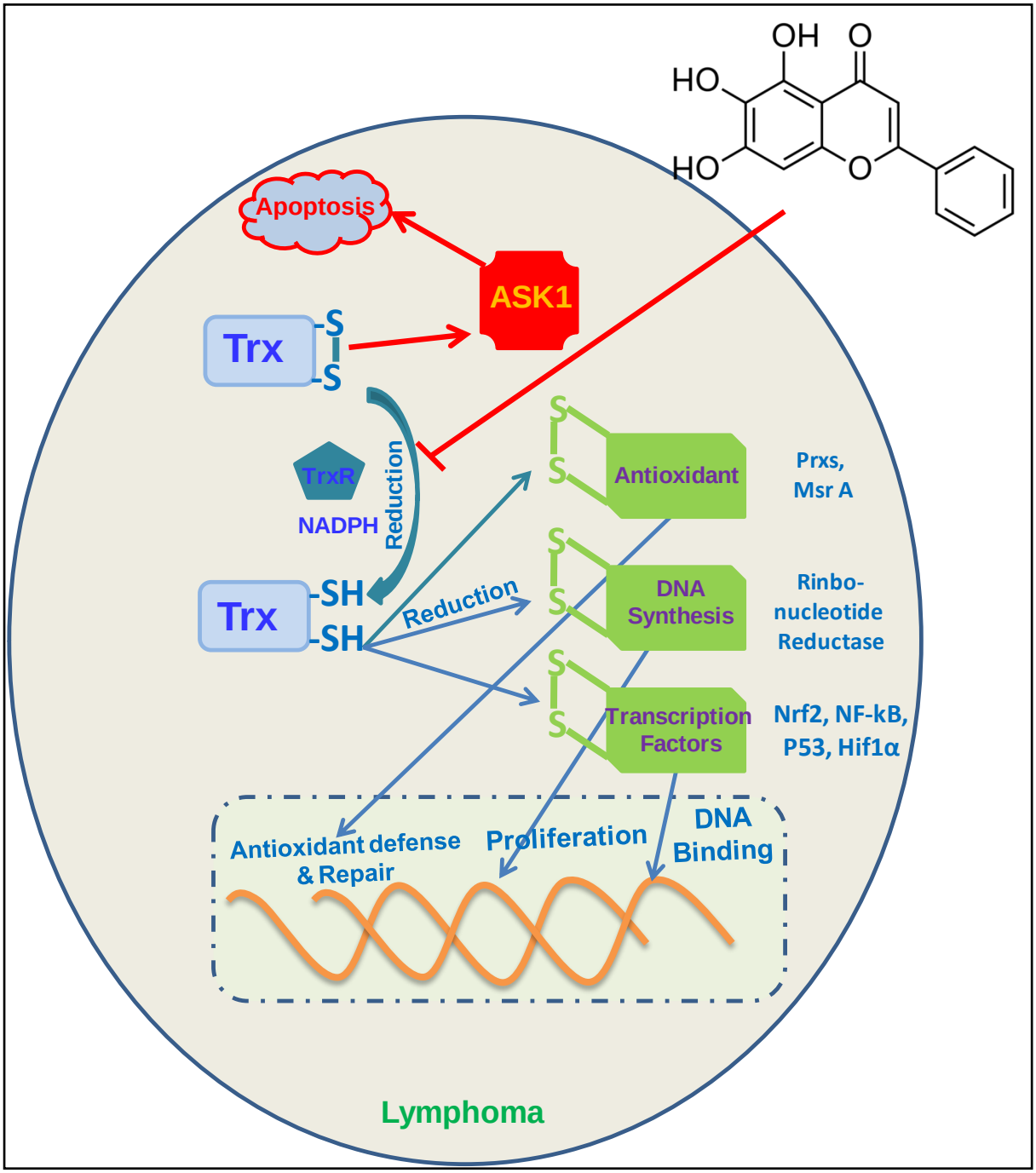


Fig. 4.12 Mechanism of anti-tumor action of baicalein

CHAPTER 5

SUMMARY & CONCLUSIONS

Summary and conclusions

This study was undertaken to evaluate radioprotective potential of a plant derived flavonoid baicalein using murine splenic lymphocytes as a model system. It was further evaluated for its radioprotective ability in vivo using mouse model (Swiss and C57BL/6). Since exposure to ionizing radiation is known to induce systemic inflammation and IR induced inflammation has been implicated in several post IR exposure pathological conditions, anti-inflammatory property of this compound was also evaluated in vitro as well as in vivo. Further, to assess clinical relevance, its effect on tumor cells was also investigated as it is expected that a radioprotector should not benefit tumor cells by offering survival advantage.

Past efforts investigating the radio-modulatory potential of phytochemicals have yielded limited success. Primary cause of this failure lies in lack of holistic understanding of effects of radio-modulator in the biological system under consideration. Improved understanding about tumor response towards these agents would substantially enhance probability of their clinical feasibility. Hence, it was planned to investigate molecular players regulating tumor radioresistance and effect of baicalein on them in the present study.

Findings from chapter 2 have specifically identified radioprotective potential of baicalein. Results from these findings revealed that although baicalein is an anti-oxidant, ROS scavenging is not the primary mechanism responsible for radioprotection. Rather baicalein influenced multiple pro-survival signaling molecules ERK/NF-κB/Nrf-2/HO-1 at cellular level amplifying the stress tolerance capacity of cells when exposed to IR. Baicalein administration to mice enhanced abundance of stem cells in hematopoietic compartment thereby preparing animals to recover from IR induced hematopoietic injury. Apart from this, similar pro-survival signaling proteins were up-regulated by baicalein administration in cells of hematopoietic compartment

(ERK, HO-1 and NF-κB in total BM-MNC whereas pNrf-2 in lineage negative BM-MNC). Thus, baicalein administration augmented cellular as well as systemic defense mechanisms and offered protection against IR induced injury to cells of the hematopoietic system in vitro and in vivo.

Administration of baicalein increased DNA binding of redox sensitive pro-survival transcription factor NF-κB in BM-MNC. Treatment of murine splenic lymphocytes with baicalein also increased DNA binding of NF-κB in vitro.NF-κB is a pro-inflammatory transcription factor that regulate inflammatory and immune responses. Mitogen induced proliferation and cytokine secretion from T cells is dependent on activation and DNA binding of NF-κB. However, administration of baicalein suppressed mitogen induced T cell proliferation and cytokine secretion. This was an interesting observation that despite activating NF-κB in vitro as well as in vivo, it could suppress mitogen induced T cell responses in vivo. Hence, study was undertaken to investigate the effect of baicalein on T cell responses. It was observed that although baicalein did not inhibit Con A induced DNA binding of this pro-inflammatory transcription factor, it suppressed Con A as well as anti-CD3/ anti-CD28 mAb induced activation, proliferation and cytokine secretion in T cells. Many anti-inflammatory compounds discovered till date act by inhibiting upstream or downstream events in NF-κBsignaling. Subsequent to DNA binding of NF-κB its dependent gene expression is regulated by NF-κB transactivation. NF-κB transactivation is a multi-step process and it is controlled by phosphorylation and acetylation of p65. Recent literature reports have implicated involvement of thioredoxin reductase as well as thioredoxin in regulation of NF-κB transactivation. Although, precise molecular mechanisms are not known it is observed that inhibition of either component of this system leads to suppression of NF-κB luciferase activity whereas nuclear overexpression of thioredoxin leads to increased

NF-κB luciferase activity. Mammalian thioredoxin reductase has been shown to be inhibited by flavonoids in cell free system. Hence, possibility of thioredoxin system suppression as mechanism of anti-inflammatory action was explored. It was observed that baicalein suppressed NF-κB trans-activation by inhibiting thioredoxin activity and thereby suppressed NF-κB dependent gene expression. Interestingly, baicalein did not inhibit homeostasis driven proliferation but suppressed induction of graft-versus-host disease in mice. This study identified yet another molecular target of baicalein inside cells in terms of thioredoxin. Thus it could be perceived from above discussion that baicalein has pleiotropic effects and it influences multiple cell survival pathways inside cells.

From the results of chapter 2, it was revealed that inhibition of ERK and Nrf-2 abrogated radioprotection offered by baicalein in murine splenic lymphocytes. Exposure to IR 4Gy induced apoptosis in ~80% lymphocytes whereas only ~5% EL4 cells underwent cell death in 24h. IR induced apoptosis in normal lymphocytes was almost completely prevented after baicalein treatment which activated ERK and Nrf-2 in these cells. Thus activation of these two key pro-survival signaling molecules can account for the difference in the radiosensitivity of lymphocytes treated with vehicle or baicalein. Further, murine T cell lymphoma which is a tumor counterpart of T lymphocytes was resistant to IR 4Gy induced apoptosis. This suggested that these two molecules may play a key role in determining radio-resistance of EL4 cells. Previous study from our laboratory have shown that EL4 cells have active antioxidant machinery and decreased basal ROS levels as compared to their normal counterpart T cells. Hence, study was undertaken to investigate the molecular mechanism responsible for the differential radiosensitivity between normal (murine splenic lymphocytes) and tumor cells (EL4 cells). Results from these study revealed that indeed basal and IR induced antioxidant machinery in EL4 cells was active as

compared to lymphocytes measured in terms of ROS, GSH/GSSG ratio and thioredoxin activity. Further, in agreement with our proposed hypothesis, inhibition of ERK or Nrf-2 by the use of pharmacological inhibitors or shRNA knockdown approach resulted in enhanced radiosensitivity of EL4 cells. These findings further highlighted the role of these crucial proteins in determining radio-resistance of both normal as well as tumor cells. It was observed that similar to murine T cell lymphoma (EL4), Jurkat cells were more resistant to IR induced cell death as compared to human PBMC. Exposure to IR 4Gy induced Nrf-2 and increased thioredoxin activity in nuclear compartment of Jurkat cells. Blocking of GSH synthesis or thioredoxin antioxidant networks enhanced sensitivity of Jurkat cells to radiation induced apoptosis. Tumor cells are highly proliferating and need constant supply of precursors of DNA. Efficient management of oxidative burden in tumor cells and maintenance of redox homeostasis depends on thioredoxin system. Interestingly baicalein could induce cell death in EL4 cells and present study unraveled a novel mechanism for anti-tumor action of baicalein by inhibition of thioredoxin system. Results of this study identified thioredoxin system as an attractive target for developing drugs with anti-tumor or radio-sensitizing potential.

This study demonstrated radioprotective, anti-inflammatory and anti-tumor potential of baicalein in in vitro and in vivo model systems.

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