PROTECTION AGAINST RADIATION INDUCED GASTRO-INTESTINAL INJURY BY 1,4-NAPHTHOQUINONE

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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.

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List of Publications arising from the thesis

Journal

1. "1,4-Naphthoquinone, a pro-oxidant, suppresses immune responses via KEAP-1 glutathionylation". **Lokesh Gambhir**, Rahul Checker, Maikho Thoh, R.S. Patwardhan, Deepak Sharma, Mukesh Kumar, Santosh K. Sandur, ***Biochemical Pharmacology.*** **2014** Mar 1;88(1):95-105.

Conferences

1. KEAP-1 glutathionylation is a critical regulator of oxidative stress induced suppression of inflammatory responses. **Lokesh Gambhir**, Rahul Checker, R.S. Patwardhan, Deepak Sharma and S. Santosh Kumar in the proceedings of conference on "Recent Trends in Free Radical and Antioxidant Research" & 13th Annual meeting of ***Society of Free Radical Research***- India held in Jan 2014 in Lonavala, Maharashtra.

2. Perturbation of Cellular Redox as a Novel Strategy to Ameliorate Radiation-induced Gastro-intestinal Syndrome. **Lokesh Gambhir**, Rahul Checker, Deepak Sharma and S. Santosh Kumar in the proceedings of conference on "Advances in free radicals, redox signalling and translational antioxidant research" at 12th Annual meeting of ***Society of Free Radical Research***- India held in Jan 2013 in Lucknow, Uttar Pradesh. **(Best Poster Award)**

Others

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(Lokesh Gambhir)

Dedicated

to my

loving parents

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Homi Bhabha National Institute

SYNOPSIS OF Ph.D. THESIS

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SYNOPSIS

PREAMBLE:

The use of ionizing radiations and radiopharmaceuticals in diagnosis and treatment is associated with occupational as well as accidental exposures. Besides these, there is an increased risk of exposure of general public during nuclear accidents. Since there are no bonafide radioprotectors for general purpose, there is an immediate requirement for the development of radioprotective agents which will be useful under a variety of operational scenarios. It is well known that, exposure to high doses of ionizing radiation is associated with the induction of acute radiation syndromes like hematopoietic syndrome and gastro intestinal syndrome. Ionizing radiation induced mortality depends on the dose of radiation and at doses higher than 7 Gy, a combination of hematopoietic and gastrointestinal syndrome causes mortality within 1-2 weeks in mice. Radiation induced denudation of luminal protrusions called villi and loss of viability in submucosal invaginations called crypts of Lieberkuhn underlines the pathogenesis of GI syndrome [1]. Each crypt in jejunum contains intestinal stem cells (ISC) at the base which are responsible to supply the differentiated enterocytes to maintain homeostasis [2; 3]. However, exposure to radiation induces apoptosis in these ISC, thus delimiting their potential to repopulate the denudated absorptive surface [4]. Though multiple modalities have been studied for protecting against radiation induced hematopoietic syndrome, very few agents have been investigated to ameliorate radiation induced GI syndrome. Earlier investigations have shown potential radioprotective efficacy of fibroblast growth factor, TLR 5 agonist CBLB502 [5], Sphingosne-1-Phosphate (S1P) [6] and anti ceramide antibody [7] by reducing apoptosis in intestinal crypts and preventing GI syndrome. Growth factors and S1P showed protection by

activating PI3k/Akt pathway whereas others decreased p53 dependent apoptosis in intestinal epithelial cells by activating survival pathways.

Intestinal tract holds the largest lymphoid tissue causing maturation and extra-thymic development of T lymphocytes and exposure to ionizing radiation is followed by an elevated inflammatory responses. Recently, several investigators have reported an important role of Nrf2 in regulating both innate as well as adaptive immune responses. Perturbation in cellular redox status is known to activate redox sensitive transcription factor Nrf2 which further induces the downstream expression of cytoprotective and anti-inflammatory genes [8].

AIM & SCOPE OF THE THESIS:

1, 4-naphthoquinone (NQ), a bifunctional para-quinone, is derived from naphthalene through the replacement of two hydrogen atoms by two ketone groups. NQ is the parent molecule for many clinically approved anticancer, anti-infective and anti-parasital drugs [9]. Earlier studies from our laboratory have shown that NQ, a pro-oxidant, induced redox alteration in splenic lymphocytes that activated the Nrf2/HO-1 pathway to protect against radiation induced hematopoietic syndrome [10]. Present studies have been undertaken to investigate the potential of NQ to ameliorate radiation induced GI injury. Inflammation is one of the major causes of radiation induced mortality. Since, activation of Nrf2 pathway has been implicated in eliciting cytoprotective and anti-inflammatory effects, it was hypothesized that NQ might suppress inflammatory responses and also prevent radiation induced GI syndrome. Sensitivity of GI tract to ionizing radiation is a major limiting factor in abdominal and pelvic radiation therapy. Application of NQ in protecting intestinal cells during chemo-radiation therapy may increase the therapeutic ratio of abdominal irradiation in GI malignancies.

Objectives:

- To investigate the effect of NQ against whole body irradiation (WBI) induced GI syndrome in mice.
- Elucidation of molecular mechanism of radioprotection offered by NQ in vivo.
- To investigate the potential of NQ to modulate the inflammatory responses in murine lymphocytes and elucidate the underlying mechanism.

ORGANISATION OF THE THESIS: The work embodied in this thesis is divided into five chapters:

(1) Introduction and Review of literature.
(2) Materials and Methods
(3) Protection against ionizing radiation induced gastro-intestinal syndrome by 1, 4-naphthoquinone.
(4) Immunomodulatory effects of 1, 4 naphthoquinone.
(5) Discussion and Conclusions
(6) Bibliography

CHAPTER 1: INTRODUCTION AND REVIEW OF LITERATURE

This chapter highlights our current knowledge in the field of radiation mediated injuries to biological systems and its protection using various strategies. The chapter describes in detail the present scenario of ionizing radiation induced gastro-intestinal (GI) syndrome, radioprotective agents and their mechanism of action. It reviews the role of inflammation in radiation injury and the different strategies that have been employed in developing novel anti-inflammatory agents.

The energy deposited by ionizing radiation (IR) can induce damage to macromolecules like DNA, protein, etc. by directly interacting with them or by generating free radicals through radiolysis of water. Research on development of radioprotective agents started more than six decades ago and identification of radioprotective agents has been an important goal for radiation oncologists and radiation biologists. Acute Radiation Syndrome is a dose dependent damage affecting the hematopoietic, gastrointestinal, cerebrovascular, cutaneous and nervous systems in the body [11]. Since a large part of IR induced damage is mediated by generation of reactive oxygen species (ROS), much of the efforts in the past were aimed to explore the potential of antioxidants to ameliorate IR induced toxicity. However, recent advances have highlighted the role of pro-survival pathways as putative targets for developing novel radioprotective drugs. Alteration in cellular redox status is known to elicit a spectrum of cellular response which can result in beneficial or deleterious effects depending upon the level of oxidative stress. Mild oxidative stress can activate pro-survival transcription factor, Nuclear factor-erythroid 2 related factor 2 (Nrf2), leading to enhanced expression of antioxidant enzymes and cytoprotective genes. Activation of Nrf2 was shown to protect against oxidative stress induced cell death implicated in pathogenesis of ischemia reperfusion, inflammation and cancer. Nrf2 protected colonic epithelial cells from IR induced loss of cell viability [7]. Exposure to IR is also marked by the induction of inflammatory responses which can further aggravate radiation injury. Inflammation is known to be regulated by cellular redox status and redox sensitive immunoregulatory transcription factors like Nrf2 and NF-κB. Nrf2 knockout mice showed prolonged inflammation during cutaneous wound healing [12], enhanced lymphocyte proliferation, impaired redox status [8] and higher secretion of Th2 cytokines [13]. NF-κB is known as a key regulator of the expression of immune cell receptors, growth factors, adhesion molecules and cytokines (IL-1, IL-2, IFN-γ, IL-6) in

lymphocytes, epithelial cells and monocytes. KEAP-1 (Kelch-like ECH-associated protein-1), a negative regulator of Nrf2 also functions as an adapter protein for CUL-3 based E3 ligase to degrade IκB kinase β (IKKβ), which results in suppression of NF-κB activation and modulation of immune responses [14]. Hence, the cross talk between NF-κB and Nrf2 is very important during inflammatory responses and these two transcription factors are potential targets for developing anti-inflammatory drugs [15].

1,4-naphthoquinone (NQ) is the parent molecule for many clinically approved like anthracycline, mitomycin, daunorubicin, doxorubicin, diospyrin and malarone [9]. Higher redox potential and electrophilic nature of quinones imparts high affinity for cellular nucleophiles like thiols of the cysteine moiety present in proteins and glutathione contributing to its biological activity. Our previous studies using NQ, showed protection against bone marrow syndrome induced by radiation. However, radioprotective ability of NQ and underlying mechanism has not been investigated in detail. Hence, the present thesis was undertaken to explore the potential of NQ to modify IR induced GI syndrome using in vivo model and to investigate its immunomodulatory effects.

CHAPTER 2: MATERIAL AND METHODS

The present chapter describes the source of chemicals, materials and animals. It includes detailed methodology employed during various experiments. For investigating the radioprotective potential of NQ in vivo, Balb/c mice were administered with NQ 2mg/kg body weight (bw) intra-peritoneally (i.p.) consecutively for 4 days with an interval of 24h. Whole body irradiation of 8Gy was given 30min after the last i.p. administration of NQ. Mice were monitored for radiation induced mortality and morbidity. In survival studies for doses above 8Gy GI syndrome

specific model was employed which involve NQ administration in conjunction with bone marrow transplant. Further histopathological, immune-histochemical and flow cytometric studies using jejunum cells were used to investigate the potential of NQ to protect against radiation induced GI injury. Isolated cells from jejunum were subjected to Western blotting, EMSA, RT-PCR and flowcytometric analysis to investigate the effect of NQ on ERK/Nrf2 pathway.

Immunomodulatory effects of NQ were investigated in terms of its potential to suppress mitogen induce T cell activation, cytokine production and proliferation of the activated T cells and macrophages. T cell proliferation and cytokine secretion were assessed by employing CFSE dye dilution method using a flow cytometer and ELISA respectively. Further insights in the anti-inflammatory action of NQ were explored in lymphocytes. Effect of cellular redox status on NQ mediated action was elucidated using thiol and non-thiol antioxidants. Effect of NQ on, redox sensitive anti-inflammatory transcription factor, Nrf2, its mode of activation and cross talk between Nrf2/NF-κB was elucidated using confocal microscopy, EMSA, immuno-precipitation and Western blotting. Lymphocytes isolated from gut associated lymphoid tissue and spleen were used to investigate the in vivo anti-inflammatory effects.

CHAPTER 3: PROTECTION AGAINST IONIZING RADIATION INDUCED GASTRO-INTESTINAL SYNDROME

The present chapter describes the potential of NQ to ameliorate radiation induced GI syndrome in terms of its ability to protect against WBI induced mortality, loss in histopathological features and cell death in intestinal epithelial and stem cells in vivo. This chapter also describes the underlying molecular mechanism of NQ mediated radio-protection.

Administration of NQ prior to radiation significantly protected (60%) against WBI 8Gy induced mortality and morbidity in mice. NQ administration rescued mice from WBI induced denudation of villi, epithelial misalignment and protected against induction of apoptosis in the crypts. The hematopoietic system is among the most sensitive and critical systems for early radiation-induced health affects that contribute to the mortality at high dose exposure. Therefore, a syndrome specific approach in combination with autologous bone marrow transplant (BMT) to mitigate the WBI induced bone marrow aplasia was employed. NQ administration along with BMT was employed to mitigate WBI (>8Gy) induced GI syndrome. NQ administration in combination with BMT showed complete protection against WBI induced mortality at 9Gy. It also offered significant protection at 10Gy (90%) and 12Gy (40%) induced mortality. NQ administration protected Lgr5+ intestinal stem cells and epithelial cells against WBI induced cell death.

Earlier results from our laboratory have shown that NQ protected against radiation induced hematopoietic syndrome by activating ERK/Nrf2 pathway in vitro. Further, mechanism underlying the NQ mediated GI protection was investigated in vivo. Jejunum cells obtained from NQ administered mice showed increased phosphorylation of ERK and activation of Nrf2. Ablation of Nrf2 using all-trans-retinoic acid (inhibitor of Nrf2) resulted in abrogation of NQ mediated protection against WBI induced mortality. These results corroborated our hypothesis that NQ, a pro-oxidant, protects against radiation induced GI syndrome by inducing Nrf2 pathway.

CHAPTER 4: IMMUNOMODULATORY EFFECTS OF 1, 4 NAPHTHOQUINONE

Inflammation is one of the major causes of radiation induced mortality. Several studies have iterated the role of inflammatory reaction and endogenous cytokine production in the

pathogenesis of radiation-induced damage to normal tissue [16]. Thus anti-inflammatory action is among the desirable attributes of a potent radioprotector. This chapter describes the immunomodulatory properties of NQ measured in terms of its ability to inhibit mitogen induced proliferation and cytokine secretion of T cells in vitro. This chapter also describes the effects of NQ on mitogen induced secretion of inflammatory mediators by macrophages and anti-inflammatory potential of NQ in vivo. Further the underlying mechanism of NQ mediated anti-inflammatory effects has been delineated. Lymphocytes isolated from spleen and gut associated lymphoid tissue of NQ injected mice showed suppression of anti-CD3/CD28 antibody induced cytokine secretion. Lymphocytes isolated from NQ administered mice were hyporesponsive towards Concanavalin A (Con A) induced proliferation and NF-κB activation. NQ inhibited Concanavalin A (Con A) and anti-CD3/CD28 antibody induced proliferation and cytokine (IL-2, IL-4, IL-6 and IFN-γ) secretion by murine lymphocytes. Lipopolysaccharide induced increase in cytokine (TNF-α, IL-1β and IL-6) secretion, nitric oxide and cyclooxygenase-2 expression by macrophages was also inhibited by NQ treatment.

NQ modulated cellular redox status by increasing the basal levels of ROS and decreasing GSH/GSSG ratio in lymphocytes. Perturbation in cellular redox status is known to induce the activation of redox sensitive anti-inflammatory transcription factor Nrf2. Several studies have shown an indispensable role of Nrf2 and its dependent gene HO-1 in the regulation of inflammatory responses via regulation of cytokines and pro-inflammatory protein and its deficiency was shown to increase susceptibility to inflammatory disorders [8]. NQ induced the activation of Nrf2 and its dependent genes (HO-1 and GCLC) in lymphocytes. NQ induced S-thiolation of proteins could be due to oxidative stress and thiol depletion. Interestingly, NQ

induced S-thiolation of KEAP-1 which is negative regulator of Nrf2 further corroborating the role of pro-oxidative nature of NQ in activation of Nrf2.

Activation of Nrf2 has been shown to inhibit mitogen induced activation of NF-κB and its dependent pro-inflammatory genes [17]. NQ inhibited mitogen induced degradation of IκBα and activation of NF-κB in murine lymphocytes. KEAP-1 functions as IKKβ E3 ubiquitin ligase by directly interacting with E(T/S)GE motif of IKKβ which is required for activation of NF-κB pathway. It was observed that NQ mediated binding of KEAP-1 to IKKβ and induced its degradation in a time dependent manner. Based on these results we propose that KEAP-1 mediated IKKβ degradation in response to NQ might be responsible for the suppression of NF-κB pathway. To corroborate the hypothesis that activation of Nrf2 pathway mediates anti-inflammatory action of NQ, pharmacological inhibitors of Nrf2 and HO-1 were used. Both all-trans-retinoic acid (ATRA) and Tin protoporphyrin (SnPP) significantly abrogated NQ mediated suppression of mitogen induced proliferation confirming the involvement of Nrf2/HO-1 pathway. These results demonstrate that NQ modulated immune responses via oxidative stress mediated KEAP-1 glutathionylation and IKKβ degradation. These results further highlight the potential of NQ to protect against radiation induced damages by suppressing inflammatory responses.

CHAPTER 5: SUMMARY AND CONCLUSION

This chapter discusses the implications of the studies described in chapters 3 and 4. The present thesis for the first time highlights the potential of a pro-oxidant 1,4-naphthoquinone to ameliorate radiation induced GI syndrome and exhibit anti-inflammatory effects by activating Nrf2 pathway. NQ induced oxidative stress leads to KEAP-1 protein modification and disruption of

KEAP-1/Nrf-2 interaction resulting in activation of Nrf-2 pathway. The potential of NQ in protecting intestinal cells during chemo-radiation therapy may increase the therapeutic ratio of abdominal irradiation in GI malignancies. The results also demonstrate that induction of mild oxidative stress in lymphocytes by NQ leads to KEAP-1 mediated IKKβ degradation and suppression of NF-κB pathway. These results further highlight the cross talk between Nrf2 and NF-κB as a potential target to develop novel anti-inflammatory agents which could be used as radioprotectors.

The major conclusions drawn from this study are:

(1) NQ protected against whole body irradiation induced GI syndrome and associated mortality in mice.

(2) NQ protected intestinal stem cells against radiation induced cell death and abrogated radiation induced denudation of villi and apoptosis of crypt cells.

(3) NQ induced perturbation in cellular redox status and induced activation of ERK/Nrf2 pathway. Ablation of Nrf2 resulted in abrogation of NQ mediated protection.

(4) NQ suppressed mitogen induced proliferation and cytokine secretion in murine lymphocytes and macrophages.

(5) NQ perturbed cellular redox status and induced activation of Nrf2 pathway in lymphocytes

(6) NQ induced S-thiolation of KEAP-1 and it induced KEAP-1 mediated degradation of IKKβ to inhibit NF-κB pathway.

NQ protected against radiation induced GI injury by activating ERK/Nrf2 pathway and suppressing inflammatory responses.

CHAPTER 6: BIBLIOGRAPHY

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ARE	Antioxidant Response Element
ARS	Acute Radiation Syndrome
ATRA	all-trans Retinoic Acid
BMT	Bone Marrow Transplant
BrdU	5-bromo-2'-deoxyuridine
CFSE	Carboxy Fluorescein Diacetate Succinimidyl Ester
Con A	Concanavalin A
CFU	Colony forming units
Cox	Cyclooxygenase
DMSO	Dimethyl Sulfoxide
DTNB	Dithiobis 2-nitrobenzoic acid
DAG	Diacylglycerol
ERK	Extracellular Signal Regulated Kinase
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
GSH	Glutathione

GSSG	Glutathione disulphide
Gy	Gray
H_2O_2	Hydrogen Peroxide
HO-1	Hemeoxygenase-1
IP3	Inositol 1,4,5-triphosphate
ITAM	Immunoreceptor-based tyrosine activation motif
i.p.	Intraperitoneally
ISC	Intestinal stem cells
IFN-γ	Interferon- γ
IL	Interleukin
IKK	IκB kinase
Kg bw	Kilogram Body Weight
KEAP-1	Kelch-like ECH-associated protein 1
LPS	Lipopolysachharide
Lck	Lymphocyte-specific protein tyrosine kinase
MAPK	Mitogen Activated protein Kinase
mM	Milimolar

NAC	N-Acetyl Cysteine
NaOH	Sodium Hydroxide
Neh	Nrf2-ECH homology
NO	Nitric Oxide
NQ	1, 4-Naphthoquinone
NQO1	NADPH-quinoneoxidoreductase1
Nrf2	Nuclear Factor -E2 related Factor 2
NF-κB	Nuclear factor kappaB
NADPH	Nicotinamide adenine dinucleotide phosphate
NSAIDs	Non-steroidal anti-inflammatory drugs
PKC	Protein kinase C
PE	Phycoerythrin
PI	Propidium Iodide
PIP2	Phosphatidylinositol (4,5) bisphosphate
PLCγ	Phospholipase C γ
PI3K	Phosphoinositide 3-kinase
ROS	Reactive Oxygen Species

SnPP	Tin-protoporphyrin
SOD	Superoxide Dismutase
SLP-76	SH2 domain-containing leukocyte phosphoprotein
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
t-BHQ	t-Butyl Hydroquinone
TNF-α	Tumor Necrosis Factor- α
TCR	T cell receptor
WBI	Whole Body Irradiation
ZAP-70	Zeta-chain-associated protein kinase 70

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 IONIZING RADIATION:

Advancement in technology has driven an increase in peaceful use of nuclear energy and radio-isotopes in the fields of nuclear industry (power production), medical imaging, diagnostics, food preservation, sanitization, sludge hygienization, smoke detectors, radio-carbon dating, treatment of cancer by external beam therapy or brachytherapy, research and many other industries. Increasing use of ionizing radiation (IR) in different human endeavours is also associated with increased risk of unwanted exposure to toxic doses of IR during planned as well as unplanned settings. Besides these, nuclear disasters or use of dirty bomb by terrorists can also put humans to high risk of exposure to IR (*1, 2*).

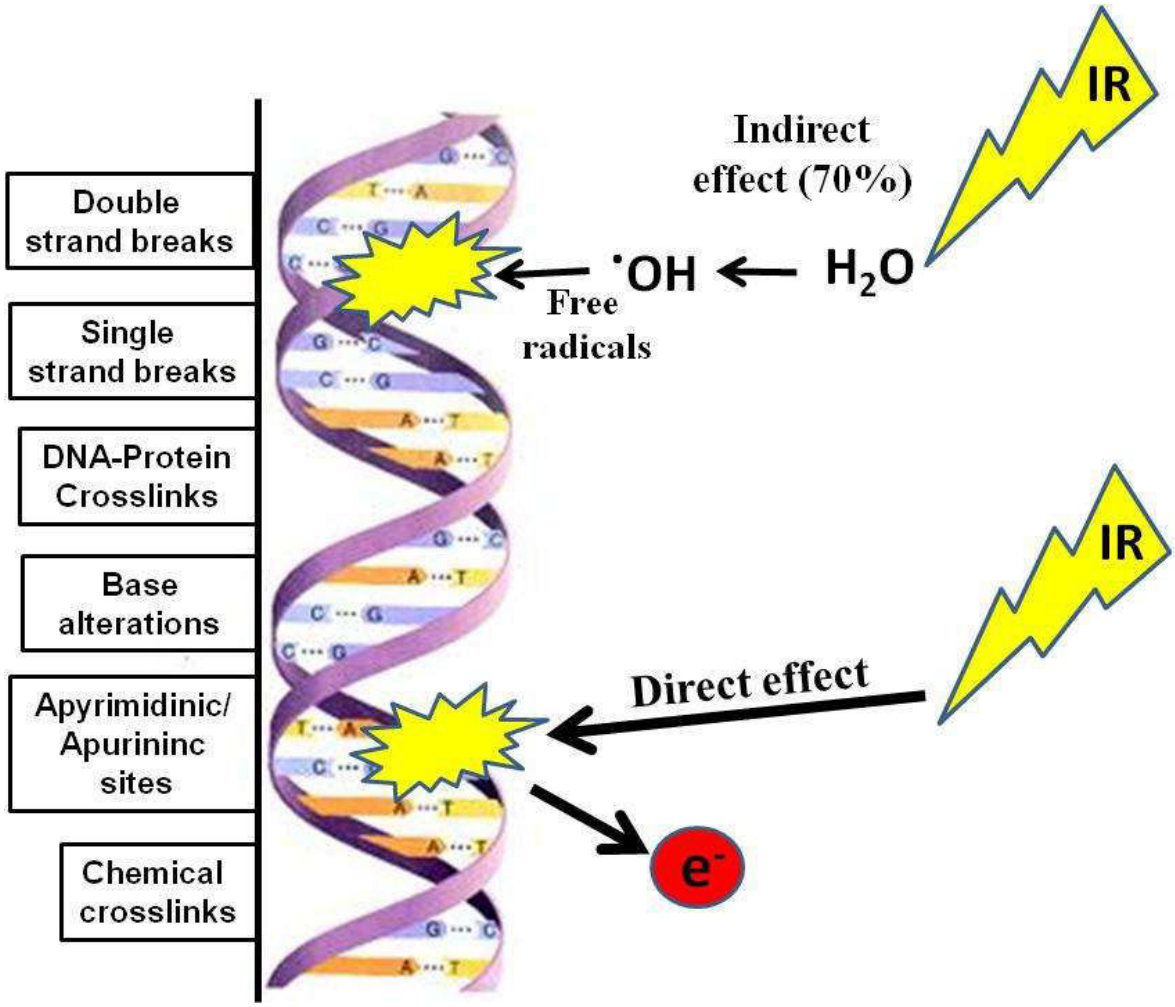
Continuous improvement in the existing procedures to utilize radiation for betterment of human life has motivated researchers across the globe to delimit its detrimental biological effects using Linear no Threshold (LNT) Model (*3, 4*). Essentially, radiation is classified into ionizing and non-ionizing radiations based on the energy and the effect it produces upon interaction with matter. Short wavelength electromagnetic radiations (X-rays, γ-rays) and particle radiations (fast electrons, α- particles, protons, neutrons, etc) are high-energy radiations (KeV to MeV range) and have ability to remove an electron from atoms or molecules from the medium through which they pass and hence they are called as ionizing radiations (*5*). Although there is an increased use of radiation in our day to day life, it has been considered an enigma to the general public and the use of radiation for therapeutic or other uses has also been associated with some cynicism. The fact that exposure to high doses of ionizing radiation causes biological damage has been known for many years. The first case of human injury was reported in the literature just a few months following the discovery of x-rays in 1895 by Roentgen. The deleterious effects of radiation on human health became evident after study of the health related abnormalities in Japanese atom bomb survivors (*1*).

Since then understanding the mechanisms of radiation induced injury to cells has been the prime research area of radiation biologists and radiation chemists (*6*).

The risk of exposure to IR may be classified into planned and unplanned exposures. Planned exposure is contributed by the application of ionizing radiation in the field of medicine for diagnosis and therapy. Planned exposures are intended to benefit patients and the benefit must outweigh the risk. Radiotherapy is frequently used to achieve local or regional control of malignancies either alone or in combination with other modalities such as chemotherapy or surgery. It is estimated that half of all cancer patients will receive radiotherapy during the course of their treatment (*7*). Under these situations, elevated doses of IR cause damage to normal tissues surrounding the tumor, primarily the hematopoietic system and the gastrointestinal tract, thus inducing detrimental side effects and reducing the therapeutic gain. Unplanned exposure can happen at low doses during occupational exposure or it may reach high doses due to the accidents at nuclear power plants (*8-10*), exposure to contaminated waste (*11*), following nuclear "dirty bomb" (*11, 12*) or due to industrial accidents during mining, milling and processing of radioactive materials (*13*). There is a risk of exposure to high doses of radiation not only for radiation workers but also for personnel participating in the emergency response.

Deleterious effects of IR on biomolecules are mediated by two distinct phenomenon, indirect effect and direct effect. Direct effect of IR is mediated via deposition of energy directly on macromolecules like DNA or other cellular components like lipids or proteins which are critical for the survival of the cell. Direct deposition of energy leads to ionization of both the nitrogenous bases and sugar to generate single strand breaks and tandem DNA damage (*14, 15*). Indirect effect accounts for 70% of the damage to cells and they are mediated via IR induced generation of reactive oxygen species (ROS) due to radiolysis of

water molecules in the cells (*16*). Radiolysis of water molecules results in the formation of hydrogen radicals, hydroxyl radicals, hydroperoxyl radicals and superoxide radicals (*17-19*).



Scheme 1.1 Radiation induced DNA damage via direct and indirect effects.

The free radicals formed as a result of radiolysis of water have very short life and are capable of interacting with biomolecules like DNA, proteins and lipids in their vicinity. These interactions initiate a cascade of signaling molecules involved in sensing and repair of DNA damage, protein-kinases, cell cycle check points and cell survival or apoptosis. This can either result in cell cycle arrest so as to facilitate repair of radiation induced damage or induce apoptosis (*20-22*).

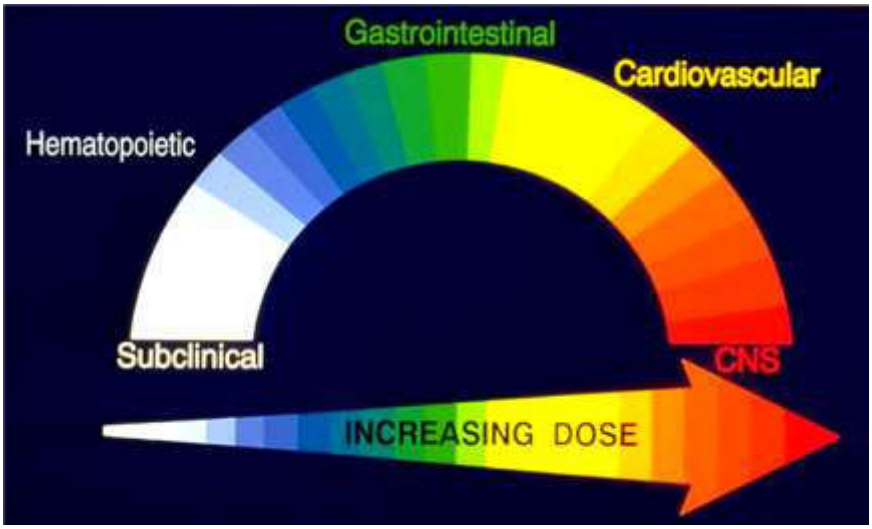
1.2 BIOLOGICAL EFFECTS OF IONIZING RADIATION:

IR induced cell death is the prime event that leads to the origin of tissue and organ damage resulting in acute radiation syndromes which contribute to deterministic effects of IR. Cells that are immature, undifferentiated and actively dividing (e.g. intestine, basal layer of skin, stem cells) are more radiosensitive. Cells that are mature, differentiated and not actively dividing are more radioresistant (*23, 24*). There are four phases of cell cycle: G1 Phase, in which cells prepare for DNA replication; S Phase, in which DNA doubles by replication; and G2 Phase, in which cells prepare for mitosis and M Phase, in which cells divide to form two daughter cells. Of these, M phase, in which the chromosomes are condensed and paired, is the most radiosensitive because of less likelihood of repair. S phase is the most radioresistant phase as the DNA is opened up for replication that facilitates easy repair (*25*). Biological effects of radiation are of two types (i) deterministic and (ii) stochastic effects. High doses of IR generate predictable outcomes that can be clinically determined. These are called non-stochastic or deterministic effects. These effects include blood cell damage, chromosomal aberrations, radiation sickness, cataract and mortality and their severity depends upon the dose, dose rate, age and immune competence of an individual and type of radiation exposure. Stochastic means random in nature. Stochastic effects of IR are those effects where the probability of occurrence, but not severity is a function of the dose without the existence of a threshold value. Most important stochastic effect of IR is induction of tumors.

Deterministic Effects	Stochastic Effects
Due to IR induced cell death	Due to damaged cell proliferation towards malignancy
Threshold dependent (Gy)	No dose threshold- applicable to very small doses
Specific for particular tissues	Probability of effect increases with dose
Severity of damages is dose dependent	Severity independent of the dose

Table 1.1 Comparison between IR induced deterministic and stochastic effects.

The pathological processes of radiation injury is initiated immediately after radiation exposure, but the clinical and histological features may not become apparent for weeks, months or even years after exposure. Radiation injury is commonly classified as acute, consequential, or late effects, according to the time of appearance of the symptoms. Acute effects are those that are observed within a few weeks after exposure. Consequential effects (consequential late effects) appear later, and are caused by persistent acute damage (*26*). If the dose of radiation involved is large enough, acute doses may result in effects which can manifest themselves within a period of hours or days. These conditions are referred as acute radiation syndrome. The acute radiation syndrome occurs after whole-body irradiation (WBI) at doses in excess of 1Gy delivered at a relatively high-dose rate. Rapidly proliferating cells are most sensitive to the acute effects of radiation, particularly lympho-hematopoietic cells and intestinal crypt cells. The inherent sensitivity of these cells results in a constellation of clinical syndromes that predominates within a predictable range of doses of WBI exposure.



Scheme 1.2: Spectrum of Acute Radiation Syndrome (*27*)

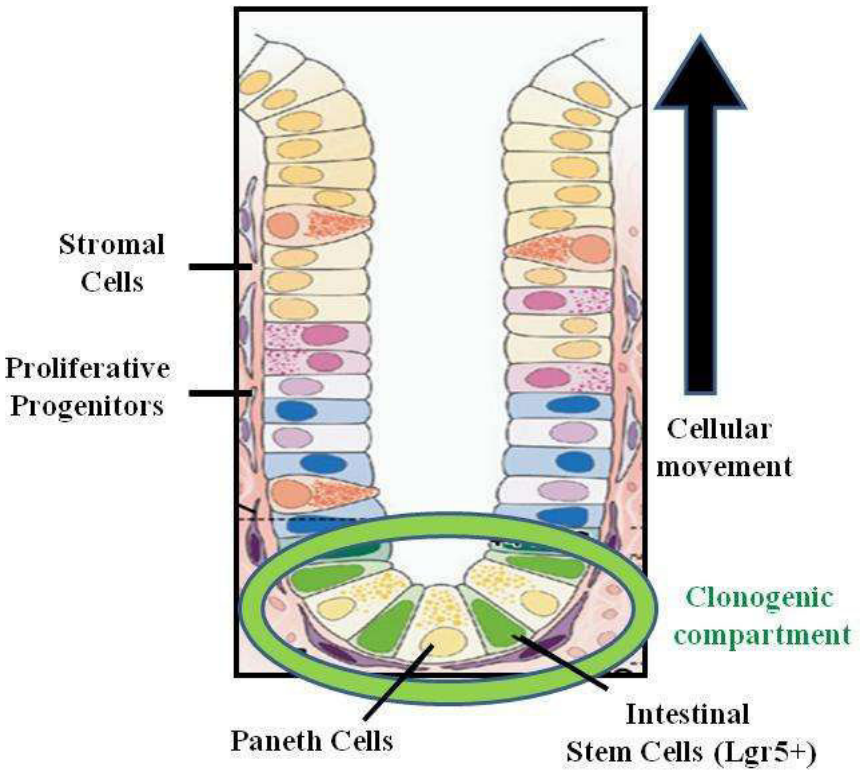
Clinical components of the acute radiation syndrome include the **hematopoietic syndrome** characterised by cellular death of bone marrow stem, progenitor cells and lymphocytes. The hematopoietic syndrome is seen with significant WBI exposures exceeding 1Gy and is rarely clinically significant below this dose. Mitotically active hematopoietic stem and progenitor cells die after a WBI dose greater than 2Gy resulting in bone marrow aplasia and lymphopenia (*28, 29*). These changes result in predisposition to infection, bleeding and poor wound healing leading to mortality within 30 days (*30, 31*). **Gastrointestinal (GI) syndrome** is seen at doses above 7Gy and is marked by the denudation of villi, apoptosis in intestinal crypts, cell cycle arrest in the intestinal epithelial cells and breakdown of the mucosal barrier. These changes result in abdominal pain, diarrhoea, nausea, vomiting and infection. The mortality due to gastrointestinal syndrome appears earlier than hematopoietic syndrome because of malnutrition from reduced absorption; bowel obstruction from ileus; dehydration, vascular damage, electrolyte imbalance, anaemia, gastrointestinal bleeding, sepsis and acute renal failure. Manifestation of symptoms of GI syndrome starts within hours of WBI exposure. **Central nervous system syndrome** occurs at doses >25Gy and it is characterized by damage to cells that do not reproduce such as neurons. Symptoms include watery diarrhoea, respiratory distress, hyperpyrexia, cardiovascular shock, septic shock, hypotension,

cerebral edema, increased intracranial pressure and cerebral anoxia leading to death within 3 days (*27, 32-34*)

1.2.1 Gastrointestinal tract:

The intestinal tract architecture is composed of three tissue layers. The outer layer contains several sheets of innervated smooth muscles that execute peristalsis. The middle layer consists of stromal tissue. The inner surface consists of a sheet of epithelial cells which process and absorb nutrients. Absorptive surface of small intestine is lined by numerous luminal protrusions, termed villi, and invaginations into the submucosa, the crypts of Lieberk¨uhn. The small intestine has three distinct regions - the duodenum, jejunum and ileum. The duodenum receives chime from stomach and digestive juices from pancreas (digestive enzymes) and gall bladder (bile). Jejunum is mid-section of the small intestine connecting duodenum to ileum and it contains plicae circulares and villi that increase the surface area. Digested food (sugar, fatty acids and amino acids) are absorbed here. Ileum also contains villi and absorbs remaining nutrients in the digested food. Three differentiated cell types (enterocytes, enteroendocrine and goblet cells) populate the villi. Enterocytes secrete hydrolases and absorb nutrients. Goblet cells provide a protective mucous lining. The rare enteroendocrine cells secrete hormones including serotonin, substance P and secretin (*35*). Paneth cells, resides at the bottom of the crypt of the small intestine and secretes antimicrobial peptides and enzymes such as cryptidins, defensins and lysozyme (*36*). Due to the rapid proliferating state of intestinal region there is a need to maintain the epithelial homeostasis. The crypt progenitors divide every 12–16 h, generating 200 cells per crypt every day. This rise in cell production is compensated by cell shedding at the tip of the villi. Further, the epithelial lining of villi is in a continuous upward movement to reach the top of the villus for compensating the shedding of cells (*37*). Only Paneth cells (which live for 20 days) and intestinal stem cells (which live for human life time) localize at the crypt bottom to

escape this flow. Proliferation in the intestine is governed by the crypt niche (*38*). Thus proliferative and differentiated compartments are maintained as cells move along the villus axis as shown in scheme 1.3 (*39*).



Scheme 1.3 Schematic presentation of intestinal crypt region (*40*).

Intestinal stem cells (ISC) present at the base of the crypt are responsible to supply the differentiated enterocytes to maintain homeostasis (*41, 42*). Retention of an undifferentiated phenotype, self-renewal capability, continuous production of all lineages and potential to regenerate upon injury underlines the characteristic features of ISC (*43-48*). ISC are extremely susceptible to apoptosis compared with other progenitor cells in the crypt (*49, 50*). Under homeostatic conditions, ISC usually divide asymmetrically resulting in a daughter stem cell and a committed cell for differentiating to mature epithelial cells. After intestinal injury, such as IR exposure, ISC undergo symmetric self-renewal division giving rise to 2 stem cells to replace damaged ISC (*45*). Multiple studies have been carried out to identify the location and presence of ISC. Earlier (in 1981), the existence of a stem cell favourable niche

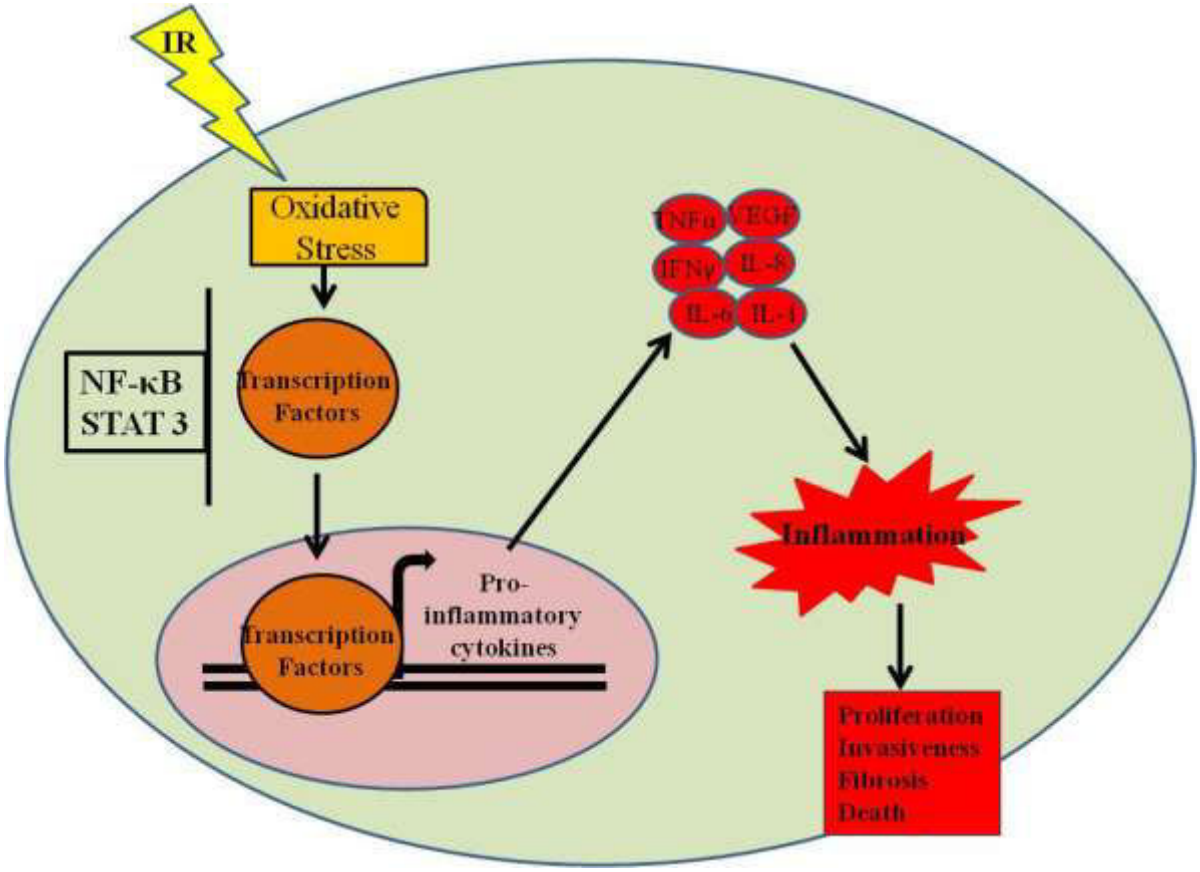
was proposed to be in the positions 1–4 of crypt. These cells, termed Crypt Base Columnar (CBC) cells were interspersed between Paneth cells at the crypt base and were found to give rise to mutant clones containing multiple cell types but subsequently failed to give rise to all 4 mature cells (*44, 51*). Subsequently, development of long-term label retention technique was employed to assist in localisation of putative stem cells. Four cells up (+4) position from the crypt base, directly above the Paneth cell zone was referred to as label-retaining cells (LRCs) or intestinal stem cells (*50, 52, 53*). Lineage-tracing experiments illustrated by Barker et al have identified a single marker, Lgr5/GPR49, a leucine-rich orphan G-protein- coupled receptor, as a putative ISC marker (*54*). Lgr5+ ISC were located as columnar base cells interspersed in between Paneth cells at the base of the crypt. A single Lgr5+ stem cell is capable of generating crypt/villus organoids in vitro containing differentiated intestinal mucosa cell lineages (*40*).

Exposure to ionizing radiation induces apoptosis in ISC leading to disruption in intestinal homeostasis. The earliest ISC response to radiation seems to be delayed progression through S-phase checkpoint and mitotic arrest, coupled with continuous epithelial cell migration along the villus axis and denudation from the villus tip (*55*). This results in progressive shrinkage of crypt after ionizing radiation exposure (*49*). Extent of cell loss and intestinal injury is radiation dose dependent. Therefore, the fate of the crypt after injury is determined by replacement of the clonogenic proliferating crypt cells by intestinal stem cell. Presence of one or more viable ISC after exposure to radiation is sufficient to regenerate the crypt within 72–96 hours with subsequent reconstitution of the villi. Hence, survival of the animal depends on the balance between crypt depopulation, and the efficiency and number of the surviving clonogenic stem cells regenerating the crypts (*56*).

1.2.2 Radiation induced inflammation:

Whole body irradiation results in systemic inflammation that also has a profound effect on the repair of radiation injury in different tissues. Apart from direct and indirect damage caused by radiation, inflammation also significantly contributes towards tissue injury. It is well known that chronic inflammation can amplify the damage inflicted to different tissues by exogenous agents (*57, 58*). Ionizing radiation activates both pro-survival and pro-apoptotic signaling pathways producing an imbalance in cell fate. Radiation induces an inflammatory response in the irradiated organs mediated by leukocyte infiltration and vascular changes. IR induced inflammation is the major limiting factor in the radiotherapy of cancer. Radiation-induced inflammatory response is initiated by the production of reactive oxygen/nitrate species, induction of apoptosis, clonogenic cell death, mucosal breakdown and increased transcription of several pro-inflammatory cytokines and chemokines by recruited immune cells and residing cells, depending on the severity of damage (*59*). The vascular endothelium is a critical target involved in tissue response to radiation exposure and participates in the initiation and development of radiation injury (*60, 61*).

Irradiation of the vascular endothelium leads to endothelial cell apoptosis and the acquisition of a pro-inflammatory, prothrombotic and antifibrinolytic phenotype, with increased secretion of soluble mediators such as cytokines, chemokines and growth factors (*62*). IR induced inflammatory process is initiated by the production of pro-inflammatory mediators such as IL-1β, IL-6, IL-8, CXCL-1, CXCL-2 and TNF-α within minutes to hours (*63-68*). Increased levels of TNF-α and IL-1 have been reported after irradiation of alveolar macrophages or tumour cells (*69, 70*). Exposure to X-ray results in over-production of IL-6 and IL-8 in keratinocytes, fibroblasts and glioma cells (*71-73*). Wu CT et al. demonstrated that IL-6 up regulation was positively linked to radiation resistance while its inhibition enhanced the radiation sensitivity in prostate cancer cells (*74*).



Scheme 1.4 Radiation induced inflammation: Radiation exposure induces activation of key immunoregulatory transcription factors. Increase in pro-inflammatory cytokines and adhesion molecules induces inflammation and its associated effects.

IR induced inflammatory responses also play a vital role in pathogenesis of late effects of IR exposure. The IR induced fibrotic tissue remodelling is a multicellular process regulated by different cytokines such as TGF-β1, TNF-α, IL-1, IL-4 and IL-13; chemokines such as MCP-1 and MIP-1β (*75-79*). Pro-inflammatory cytokines like IL-1 and TNF-α have been implicated in development of fibrosis. IL-1β is directly up-regulated by radiation which activates other inflammation related molecules such as the matrix metalloproteinases (MMPs) that further degrade the extracellular matrix components (*80*). IR exposure leads to the activation of immunoregulatory transcription factors which control the expression of numerous immune mediators involved in cancer promotion and progression. Thus, targeting

the IR induced inflammatory signaling pathways may improve the clinical outcomes of radiotherapy (*81, 82*). It has been observed that suppression of pro-inflammatory transcription factor NF-κB by over-expression of IκBα leads to sensitization of human glioblastoma, fibroblast and intestinal epithelial cells to radiation (*83*). In recent years, the NF-κB inhibition by synthetic compounds as well as nutraceuticals has been used as a strategy for tumour radio-sensitization (*84*). Curcumin has been shown to down-regulate NF-κB expression and STAT-3 phosphorylation resulting in better therapeutic gain (*85*). Infection associated inflammation is the primary cause of death during acute radiation syndromes. The affected patients with bone marrow aplasia experience reduced defence against exogenous and endogenous pathogens leading to infection and inflammation, and consequently suffer from invasive infection and organ dysfunction resulting in death (*86*).

1.3 INFLAMMATION:

Inflammation is a complex localized protective reaction initiated by host cells/tissues of the body to allergic or chemical stimulation, injury and infections. The five classical signs of inflammation are characterized by pain, heat, redness, swelling and loss of function. Initiation of inflammation is a multifactorial signaling process that result in increased movement of leukocytes, protein and fluids into the affected region due to dilated blood vessels (*87-89*). Outcome of inflammation is mediated by secretion of chemical mediators from cells like mast cells, platelets, neutrophils and monocytes/macrophages. These mediators are termed as pro-inflammatory factors that determine the severity of inflammation (*90*).

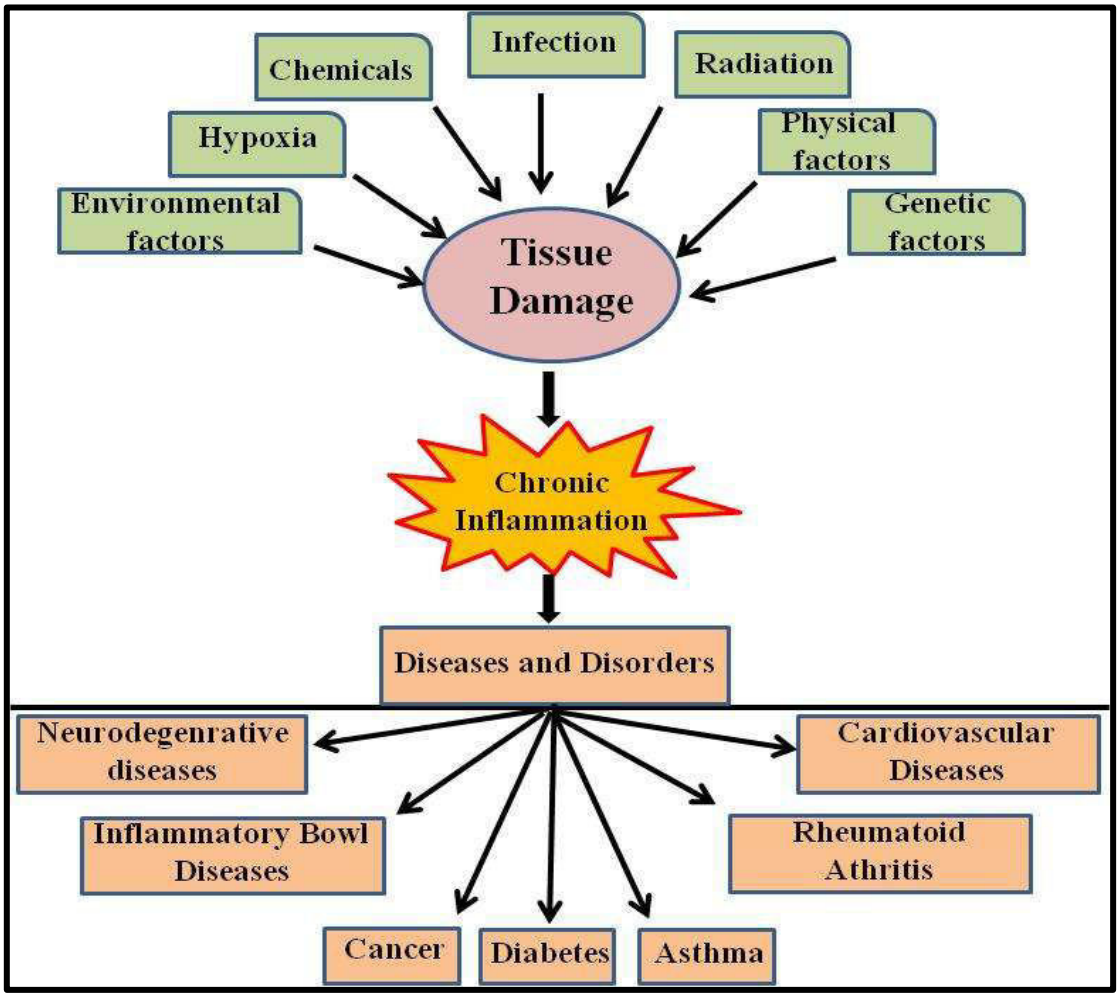
Even though the process of inflammation is complex, it is mainly divided into two types i.e. acute or chronic based on the duration.

- **Acute inflammation** is characterized by rapid onset within minutes to few hours and it lasts for a short duration (less than a week). It is exemplified by the exudation of fluids and plasma proteins; and the recruitment of leukocytes, most notably neutrophils to the site of injury/infection. Acute inflammatory response plays a major role in combating against invading bacteria, virus and parasites while still facilitating wound repairs.

  Components of acute inflammation:

  - Vascular dilation: relaxation of vascular smooth muscle leading to enlargement of tissue with blood (hyperaemia).
  - Endothelial activation: increased endothelial permeability allows plasma proteins to pass into tissues and increased expression of adhesion molecules on the endothelial surface mediates neutrophil adherence.
  - Neutrophil activation: expression of adhesion molecules causes neutrophils to adhere to endothelium and increased motility allows emigration from vessels into surrounding tissues.

- **Chronic inflammation** is of a more prolonged duration (months to years) and manifests histologically by the presence of lymphocytes and macrophages in the tissues, resulting in fibrosis and tissue necrosis. Importunate and prolonged chronic inflammation is the underlying cause of multiple oxidative stress associated diseases such as rheumatoid arthritis, atherosclerosis, heart disease, Alzheimer, asthma, cancer, congestive heart failure (CHF), multiple sclerosis (MS), diabetes, infections (bacteria, fungi, parasites), gout, IBD-inflammatory bowel disease (*91, 92*).



Scheme 1.5 Sketch diagram illustrating the tissue damage induced chronic inflammation resulting in diseases.

The major events involved in inflammatory processes are as follows:

- Leukocyte migration (*93, 94*)
- NO release and Arachidonic acid metabolism (*95-97*).
- Excessive generation of reactive oxygen species (ROS) (*98-102*)
- NF-κB activation (*103, 104*)
- Release of pro-inflammatory cytokines (*105*)

The immune system is a highly complex, intricately regulated group of cells and secreted proteins whose coordinated function is essential to guard the body from infection. Cells of the

immune system initiate a cascade of reactions in response to inflammatory signals through release of hormones, cytokines and autacoids (*106*). Lymphocytes (T cells, B cells), macrophages and neutrophils are the major immune cells that are involved in the manifestation of inflammation.

1.3.1 Macrophages in mounting inflammatory responses:

Macrophages are a type of phagocytic cells of the innate immune system which play a key role in host defence and inflammatory responses. Apart of acting as host armour, these cells also contribute to diverse functions like tissue remodelling, repair and development etc. (*107, 108*). Macrophages are found in all tissues. Though macrophages in different tissues differ in their functional capabilities, these cells are required for maintaining tissue homeostasis (*109*). Dysregulation of macrophage mediated immune responses has been implicated in pathogenesis of many chronic diseases, including atherosclerosis, asthma, inflammatory bowel disease, rheumatoid arthritis and fibrosis (*110*). When tissues are damaged following infection or injury, monocytes are recruited from the circulation and differentiate into macrophages as they migrate into the affected tissues. These recruited macrophages often show a pro-inflammatory phenotype in the early stages of the wound healing response. In tissues, mononuclear phagocytes respond to the surrounding niche (e.g., microbial products, damaged cells, activated lymphocytes) by gaining distinct functional phenotypes (*111*).

In response to various signals, macrophages may undergo classical activation (stimulated by TLR ligands and IFN-γ) or alternative activation (stimulated by IL-4/IL-13). Upon activation, they secrete a variety of inflammatory mediators including TNF-α, IL-1β, prostaglandin E2 (PGE2) and nitric oxide (NO) which activates anti-microbial defence mechanisms, including ROS that contribute to the killing of invading organisms (*109, 111*). They also produce IL-12 and IL-23, which direct the differentiation and expansion of anti-microbial TH1 and TH17 cells involved in inflammation (*112*). Indeed, if the inflammatory

macrophage response is not quickly controlled, it can become pathogenic and contribute to disease progression, as is seen in many chronic inflammatory and autoimmune diseases. In these diseases, macrophages are an important source of multiple key inflammatory cytokines that have been identified as drivers of autoimmune inflammation, including IL-1β, IL-12, IL-23 and TNF-α (*113, 114*). PGE2 is one of the main prostaglandin secreted in large quantities by macrophages and acts as an autocrine regulator of their activity. Exposure to LPS and IFN-γ induces an increase in the expression of cyclooxygenase-2 (COX-2) that leads to formation and subsequent secretion of prostaglandins by macrophages. COX-2 is inducible in cells that play a role in mediating inflammation such as macrophages, fibroblasts, and endothelial cells (*115*). Apart from macrophages, T cells are the other major cell type that mediates inflammatory responses.

1.3.2 T cell activation:

Naïve T cells recognize foreign antigen derived peptides presented on self MHC molecules by the antigen-presenting cells (APCs) leading to their activation. The activated CD4+T cells differentiate into different lineages of helper T cells like TH1, TH2, TH17, T(FH) or Treg cells. Activated CD4+ T helper lymphocytes release cytokines which stimulate antibody production by B cells and the killing of microbes inside the cytosolic vesicles by phagocytes. The CD8+ T lymphocytes, called as cytotoxic T lymphocytes kill any type of host cells that are harbouring infectious microbes (virus) in the cytoplasm. The antigen specific antibodies produced by B lymphocytes recognize extracellular microbial antigens and facilitate their recognition, neutralization, uptake and clearance by phagocytes and complement components.

T cell activation and downstream signaling is a highly regulated cascade of multiple signaling molecules activated by engagement of T cell receptor with cognate peptide MHC complex and costimulatory receptors (CD28). The duration of interaction of peptide-MHC on

antigen presenting cells with cognate T cell receptor is dependent on the affinity which decides the fate (activation, clonal expansion, differentiation or anergy) of naïve T cells. T cell receptor (TCR) is a complex of six (α, β, γ, ζ, €, θ) polypeptide units called CD3. Clonotypic TCR α and β chain, generated from genetic rearrangement, contributes to the specificity of the ligand binding. Interaction of cognate peptide-MHC with TCR is conveyed to the intracellular signaling machinery via three CD3 chains (θ€, γ€, ζζ) containing immunoreceptor tyrosine based activation motif (ITAM) present in the cytosolic domain of these proteins (*116, 117*). Due to lack of intrinsic enzymatic activity in TCR, these motifs are phosphorylated by SRC family members Lck and Fyn (*118, 119*). After TCR-peptide-MHC interaction, TCR co-receptor CD4/CD8 assists in localisation of Lck in close proximity to the ITAMs of CD3 and ζ chain and exclusion of CD45 (inhibitory phosphatase) from central part of the supra molecular activation complex (cSMAC) (*120, 121*). Lck dependent phosphorylation of ITAM results in recruitment and aggregation of ZAP70 (ζ chain associated protein kinase 70) which is further phosphorylated by Lck at tyr 493 residue. Phosphorylation of ITAM serves as binding sites for SH2 domain of ZAP70 (*122, 123*). Activated ZAP70 auto-phosphorylates at tyr 292, 315 and 319 residue and also phosphorylates LAT and SLP-76 which act as adapter proteins for phospholipase C γ (*121, 124, 125*).

This proximal signaling complex results in the activation of PLCγ1 dependent pathways including Ca^{2+} and DAG-induced responses, cytoskeletal rearrangements, and integrin activation pathways (*126*). Activated PLCγ1 hydrolyzes the membrane lipid PI(4,5)P2 (phosphatidylinositol 4,5-bisphosphate) producing the second messengers IP3 (inositol trisphosphate) and DAG (diacylglycerol). The IP3 generated by PLCγ1 stimulates the release of Ca^{2+} from endoplasmic reticulum (ER) stores into the cytoplasm. TCR-induced increase in intracellular Ca^{2+} levels result in activation of calcineurin and calmodulin-

dependent kinase (CaMK). Activated Calcineurin dephosphorylates nuclear factor of activated T cells (NFAT), leading to nuclear translocation and expression of genes important for T cell activation including IL-2 (*127, 128*). On the other hand, TCR-induced production of DAG results in the activation of two major pathways involving Ras and PKCθ. Ras is a guanine nucleotide–binding protein and is required for the activation of the serine-threonine kinase Raf-1. Activation of Raf-1 further initiates the signaling cascade leading to ERK dependent activation of signal transducer and activator of transcription 3 (STAT3) (*129*). The other major signaling pathway regulated by DAG is mediated by PKCθ. DAG is essential for recruiting PKCθ to the plasma membrane following T cell activation. PKCθ and Zap70 regulate NF-κB activation which plays a central role in T cell mediated immune response (*130*).

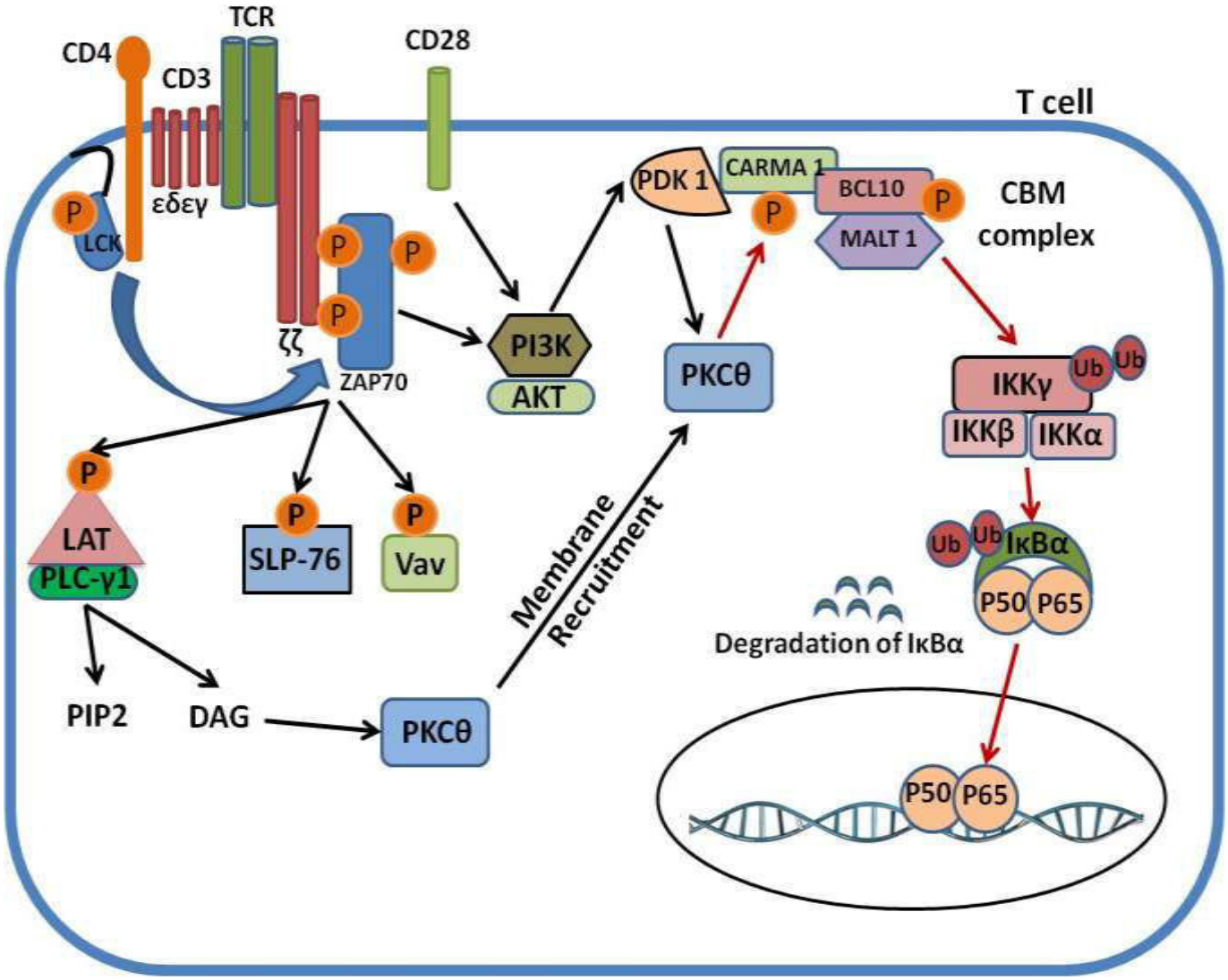
1.3.3 Role of NF-kappaB in T cell mediated response:

NF-κB is a ubiquitous heterodimeric Rel family transcription factor whose activation is controlled by cytoplasmic partitioning. NF-κB was first identified as a transcription factor that binds to the intronic enhancer of the kappa light chain gene (the κB site) in B cells (*131*). The NF-κB family of transcription factors comprises of 5 different proteins cRel, p65 (RelA), RelB, p50 and p52 which interact to form homo or heterodimer to perform different regulatory functions (*132-134*). N-terminal region of NF-κB protein contains a Rel homology domain (RHD) which is essential for binding to consensus motif in DNA known as κB element and also for dimerization and interaction with inhibitory protein, IκBα (*135-137*). In the absence of stimulus, NF-κB is sequestered in cytoplasm by IκBα which contains ankyrin repeats that bind at RHD domain of NF-κB protein masking the nuclear localisation signal and DNA binding motif (*138*). NF-κB can be activated by an array of stimuli like oxidative stress, IR, microbial injection, TNF-α, IL-1, MHC-peptide-TCR interaction or CD3/CD28 stimulation (*139*). Cytoplasmic events in response to stimuli lead to activation of IKK

complex which consists of IκB kinase (IKK) α, β and NEMO/γ. α and β subunits constitute the catalytic subunit whereas γ subunit forms the regulatory and structural subunit of this complex (*140*). IKK complex further phosphorylates ser residue on IκBα at N terminal region leading to its ubiquitination mediated degradation by 26S proteosome machinery. NF-κB translocates to the nucleus where further post translational changes govern its activity (*141*).

Engagement of antigen receptors on B and T lymphocytes also results in activation of IKK and NF-κB. Although TCR can induce limited NF-κB activation, but costimulation through CD28 is required for its efficient activation (*142*). Multiple mediators are involved in NF-κB activation by TCR including ZAP70, SLP76, PLCγ-1, SAP, PKCθ, Vav-1, Carma-1 etc. (*143-147*). A sequential order exists for TCR based NF-κB activation in which CBM (Carma-1, Bcl10, MALT-1) present downstream to PKCθ is required to form supra-molecular activation cluster (SMAC) to promote activation of IKK complex. PKCθ is a Ca^{2+} independent PKC isoenzyme expressed in lymphoid tissues and is required for activation of NF-κB in mature T cells. It is the only isoform in PKC family which translocates to the membrane during TCR activation induced signaling (*130, 148*).

Activated PKCθ phosphorylates Carma-1, initiating the assembly and recruitment of CBM complex to the membrane, serving as a connecting link between TCR and IκBα kinase (IKK) complex. Once CBM gets phosphorylated, it promotes the generation of lys 63 link polyubiquitin chains of NEMO. These polyubiquitin chains are required for the activation of TAK-1 a MAPKKK responsible for activation of IKKβ (*149-152*).



Scheme 1.6 Activation of NF-κB pathway following MHC-TCR interaction in T cells. Adapted with modification from Vallabhapurapu S et al., 2009 (*153*).

The activated IKK complex phosphorylates IκBα on ser32 and ser36, leading to its polyubiquitination at lys19 by the Skp1, Cdc53/Cullin1, and F-box protein β transducin repeat-containing protein (βTRCP). The ubiquitinated IκBα is degraded via the 26S proteasome, thereby exposing the NLS on RelA and inducing nuclear translocation of RelA:p50 dimers and transcription of its dependent genes. NF-κB is involved in the control of transcription of many genes whose functions extend beyond the immune response. The

NF-κB/Rel target genes include cytokines, chemokines, cytokine/chemokine receptors, adhesion molecules, survival genes, cell cycle regulators, acute phase proteins and inducible effector enzymes (Table 1.2). The majority of proteins encoded by NF-κB target genes participate in the host immune responses. Blocking of NF-κB activation, thereby inhibiting T cell responses, is used as an important strategy for curbing inflammation using small molecules by several investigators (*154-157*).

Category	Target gene
Cytokines and growth factors	➢IFN-γ, TNF-α, TNF-β, IL-1α, IL-1β, IL-2, IL-6, IL-8, IL-12, Eotaxin, G-CSF (Granulocyte Colony Stimulating Factor), M-CSF (Macrophage olony Stimulating Factor), VEGF (Vascular Endothelial Growth Factor) and G-CSF (granulocyte colony-stimulating factor)
Immunoreceptors	➢Immunoglobulin κ light chain, Interleukine 2 receptor α-chain, Major histocompatibility complex class I ➢B7.1 (CD80),CCR5 (Chemokine receptor), CD48 Antigen of stimulated lymphocytes ➢Fc epsilon receptor II (CD23), Immunoglobulin Cgamma1 IgG heavy chain 1 ➢Immunoglobulin e heavy chain IgE heavy chain ➢Invariant Chain, β2 Microglobulin, T-cell receptor β chain
Regulators of apoptosis	➢CD95 (Fas) (Pro-apoptotic receptor), Fas-ligand (Inducer of apoptosis), IAPs (Inhibitors of Apoptosis)

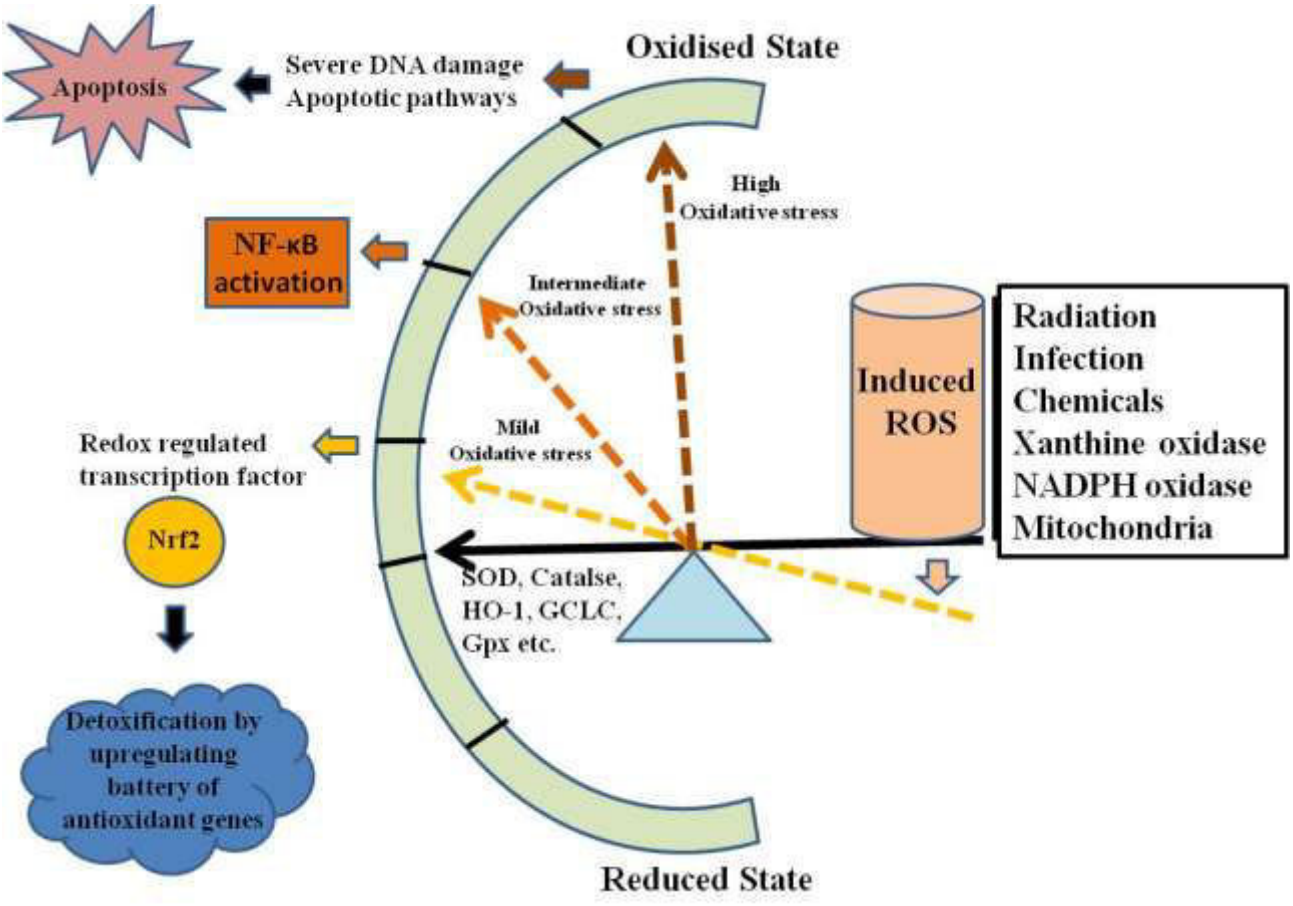
Table 1.2 Some of the NF-κB dependent genes that regulate immune response.

NF-κB activation is also dependent on the oxidative stress and cellular redox environment. Perturbation in cellular redox can thus be used as an alternative strategy to regulate T cell mediated inflammation.

1.3.4 Cellular redox homeostasis and T cell activation:

Cellular redox status is determined by the ability of a cell to maintain the balance between the magnitude of generated oxidative stress and the rate of its detoxification (*158*). Maintaining the redox balance is important for proper function and responses of cells. Any disturbance in the redox homeostasis induces oxidative stress mediated signaling cascade that could lead to cell death or induce adaptive survival responses. Outcome of perturbation in the redox balance depends on the magnitude of oxidative stress induced inside the cell (*159*). Alteration in the cellular redox could be due to excess generation of reactive oxygen species or depletion of endogenous antioxidants.

Reactive oxygen species include free radicals and pro-oxidants with a strong oxidizing property. Free radicals are atoms or molecules having unpaired electrons in their outer orbits which make them highly reactive in nature. Oxidative stress is the outcome of imbalance in the ratio between ROS production and ROS elimination by cell's antioxidant machinery (*160*). The intracellular "redox homeostasis" or "redox buffering" capacity is maintained primarily by glutathione (GSH oxidized / reduced) and thioredoxin (TRX oxidized / reduced) redox couples. GSH/GSSG ratio represents the major cellular redox buffer and it is therefore used as an indicator of the redox environment of the cell (*161, 162*). Basal levels of ROS are endogenously produced in the mitochondria due to partial reduction of oxygen, inflammatory reactions and enzyme linked reactions (NAPH oxidase, Xanthine oxidase, cytochrome c oxidase) (*163, 164*). Exposure to exogenous stressors including heavy metals, ionizing radiation, drugs and microbial infections can induce ROS inside the cells (*165*).



Scheme 1.7 Perturbation of cellular redox homeostasis: Induction of mild oxidative stress activates redox sensitive pro-survival pathways like Nrf2 that protects against the oxidative damage. High oxidative stress leads to induction of apoptosis.

Being highly reactive in nature, ROS, when produced at high rate can interact in a nonspecific manner with a wide range of macromolecules including proteins, carbohydrates, lipids and DNA leading to the alteration and impairment of function of cellular components leading to apoptosis (*166*). Exposure to IR induces high level of oxidative stress which can induce cellular apoptosis either by inducing death receptors which is exemplified by Fas-mediated caspase-8 activation or by mitochondria-mediated caspase-9 activation pathway. Both pathways converge on caspase-3 activation. However, generation of low levels of ROS can activate several redox-sensitive pro-survival signaling pathways and function as secondory messengers in the signal transduction (*158*).

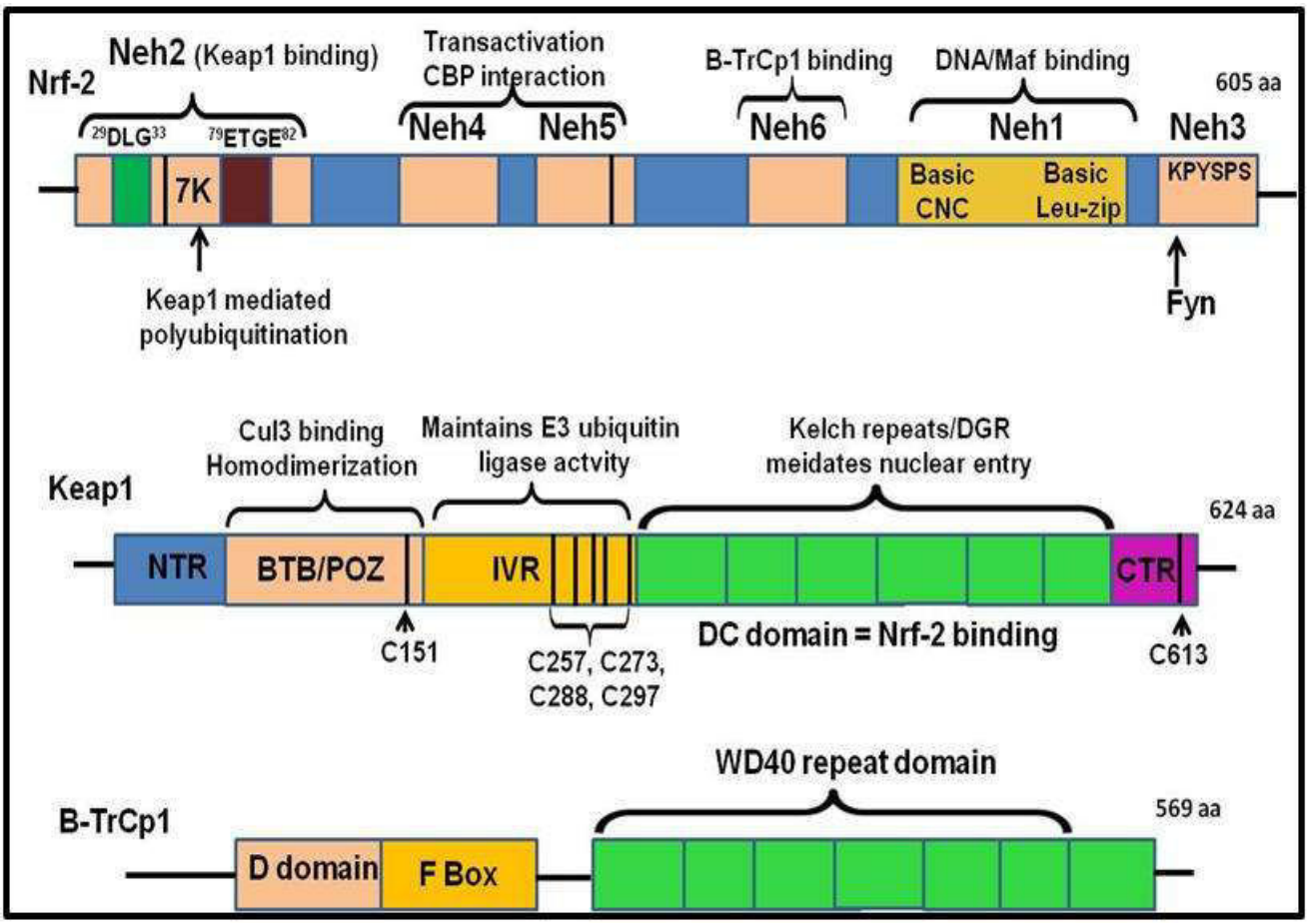
ROS also play an eminent role in T cell mediated immune responses. Hydrogen peroxide (H_2O_2) and superoxide (O_2^-) have been shown to exert diverse effects on the target proteins depending upon the concentration and time of exposure (*167*). High levels of ROS are associated with inhibition of cellular functioning whereas low levels are demonstrated to elicit beneficial effects. ROS have very short half-life and are tightly regulated via a complex network of antioxidants (*168*). Controlled and organised production of ROS underlines their potential to act as secondary messengers involved in signal transduction from cell surface to nucleus. Binding of ligands to receptor has been shown to generate low levels of ROS which can suppress phosphatase activity leading activation of kinases and subsequent signal transduction pathways (*169-171*). Scavenging of endogenous ROS can impair normal cellular response like production of cytokines and growth factors by T cells (*172-175*). All three redox couples in the cells (GSH/GSSG, cys/cyss and thioredoxin oxidised/reduced) have been shown to play a pivotal role in regulation of immune functions. GSH/GSSG is the major player in the antioxidant repertoire of redox buffer (*176-178*).

Redox state of many proteins plays an important role during immune responses (*164*). Critical cysteine residues present on proteins act as redox sensors and are prone to oxidation into sulfenic acids or disulphide formation or glutathionylation resulting in the modulation host immune responses (*179-181*). The effect of oxidative stress on functions of these proteins depends on the concentration, duration and location of ROS generated inside the cell. Mild oxidative stress (low levels of ROS) can activate redox-sensitive transcription factors like Nrf2.

1.4 Nrf2 PATHWAY:

The transcription factor, nuclear factor [erythroid-derived 2]-like 2 (Nrf2) was first identified as NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-

E2/AP1 repeat of the β-globin locus control regions. The Nrf2 gene was first cloned and characterized by employing the tandem repeats of nuclear factor like erythroid factor-2 (NF-E2) / activator protein-1 (AP1) of the β-globulin locus as a recognition site probe. Nrf2 contains a basic leucine zipper DNA binding domain at the C-terminus and has N-terminal acidic domain (rich in glutamic and aspartic acid residues). It was later characterized as Cap`n`Collar (CNC) protein involved in the control of development of *Drosophila* head segment by basic leucine zipper DNA binding domain (bZip) homeotic gene (*182*). Nrf2 knockout mice are viable and exhibit no phenotypic defects but are sensitive to oxidative stress (*183-188*).



Scheme 1.8 Structures and functions of Nrf2 and its repressors KEAP-1 and β-TrCP1: The relative position of the Neh domains is shown. The DLG and ETGE motifs present in Neh2 domain that bind to KEAP-1 are represented above with the numbering of amino acids based on the human cap'n'collar (CNC)-basic-region leucine zipper (bZIP) protein (*189, 190*).

Human Nrf2 is homologous to mouse and has six highly conserved domains called Nrf2-ECH homology domains (Neh). Neh1 domain contains a nuclear localisation signal and CNC-type basic leucine zipper that is necessary for DNA binding and dimerization. The Neh2 domain contains a KEAP-1 (Kelch-like ECH-associated protein 1, a negative regulator of Nrf2) binding pocket and has seven lysine residues that direct ubiquitin mediated proteasomal degradation of Nrf2 (*191, 192*). Neh3 is essential for interaction with CHD6 (a chromo-ATPase/helicase DNA binding protein) suggesting its involvement in interaction with co-transcription factors (*193*). Neh4 and Neh5 are transactivation domains that interact with the CREB-binding protein (CBP) (*194*). Neh6 domain has been attributed to interact with β-transducin repeat-containing protein (β-TrCP) (*195*). KEAP-1 binds to Nrf2 which brings it in close proximity of E3 ligase complex through two major domains: the BTB (Bric a Braˇc, tramtrack, broad complex) domain which interacts with Cul3 and the kelch domain which binds to Nrf2. Interaction of Neh2 domain with KEAP-1 depends on its low-affinity binding via DLG motif and the high-affinity binding of an ETGE motif which results in a hinge and latch mechanism of binding. The N-terminal BTB / POZ (Pox virus Zinc finger) domain forms homodimers that enables KEAP-1 to interact with Nrf2 (*196-200*).

Exposure of cells to low levels of oxidative stress, electrophiles or chemopreventive agents leads to activation of Nrf2. Upon activation, Nrf2 dissociates from its inhibitory protein KEAP-1 and translocates to the nucleus, forms a heterodimer with its co-transcription factor Maf and binds to the antioxidant response element (ARE) sequence to induce transcription of several different types of genes (*190*). ARE sequence was characterised as 5`-RTGACnnnGCR-3` using murine GST-Ya ARE and this sequence was used to identify genes that contain ARE sequence in their promoters (*201*). The Nrf2 downstream genes include phase II detoxifying enzymes like glutathione S-transferase (GST), NAD(P)H quinone oxidoreductase-1 (NQO1) (*202*), and UDP-glucuronosyl transferase (UGT),

intracellular cytoprotective proteins like glutamate cysteine ligase (GCL) (*203*), glutathione peroxidase (GPx) (*204*), thioredoxin (Trx) (*205*), thioredoxin reductase (TrxR) (*206*), peroxiredoxin (Prx) (*207*), hemeoxygenase-1 (HO-1) and transporters like multidrug resistance-associated protein (MRP) (*208*). Phase II enzymes reduce the toxicity of xenobiotics by making them water soluble thereby facilitating their elimination. Efflux of endogenous molecules and xenobiotics is also governed by Nrf2 mediated expression of transporters.

1.4.1 Mechanism of Nrf2 Activation:

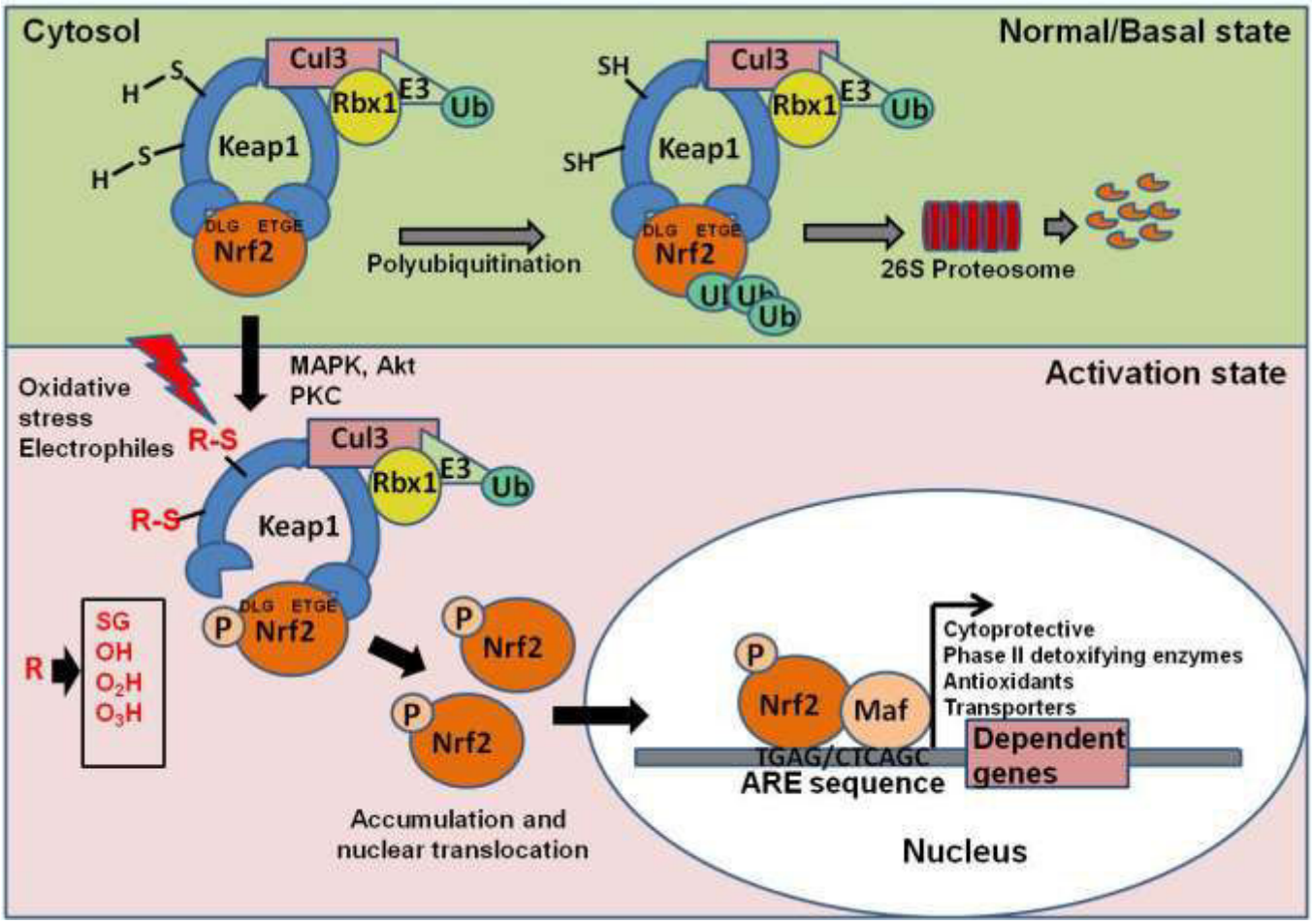
Nrf2 is sequestered in the cytoplasm by its repressor KEAP-1 which strictly regulates Nrf2 stabilization and maintain the levels inside the cell. This interaction between the two proteins is a dynamic process which is regulated in such a manner that enables Nrf2 to control both the basal and inducible expression of its dependent genes. Under normal conditions, Nrf2 is maintained at low basal levels sufficient enough for the expression of cytoprotective genes (*191*). Nrf2 is kept at low levels when bound to a homodimer of KEAP-1 through its kelch repeats domains at C terminal, leading to Cullin3/Rbx1-mediated polyubiquitination and subsequent proteasomal degradation of Nrf2. A characteristic distinguishing feature of KEAP-1 protein is that it contains numerous cysteine (cys) residues that encrypt its potential to act as a redox sensor (*209*). The significance of KEAP-1 as a central regulator of Nrf2 activation was revealed while addressing the negative regulation of antioxidant machinery by KEAP-1 dependent proteasomal degradation of Nrf2 (*210*). The half life of Nrf2 was found to increase from 15min to 30min in cells expressing mutated ETGE motif containing Nrf2 and wild type KEAP-1 (*211*)**.** Using *in vitro* alkylation and *in vivo* site-directed mutagenesis, cys151 was identified as the major site that is directly alkylated by Nrf2 inducers along with two other critical residues cys273 and cys288 (*212-215*). Mutation at cys151 abolished the induction of Nrf2 by activators like sulforaphane and *tert*-butylhydroquinone but had no

impact on KEAP-1:Nrf2 binding (*216*). On the contrary, Wang et al reported activation of Nrf2 by arsenite in cys151 KEAP-1 mutant cells indicating a possible redox independent mode of Nrf2 induction (*217*). Further, mutations at cys273 and cys288 showed abrogation of repression of Nrf2 by KEAP-1 despite binding to Nrf2 (*215, 216*). These observations demonstrated that modifications of cys273 and cys288 are essential for Nrf2 activation. Several cellular redox modifiers have been shown to modulate the activation of Nrf2 via modification of these critical cysteine residues in KEAP-1.

Exposure of cells to oxidative, xenobiotic or electrophilic stress abrogates KEAP-1 induced degradation of Nrf2. Perturbation in the cellular redox status results in modifications of critical cysteine residues present in the KEAP-1 resulting in the conformational change rendering the release of Nrf2 from the low affinity binding motif (*218, 219*)**.** This further leads to Nrf2 stabilisation and accumulation in the cytosol followed by its nuclear translocation. According to hinge and latch model, ETGE motif remains bound to the KEAP-1 following activation stimuli. This results in saturation of KEAP-1, which is no longer able to compete with the free Nrf2, inducing the translocation of Nrf2 to the nucleus and binding to ARE leading to expression of cytoprotective machinery of the host cell (*220*). An alternate model of induction is attributed to the polyubiquitination of KEAP-1 at lys63. This leads to subverted Cullin3 interaction and dissociation of Nrf2 from KEAP-1 (*221*). This observation was further supported by employing the ubiquitin-specific protease-15 deubiquitinase which restored KEAP-1 activity (*222*).

Post translational modification also governs the Nrf2 activation. Nrf2 contains multiple serine, threonine and tyrosine residues which can serve as potential sites for phosphorylation. Different kinases have been identified including protein kinase C (PKC), mitogen-activated protein kinases (MAPK), phosphatidylinositol 3-kinase (PI3K) and RNA-dependant protein kinase-like endoplasmic reticulum kinase (PERK) (*223-226*). tBHQ was

shown to enhance NQO1 protein expression and activity in a PI3K dependent manner in human neuroblastoma cells. tBHQ elicited ARE mediated induction of GST in hepatoma cells in a PI3K dependent manner. PI3K inhibitor (Ly294002) abrogated tBHQ mediated NQO1 induction, indicating a possible role of PI3K in Nrf2 activation (*225*). Important role of MAPK in activation of Nrf2 via phosphorylation has been demonstrated by multiple investigators. Jeong et al studied MAPK mediated activation of phase II detoxification enzymes using multiple inducers (*227*). In hepatoma cells, sulforaphane and tBHQ induced the activation of ERK and Raf-1 to mediate the induction of phase II detoxification enzymes via Nrf2/ARE pathway (*228, 229*). MAPK/ERK upon activation serves as the initiator of a phosphorylation cascade that modulates the activity of multiple downstream transcription factors (*230, 231*). Dithiolcarbamate was shown to activate ERK and p38 resulting in transcriptional up-regulation of Nrf2 dependent γ-glutamylcysteine synthetase (*232*). Shen et al while investigating the transactivation potential of different Nrf2 domains described the differential effects of multiple MAPKs in activating Nrf2. They further demonstrated that Raf-1 mediated activation of Nrf2 is due to up-regulation of the co-activator CREB binding protein (*230*).



Scheme 1.9 Schematic model of Nrf2 activation under normal and oxidative stress conditions:

Apart from KEAP-1, Cul3/Rbx1 and other mediators also contribute in regulating the low basal levels of Nrf2. Phosphorylation status of tyr568 on Nrf2 is governed by Src subfamily kinases like Fyn, Src and Fgr which influence the nuclear export of Nrf2. Under oxidative stress conditions, glycogen synthase kinase-3 beta (GSK-3β), a serine / threonine protein kinase plays an important role in the nuclear export of Nrf2 by phosphorylating Fyn. Another Src member Bach1 has been shown to govern the export of Nrf2 from nucleus and thereby negatively regulate the expression of its dependent genes. It has also been shown that Bach1 competes with Nrf2 for binding to ARE sequence resulting in suppression of ARE mediated expression of Nrf2 dependent genes (*220, 233*)**.**

Multiple studies have also highlighted the KEAP-1 independent activation of Nrf2. Along with KEAP-1 dependent degradation of Nrf2 there is an alternate mechanism that controls the activation and stabilisation of Nrf2, mediated by β-transducin repeat-containing protein (β-TrCP) (*234*). Mouse Nrf2 contains two binding sites for β-TrCP which acts as an adapter for the Skp1-Cul1-Rbx1 ubiquitin ligase complex. GSK-3β phosphorylates ser residue in SCF/β-TrCP destruction motif "DSGIS" in Neh6 domain leading to KEAP-1 independent degradation (*195*).

Apart from the cytoprotective nature of Nrf2, its role as a redox sensitive anti-inflammatory transcription factor has been well documented. Recent reports reveal an important role of Nrf2 in modulating immune responses and ameliorating immune disorders. Nrf2 knockout mice showed enhanced bronchial inflammation (*235*), prolonged inflammation during cutaneous wound healing (*236*), high susceptibility for lupus like autoimmune syndrome, enhanced lymphocyte proliferation and impaired redox status (*237*). Stimulated lymphocytes from Nrf2 deficient mice showed higher secretion of Th2 cytokines like IL-4 and IL-13 (*235*). Further, Nrf2 deficient mice showed higher mortality to septic shock caused by lipopolysaccharide or cecal ligation or puncture (*238*). Nrf2 dependent protein, HO-1 and its degradation product carbon monoxide, have been reported to show anti-inflammatory effects by inhibiting pro-inflammatory cytokine production, leukocyte migration, adhesion (*239-241*) and suppressed LPS induced production of tumour necrosis factor-*a* (TNF-*a*) and nitric oxide (NO) in murine macrophages (*242*). Several studies have shown an indispensable role of Nrf2 and its dependent gene HO-1 in controlling inflammatory responses via regulation of cytokines and pro-inflammatory proteins and its deficiency was shown to increase susceptibility to inflammatory disorders (*197, 243-248*). Increased expression of HO-1 was shown to protect against airway inflammation (*243*), brain infections (*245*), skin inflammation (*247*) and gastro-intestinal inflammation (*244*). Further,

CO was shown to attenuate LPS induced activation of human umbilical vascular endothelial cells (*249*) and ameliorated murine joint inflammation. Several investigators have highlighted the role of KEAP-1/Nrf2 pathway in regulating NF-κB activation as well as NF-κB mediated inflammatory response (*250-253*).

Apart from the anti-inflammatory action of Nrf2 dependent genes, activation of Nrf2 has been shown to suppress activation of NF-κB there by inhibiting inflammatory reactions. Multiple studies have highlighted the cross talk between Nrf2 and NF-κB as prime target for development of novel anti-inflammatory agents. Several anti-inflammatory or anti-carcinogenic phytochemicals suppress NF-κB signaling and activate the Nrf2-ARE pathway. Nrf2-deficient mice, subjected to a moderately severe head injury, show a greater cerebral NF-κB activation compared with their wild-type Nrf2 counterparts (*254*) while Nrf2 over expression suppresses NF-κB- DNA binding activity (*255*). Cinnamaldehyde activates Nrf2 and inhibits the degradation of IκBα leading to suppression of NF-κB pathway (*256*). Curcumin, a polyphenol, was shown to activate Nrf2 which further mediated the modulation of expression and transactivation of NF-κB (*257*). Recently, Kim et al showed the ability of KEAP-1 to suppress NF-κB pathway by inhibiting phosphorylation and inducing degradation of IKKβ (*258*). These studies clearly demonstrate the potential of the cross talk between these two transcription factors for therapeutic interventions.

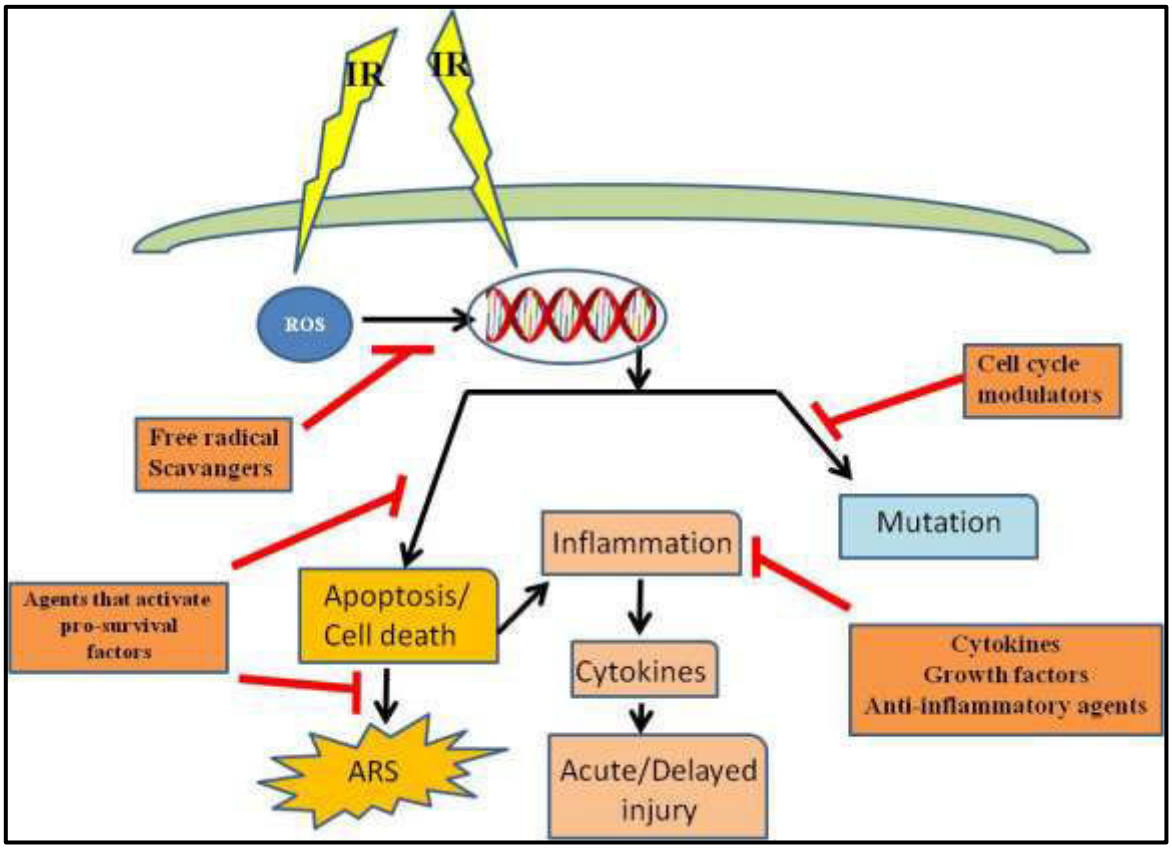
1.5 STRATEGIES EMPLOYED FOR PROTECTING AGAINST IONIZING RADIATION INDUCED DAMAGE:

Ionizing radiation mediated tissue injury is not mediated through direct and indirect effects and also through systemic inflammation. Plethora of studies has shown that a variety of agents can mitigate the radiation induced damage by different mechanisms. These agents can be classified on the basis of time of administration (*259, 260*).

- Prophylactic radioprotectors are agents given prior to irradiation to prevent injury from ionizing radiation induced free radicals.
- Radiation mitigators are given just before or immediately after radiation exposure with the aim of preventing or reducing the radiation damage on tissues before the appearance of symptoms. These agents also aid in accelerating the recovery and repair process of injured tissue.
- Therapeutic agents are given after irradiation where in appearance of obvious symptoms of IR injury has set in. These agents aid in decreasing ionizing radiation induced injury by reducing inflammation or oxidative stress or enhancing DNA repair or upregulating pro-survival pathways.

Work on development of radioprotective agents started more than six decades ago and identification of radioprotective agents has been an important goal for radiation oncologists as well as radiation biologists. The potential application of prophylactic agents is to protect normal tissues, but not tumors, during planned exposure settings like cancer radiotherapy. However, therapeutic agents find applications mainly during accidental exposures. Multiple strategies have been employed to develop putative agents that can protect against radiation induced damage like free radical scavengers, inhibitors of radiation induced cell death

signaling pathways, cell cycle modulators, cytokines, growth factors and activators of pro-survival pathways.



Scheme 1.10 Strategies employed to develop a potent radioprotector.

Since radiation induced damages are mediated by the induction of free radicals, employing a free radical scavenger was the first amenable strategy. A landmark paper by Patt et al demonstrated the efficacy of cysteine in ameliorating X-ray induced lethality in rats (*261*). Thereafter, several studies using free radical scavengers such as N-acetyl cysteine, glutathione, β-mercaptoethylamine (cysteamine, MEA), propylgallate, nordihydroguiaretic acid, vitamin-E & vitamin-C showed their ability to protect mice against IR induced toxicity (*262-266*). Endogenous antioxidants such as superoxide dismutase, catalase, peroxiredoxins, thioredoxin and glutathione have been used to develop potent radioprotectors (*267*). Thiol group containing molecules have been investigated extensively due to their ability to donate reducing equivalents to unstable molecules such as reactive oxygen species (*212, 268, 269*).

The most effective FDA approved radioprotective drug developed till date for use in the protection of normal tissues in patients treated with radiation is amifostine (S-2-[3-aminopropylamino] ethylphosphorothioic acid) (*270*). It is a complex aminothiol, which is reported to exhibit multiple biochemical properties like free radical scavenging activity, high affinity for DNA and the structural similarity with cellular polyamines (*271*). Amifostine is a clinically approved radioprotector only for head & neck cancer patients in preventing radiation induced xerostomia. However, it exhibits considerable toxicity at radioprotective doses due to which its use is limited (*272*).

Apart from antioxidants, other agents have been investigated including granulocyte colony-stimulating factor (G-CSF) which improved bone marrow recovery and reduced WBI induced lethality (*273*). Keratinocyte growth factor (KGF) is another radio-mitigator and it has been shown to stimulate a number of processes such as differentiation, DNA repair, as well as scavenging reactive oxygen species (*274*). KGF has been recently approved by FDA for mitigating oral mucositis (*275*). Genistein, an isoflavone, has also been shown to exhibit radioprotective effects through antioxidant action and by modulating cell cycle (*212, 269*). Genistein offered protection to normal cells while promoting G2/M cell cycle arrest in cancer cells (*276*).

Compound	Property	Clinical uses
Amifostine	Antioxidant	Adjuvant for radiation therapy to reduce xerostomia in Head & Neck cancers
Curcumin	Antioxidant	Pharmacological effects seen in intestine of cancer patients
Geinstein	Cell cycle modulator	Reduced pain/diarrohea In patients undergoing radiotherapy of the abdomen
Palifermin	Growth factor	Reduce mucositis in patients receiving radiotherapy
Halofuginone	TGF-β inhibitor	Reduce fibrosis in patients undergoing radiotherapy

Table 1.3 Radiation countermeasures investigated under clinical scenarios (*259, 269, 274, 277-280*).

Activation of NF-kB signaling has been exploited to induce radioprotective cytokines and thereby suppress apoptosis (*281*). CBLB502, a polypeptide derived from *Salmonella* flagellin, which binds to toll-like receptor 5 and subsequently activates NF-kB signaling was shown to protect against WBI induced hematopoietic and GI syndrome associated mortality while it did not alter radiosensitivity of the tumors (*282*). Though multiple modalities have been studied for protecting against radiation induced hematopoietic syndrome, but very few agents have been investigated to ameliorate radiation induced GI syndrome. Recent advances have highlighted that activation of pro-survival pathways could be used to develop potent radioprotectors. An anti-ceramide monoclonal antibody protected against WBI induced apoptosis in the small intestine and mortality in mice (*283*). *Lactobacillus* probiotic was shown to protect against WBI induced apoptosis in intestinal epithelial cells in a TLR-2 and cyclo-oxygenase-2 dependent manner (*284*). Sphingosine-1-phosphate (S1P), a ceramide antagonist, protected against WBI induced endothelial apoptosis in intestine by activating Akt

pathway (*285*). R-spondin 1 improved survival percentage in mice exposed to WBI doses inducing GI syndrome by activating Wnt/β-catenin pathway (*56*). Despite the potent efficacy of several investigated agents, none of them could reach clinic to be used as general radioprotector leaving this area of research wide open to develop newer agents which can surpass some of the drawbacks of previously developed radioprotective agents.

1.5.1 Cellular Redox modulation as novel strategy for development of radioprotective compounds:

The strategy of using antioxidants/free radical scavengers as radioprotectors has not yielded expected results in the clinic. This could be due to need for its presence systemically in cells with uniform distribution among the cellular compartments in sufficient concentrations to nullify the toxic effects of ROS. Achieving such high concentration of antioxidant throughout the living system to effectively detoxify free radicals is less plausible. Further, the efficacy of antioxidants to neutralize free radicals also depends on its rate of reaction with the free radicals. Thus, antioxidant property alone may not be sufficient to neutralize radiation induced toxicity. We hypothesized that activation of pro-survival pathways by employing a pro-oxidant prior to radiation exposure may prime the cells to counterbalance the radiation insult. Activation of antioxidant and cytoprotective machinery under the regulation of Nrf2 would prepare the cell to combat against radiation induced oxidative stress.

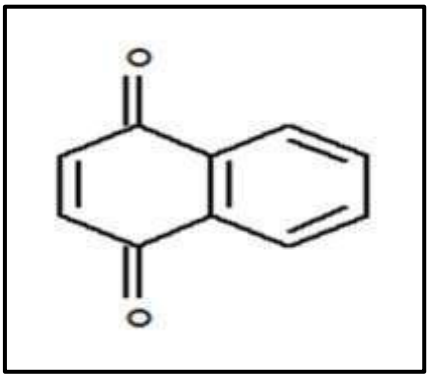
Hence, we have employed a novel strategy of tickling cellular redox using pro-oxidants to activate pro-survival transcription factor, Nrf2, to obtain protection against radiation induced GI damage. This is in contrary to the classical belief that radical scavengers could be potent radioprotectors. Multiple studies have highlighted the potential of pro-oxidants to protect against oxidative stress induced cellular damage (*286, 287*). Plumbagin, a

naphthoquinone derivative and a pro-oxidant, was shown to protect oxidative stress induced spinal injury (*288*). Further, sulforaphane, a redox modifier and potent Nrf2 activator agent, has been shown to protect against oxidative stress induced focal cerebral ischemia, brain inflammation, ischemia and acute renal failure (*289*). It was also shown to prevent doxorubicin-induced oxidative stress and cell death in rat H9c2 cells (*290*). Tert-butylhydroquinone (tBHQ) has been shown to confer protection against H_2O_2 induced cell death in the neural stem cells (*291*). Similarly, 15d-PGJ(2) protected astrocytes against Mn-induced inflammation and oxidative stress by modulating the activation of the NF-κB and Nrf2 pathways (*292*).

We have earlier shown that 1, 4-naphthoquinone (NQ), a well-known pro-oxidant, offered protection against radiation induced hematopoietic syndrome in mouse model via activation of Nrf2 pathway (*287*). Cross talk between Nrf2 and NF-κB has been the prime target for developing novel anti-inflammatory agents. Multiple Nrf2 activators have been shown to suppress NF-κB activation and its dependent pro-inflammatory genes. Previous studies from our laboratory employing a derivative of 1, 4-naphthoquinone i.e. plumbagin, have elucidated the potent anti-inflammatory activity both in vitro and in vivo model systems (*154, 293*). These studies were further extended using NQ to explore the possibility of protecting against radiation induced GI syndrome. Since NQ was able to activate Nrf2 (*287*), an immunoregulatory transcription factor and a negative regulator of NF-κB, we also investigated the anti-inflammatory effects of 1,4 NQ in innate and adaptive immune responses.

1.6 1, 4 NAPHTHOQUINONE (NQ):

1, 4-naphthoquinone (NQ), is a bifunctional para-quinone, and it is derived from naphthalene through the replacement of two hydrogen atoms by two ketone groups. NQ is also known as 1,4-naphthalenedione, alpha naphthoquinone, p-naphthoquinone, 1,4-naphthylquinone, 1,4-dihydro-1,4-diketo naphthalene. It is one of the simplest quinones studied and is a parent molecule of multiple bioactive quinones with biological significance like vitamin K_3. Naphthoquinone is a class of natural phenols based on the C6-C4 skeleton, sometimes referred to as naphthyl functionality. The physicochemical properties of NQ are listed in table 1.4.



Scheme 1.11 Chemical structure of NQ

1,4 Naphthoquinone	Properties
Molecular Formula	$C_{10}H_6O_2$
Molecular Weight	158.15
Appearance	Yellow to brown powder
Solubility	DMSO
Melting Point	125-128°C
Density	1.422
Photosensitivity	Yes

Table 1.4 Physical and chemical properties of NQ.

1.6.1 Chemical synthesis of NQ and its derivates:

Chemical structure of naphthoquinones is based on bicyclic system in monomeric form– naphthalene skeleton substitute in position C1 and C4 (1, 4-naphthoquinones) or C1 and C2 (1, 2- naphthoquinones). Most of them are coloured compounds and their appearance varies between yellow, orange and brown. The simplest and most abundant naphthoquinones in plants are juglone, lawsone, plumbagin and lapachol. Almost all naphthoquinones are soluble in alcohol, acetone, chloroform, benzene, DMSO and acetic acid. 1, 4-naphthoquinone is the common centre point of these quinones and shares structural homology with wide range of quinoid compounds.

Naphthoquinones are present in reduced and glycosidic form among the higher plant families like Avicenniaceae, Bignoniaceae, Boraginaceae, Droseraceae, Ebenaceae, Juglandaceae, Nepenthaceae and Plumbagnaceae. They are biosynthesized via a variety of pathways including

- Acetate and malonate pathway (plumbagin)
- Shikimate/succinyl CoA combined pathway (lawsone)
- Shikimate/mevalonate pathway.

Quinones are the second largest class of anti-tumor agents currently in use. Quinones are ubiquitous in nature and probably found in almost all respiring animals and plants, and have important role in biological functions (*294, 295*). Quinones, including ubiquinone (coenzyme Q10), acts as electron carrier in mitochondrial electron transport chain and phylloquinone (vitamin K1) take part in the blood clotting (*296*). NQ is a parent molecule of many clinically approved anticancer, anti-infective and anti-parasital drugs. There are many clinically important agents containing a quinone nucleus with admirable anticancer activity (e.g. anthracycline, mitoxantrones, mitomycin, diaziquone, saintopin daunorubicin and

doxorubicin). Plastoquinone is known to play a key role in photosynthesis cycle in plants and bacteria (*296*). Multiple clinically important quinones belong to the group of DNA intercalators, alkylating agents, topoisomerase inhibitors and inhibitor of nitric oxide synthase. In addition to these biological roles, quinones have wide variety of industrial usage, as herbicide, bleaching reagent and cosmetic (*297, 298*).

There are many clinically approved drugs which are chemical derivatives of naphthoquinone either in monomeric, dimeric or trimeric form.

- **Atovaquone** is a hydroxyl derivatives of 1,4 NQ that has been shown to possess anti-malarial activity (*299*).
- **Mepron** (GlaxoSmithKline) is a formulation of micro-fine particles of atovaquone that is currently used as an effective antiprotozoal agent against *Pneumocystis carinii*, and *Plasmodium species* by inhibiting dihydroorotate dehydrogenase (*299*).
- **Nanaomycin** is an antibiotic produced by a strain of *Streptomyces*. It is a hydroxyl derivative of 1,4 NQ involved in heme dependent radical generation and is reported to inhibit coupling of oxidative phosphorylation (*300*).
- **Malarone** (Glaxo Wellcome, Inc., Reasearch Triangle Park, NC), is a part of fixed-dose formulation of atovaquone and proguanil hydrochloride. It has been shown to be effective against strains that are resistant to a variety of other antimalarial drugs and has a favourable safety profile (*300*).
- **Diospyrin** is a dimer of hydroxyl derivative of NQ that is capable of inhibiting the growth of *Leishmania donovani* promastigotes by inhibiting type I DNA topoisomerase (*301*).

1.6.2 Prooxidant effects of NQ:

Quinones undergo metabolism via redox cycling and form semiquinone radicals either by one electron reduction in the presence of NADPH–cytochrome P450 reductase and NADH–cytochrome b reductase (*302, 303*). Although the hydroquinones are generally more stable than their semiquinones with respect to reactions with O_2, they can undergo autoxidation to yield H_2O_2 (*304*). All quinones are redox-cycling agents that generate ROS. Partially substituted quinones also function as arylating agents, reacting with cellular nucleophiles such as thiols on cysteine residues of proteins and glutathione (GSH). These covalently linked quinone-thiol adduct called Michael adducts also retain the ability to function as redox-cycling agents (*305*).

Quinones are also generated in the body as a result of xenobiotic metabolism via the cytochrome P450 system. In general, biological functions of quinones have been attributed to the ability to undergo reversible oxidation-reduction reactions, as well as their electrophilic nature leading to the formation of free radicals (*306, 307*). Because of their ability to generate reactive oxygen species (ROS) and induce mild oxidative stress, quinones possess broad spectrum of biological activities.

High amount of ROS are toxic and induce cell death, whereas low levels behave as second-messenger and induce pro-survival pathways by activating redox-sensitive transcription factors like Nrf2. Exposure to ionizing radiation is followed up by elevated inflammatory responses. Intestinal tract holds the largest lymphoid tissue causing maturation and extra-thymic development of T lymphocytes. Thus we hypothesized that a pro-oxidant may not only protect against radiation induced toxicity by activating Nrf2 but also suppress inflammation by inhibiting pro-inflammatory transcription factor NF-κB. Role of Nrf2 has been highlighted in inducing anti-inflammatory responses, maintaining intestinal redox

homeostasis, regulating proliferation of intestinal stem cells and protecting colonic cells from radiation induced damage. Hence, the present study was undertaken to assess the efficacy of NQ to ameliorate radiation induced GI syndrome and also examine its potential as anti-inflammatory agent. The present thesis describes the effect of NQ on radiation induced gastrointestinal injury, modulation of immune responses and delineating the underlying mechanism(s).

1.7 AIMS OF THE STUDY:

1. To investigate the effect of NQ against whole body irradiation (WBI) induced GI syndrome in mice.
2. Elucidation of molecular mechanism of radioprotection offered by NQ in vivo.
3. To investigate the potential of NQ to modulate the inflammatory responses in murine lymphocytes and elucidate the underlying mechanism.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS AND METHODS:

2.1.1 Reagents and Chemicals:

NQ, RPMI 1640, DMEM, HEPES, EDTA, EGTA, phenylmethanesulfonyl fluoride (PMSF), leupeptin, aprotinin, benzamidine, dithiothreitol (DTT), N-acetyl cysteine (NAC), Trizol reagent, propium iodide (PI) and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (MO, USA). Fetal calf serum (FCS) was obtained from GIBCO BRL (MD, USA). Concanavalin A (Con A), tin-protoporphyrin (SnPP) and all-trans-retinoic acid (ATRA) were purchased from Calbiochem (Darmstadt, Germany). cDNA synthesis kit, In situ cell death detection kit (TUNEL) and SYBR green PCR mix were procured from Roche Chemical Co (USA). Carboxyfluorescein succinimidyl ester (CFSE), LIVE/DEAD fixable dead cell stain kit and Hoechst33342 were procured from Molecular Probes (NY, USA). Antibodies against pERK, ERK, IκBα, β-actin, IKKβ and KEAP-1 were procured from Cell Signaling Technologies (CA, USA). Antibody against GSH was procured from Abcam (CA, USA). Lgr5 antibody was procured from Origene (MD, USA). Antibody against Nrf2, FITC labelled anti-BrdU antibody, oligonucleotide probes for NF-κB, Nrf2 and anti-Cox-2 antibody were purchased from Santacruz Biotechnology (CA, USA). ELISA sets for detection of cytokines (IL-2, IL-3, IL-4, IL-6, IL-1β, TNF-α and IFN-γ) and monoclonal antibodies against CD3 and CD28 were procured from BD Pharmingen (CA, USA). All other chemicals were purchased from reputed local manufacturers.

2.1.2 Animal maintenance:

Eight week old inbred BALB/c male mice, weighing approximately 20-25g, reared in the animal house of Bhabha Atomic Research Centre were used. They were housed in plastic cages at constant temperature (23°C) with a 12/12 h 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.

Among several commonly used inbred mouse strains, BALB/c has been found to be sensitive to exposure of ionizing radiation due to natural genetic variation in nonhomologous end joining (NHEJ) repair pathway which is primary mode of DSB repair in mammalian cells (*308*). MHC haplotype antigen of BALB/c mice is H-2^d. Therefore, BALB/c mice were used in the present study to examine effects of radiation exposure and its amelioration by administration of NQ.

2.1.3 Cell line and culture conditions:

RAW 264.7 cells obtained from Health Protection Agency Culture Collections (HPACC, UK) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) at 37°C in an atmosphere of 5% CO2.

2.1.4 Treatment with NQ:

For *in vivo* studies, NQ was dissolved in DMSO and final concentration was adjusted to 0.5mg/ml with 1X-PBS. Four doses of NQ (2mg per kg body weight or 2mg/kg bw) were injected intra-peritoneally (i.p.) to mice consecutively for 4 days at an interval of 24h using 26.5 gauge size needle. Vehicle treated mice served as control. For in vitro studies in lymphocytes, a 100mM solution of NQ was prepared in DMSO, stored as small aliquots at −20°C, and then diluted as needed in cell culture medium. Cells were treated with NQ 5µM or different concentrations of NQ for 2h in RPMI medium and were further stimulated with mitogen without washing the cells. DMSO was used as vehicle control in vitro.

2.1.5 Irradiation schedule:

Whole body irradiation (WBI) was carried out using ^{60}Co γ-irradiator Bhabhatron at a dose rate of 1 Gy/min (BRIT, Mumbai, India). BALB/c mice were placed in ventilated perspex boxes and exposed to 8Gy-12Gy whole body γ-radiation. Radiation dose of 8Gy and above were selected to induce gastro-intestinal syndrome associated mortality (*309*).

2.1.6 Preparation of bone marrow cell suspension:

Exposure to higher doses of ionizing radiation (>2Gy) induces bone marrow aplasia and also contributes to radiation induced mortality at doses >6Gy by increasing susceptibility to infections. The hematopoietic syndrome associated death occurs within 3-5 weeks. At doses >8Gy, IR causes damage to GI system resulting in lower nutrient absorption, diarrhoea and death within 2 weeks. In order to study the effect of NQ on WBI induced GI syndrome in mice exposed to doses >8Gy, bone marrow transplant (BMT) along with NQ administration was employed. For bone marrow isolation, both femur bones of mice were aseptically removed with help of scissor and cell suspensions were prepared by flushing with 1ml cold RPMI-1640 through the marrow of the femur using 1ml syringe. Cells from both femurs of each mouse were centrifuged and suspended in a known volume of medium. The viability of bone marrow cells were estimated by trypan blue dye exclusion method.

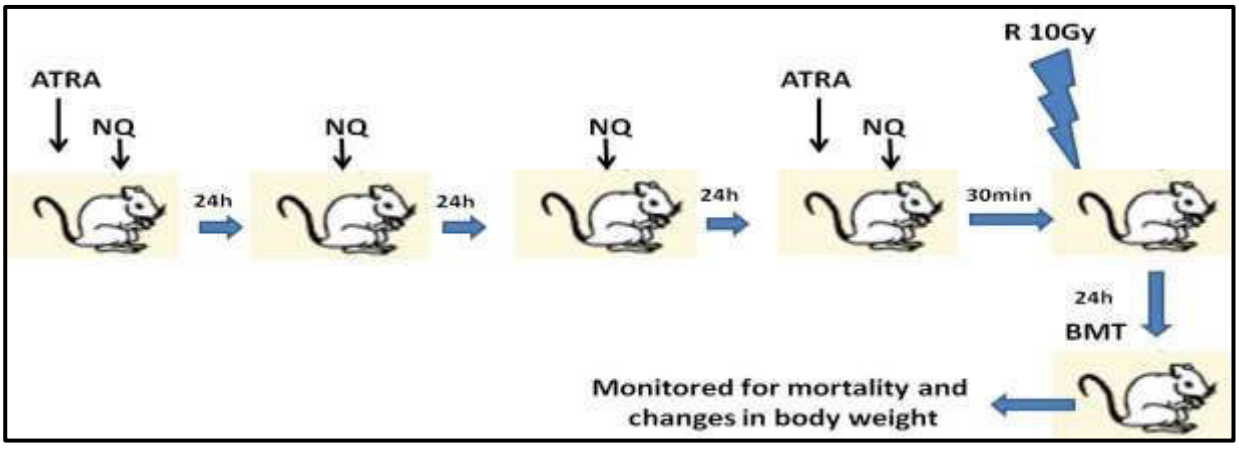
2.1.7 Survival studies:

Mice were administered NQ 2mg/kg body weight (kg bw) intra-peritoneally (i.p.) consecutively for 4 days at an interval of 24h. Whole body irradiation (WBI) dose of 8Gy was given 30min after the last dose of NQ. In survival studies for doses > 8Gy, syndrome specific strategy was employed. Mice were administered with four doses of NQ as mentioned above and were exposed to WBI dose of 9 or 10 or 12Gy. Vehicle treated mice served as

control. Mice were injected 2 million autologous bone marrow cells in the lateral tail vein 24h after WBI. Mice (10 mice per group) were monitored for radiation induced changes in body weight and mortality up to 30 days.

For investigating the therapeutic effect of NQ administration on WBI induced mortality, NQ was administered at different time intervals after radiation exposure. Mice were exposed to WBI 8Gy and NQ (2mg/kg bw) was administered i.p. 1h after radiation treatment. In order to achieve effective therapeutic mitigation of radiation induced mortality, administration of NQ was continued for 5 days in a week at an interval of 24h for 3 weeks. 10 mice per group were taken. Mice were monitored for radiation induced changes in body weight and mortality for 30 days.

In order to study the role of Nrf2 in NQ mediated radioprotection, ATRA, an inhibitor of Nrf2, was administered to mice at a dose of 5mg/kg bw twice a week i.p. Mice were administered with NQ 2mg/kg bw i.p. consecutively for 4 days at an interval of 24h. Mice were given 10Gy WBI 30min after the last dose of NQ followed by BMT 24h after WBI. 10 mice per group were taken. Mice were monitored for radiation induced mortality for 30 days.



Scheme 2.1: Treatment regimen employed to investigate the effect of Nrf2 inhibition of NQ mediated protection against WBI induced mortality. All trans retinoic acid (ATRA) was used to inhibit Nrf2.

2.1.8 Histological studies:

Mice (3 animals per group) were administered NQ (2mg/kg bw consecutively for 4 days with an interval of 24h) or vehicle and were exposed to WBI (8Gy) after 30min. These mice were sacrificed on day 4 after WBI and necropsy was performed to isolate different organs. The intestine was washed in PBS and the jejunum was fixed in 10% neutral buffered formalin prior to paraffin embedding. Fixed tissue was processed by progressive dehydration of tissues in ethanol, clearing in xylene, paraffin vacuum infiltration and embedded in to paraffin blocks at pathology section of Medical Division, BARC. The sections of 5µm thickness were cut using a rotary microtome (AO 820, USA). The sections of tissues were stained in Eosin and Harris haematoxylin and examined using upright trinocular microscope attached to CCD camera.

2.1.9 BrdU staining of jejunum:

Bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU) is an analog of thymidine commonly used for detection of proliferating cells. BrdU is incorporated into the newly synthesized DNA during the S phase of the cell cycle, substituting for thymidine during DNA replication (*185*). Antibodies specific for BrdU are used to detect cells with actively replicating DNA. The mice were given BrdU (200mg/kg bw, 2h before isolation of jejunum) and the paraffin-embedded jejunum sections were used for immunohistochemical analysis using anti-BrdU antibody. The sections were deparaffinized in xylene, dehydrated in 100% ethanol and rehydrated by sequentially immersing the slides through graded ethanol washes (95%, 90%, 80% and 70%) for 3min each at room temperature. DNA was denatured by incubating sections in 2N HCl for 30min at 37°C and neutralized by immersing sections in 0.1M borate buffer twice for 5min. The sections were washed, permeabilized and incubated with FITC- anti-BrdU antibody for 4h at 37°C. Then sections were counterstained with

Hoechst33342. The fluorescence was observed under a Zeiss Axio Observer D1 fluorescence microscope with Axiovision 4.7 software (Go¨ ttingen, Germany).

2.1.10 Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay:

The paraffin-embedded jejunum sections were used to detect nicked DNA in the nucleus of intestinal epithelial cells to determine apoptotic cells. Jejunum sections were stained with a TUNEL apoptosis detection kit (Roche) following the manufacturer's protocol.

2.1.11 Preparation of jejunum cell suspension:

Jejunum is the second part of the small intestine which lies between duodenum and ileum and it is the main absorptive surface of small intestine. Three differentiated cell types (enterocytes, enteroendocrine, and goblet cells) populate the villi in jejunum. Jejunum cell suspension was made following the protocol described by Sato et al (*40*). Isolated jejunum was washed with ice cold 1X PBS, cut open into small pieces followed by gentle squeezing on 100µ cell strainer to prepare a cell suspension. Cell suspension was further incubated for 30min at 4°C in PBS containing 2 mM EDTA. Dissociated cells were passed through cell strainer with a pore size of 70µ. Collected single cell suspension was washed twice with 1X PBS.

2.1.12 Intracellular antibody staining:

For BrdU staining of intestinal epithelial cells, the mice were administered with four doses of NQ (2mg/kg bw) or vehicle consecutively for 4 days at an interval of 24h and were exposed to WBI (8Gy) after 30min. These mice were given BrdU (200mg/kg bw) 2h before sacrificing on day 4 and the jejunum cells were isolated from the respective treatment group. Cells were fixed with 4% paraformaldehyde for 10min at room temperature and excess

paraformaldehyde was removed by washing once with wash buffer (PBS containing 1% bovine serum albumin). DNA was denatured by incubating cells in 2N HCl for 20min at 37°C and neutralized by 0.1M borate buffer for 5min. Cells were washed and permeabilized with PBS containing 0.02% Tween 20 three times for 5min each at room temperature followed by two washes with wash buffer and then incubated with FITC-labeled BrdU mAb for 1h at room temperature. Cells isolated from vehicle treated mice served as control. Cells stained with isotype control were used to analyse changes in protein expression using a flow cytometer (Partec CyFlow) and analysed using Flowjo 7.6.5 software (Treestar Inc).

For investigating the effect of NQ administration on pERK and Nrf2 levels in jejunum cells, mice were administered four doses of NQ (2mg/kg bw) or vehicle consecutively for 4 days at an interval of 24h. Jejunum cells were isolated at the indicated time points and were processed for intracellular antibody staining using PE-labeled pERK and Nrf2 mAb as described above. For investigating the effect of NQ on COX 2 in macrophages, two million RAW 264.7 cells in the presence or absence of NQ were stimulated with LPS (1µg/ml) for 24h at 37°C. Cultured cells were fixed with 4% paraformaldehyde for 10min at room temperature and further processed with intracellular staining with PE conjugated anti-COX 2 mAb as described above.

2.1.13 Surface antibody staining:

Direct immunofluorescence staining technique involves the incubation of live cells with fluorochrome-conjugated antibody to decipher the subpopulation in the given sample on the basis of expression of surface proteins. Jejunum cells were used for direct immunofluorescence staining. Cells were resuspended in 50µl buffer (PBS containing 10% FCS) and incubated on ice for 10min for blocking Fc receptors. The cells were further incubated with 0.2µg of FITC conjugated anti-CD45 antibody in 100µl of blocking buffer for

30min on ice in dark, washed three times, resuspended in 1ml 1X PBS and fixed with 4% paraformaldehyde for 20min on ice. Cells were further washed and stained with hoechst33342. Stained cells were acquired using Partec Cyflow Space flowcytometer and analyzed using FlowJo software (Treestar Inc).

2.1.14 Intestinal stem cell staining:

Multiple elegant lineage studies have identified Lgr5 as a marker of intestinal stem cells (ISC) (*54*). Exposure to IR induces cell death in ISC. Mice (3 animals per group) were administered with four doses of NQ (2mg/kg bw) or vehicle consecutively for 4 days at an interval of 24h and were exposed to WBI (8Gy) 30min after last injection. These mice were sacrificed on day 4 after WBI and the isolated jejunum cells were stained with anti-Lgr5 antibody as described in section 2.1.13 and then stained with Hoechst 33342. Single viable epithelial cells were gated by forward scatter, side scatter and pulse width parameter. Lgr5+ cells were gated with reference to isotype control. Percent cell death and S+G2+M cells were measured using a flow cytometer (Partec CyFlow) and analysed using FlowJo software.

2.1.15 Monitoring gut bacterial translocation:

The intestinal epithelium provides an effective barrier against the translocation of bacteria, bacterial products and antigens from the lumen. Enteric bacteria have been implicated in radiation induced intestinal injury. Infection with an enteric pathogen exacerbates radiation-induced injury and leads to systemic infection (*310*). To evaluate the effect of NQ on WBI induced bacterial translocation from intestine, mice were administered with 4 consecutive doses of NQ (2mg/kg bw) i.p. at an interval of 24h and were given WBI 10Gy 30min after the last dose. BMT was done with two million bone marrow cells in lateral tail vein of the mice 24h after WBI. Mice were sacrificed on day 4, liver, spleen and jejunum were removed from each mouse under sterile conditions (*311*). Each tissue was then weighed and

homogenized in 2ml of sterile PBS. Two serial 10-fold dilutions were made for each sample, and 0.1-ml aliquots of diluted sample were plated onto Luria agar plates. Following incubation at 37°C for 24h, bacterial colonies were counted.

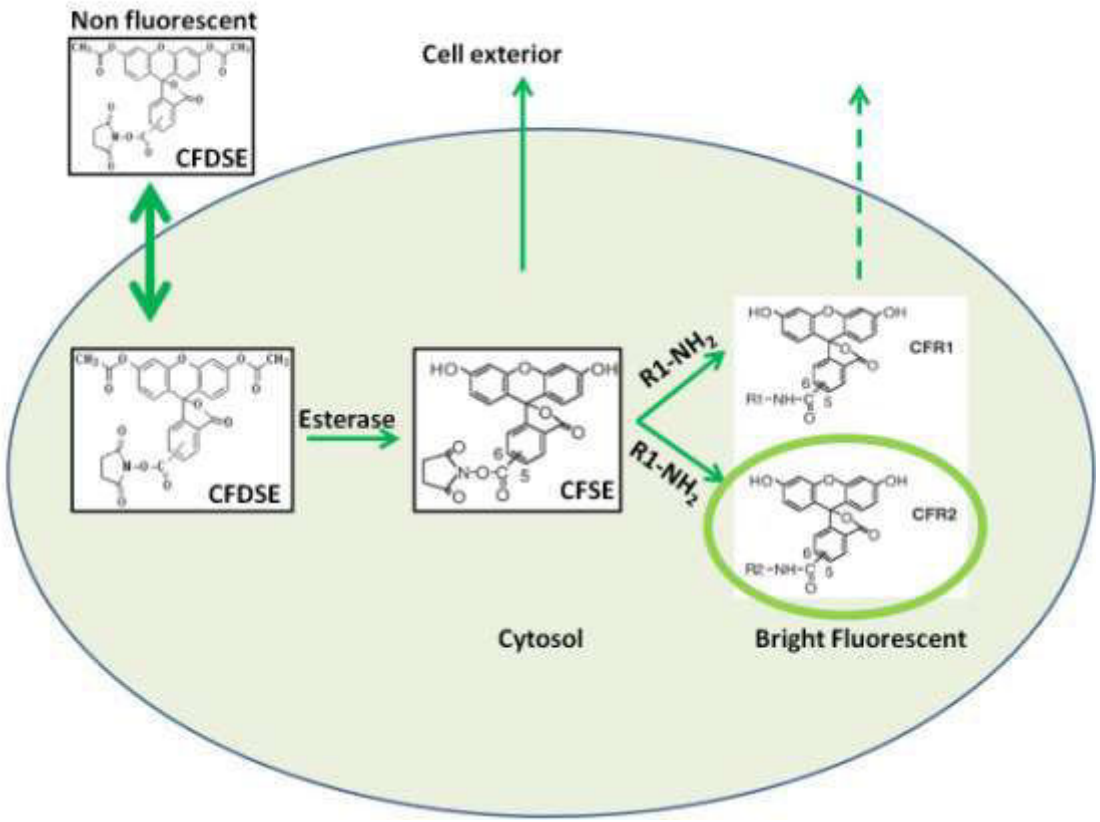
2.1.16 Splenic lymphocyte preparation:

Spleen is an organised secondary lymphoid organ which is a major source of lymphocytes and other accessory immune cells in mice. Splenic lymphocytes from disease free young mice are naïve and a very few lymphocytes show activated or memory phenotype. Spleen was aseptically removed from the mice and placed in sterile petri dish containing RPMI 1640 medium. Single cell suspension was prepared by gently squeezing the organs on a sterile wire mesh (100μ) placed in the petri dish. Cell suspension was carefully transferred to 15ml sterile tubes and centrifuged at 3000 rpm for 5min. Red blood cells (RBC) were lysed by brief hypotonic shock using ice cold water (5ml, 10sec) followed by ice cold 2X PBS (5 ml). The RBC membranes were allowed to pellet by centrifuging the tubes at 1000 rpm for 30sec and lymphocytes were carefully decanted into a fresh tube. Lymphocytes were further centrifuged at 3000 rpm for 5min and the cell pellet was resuspended in complete medium (RPMI 1640 medium containing 10 % heat inactivated fetal bovine serum). Trypan blue staining was done to enumerate the viable cells in haemocytometer using a microscope.

2.1.17 CFSE staining:

Proliferation of lymphocytes in response to stimulation by infections or tumors is one of the prime events of immune system response required to eradicate the stimuli. Cell proliferation can be measured by techniques which quantifies rate of DNA replication like ^{3}H-thymidine or BrdU-based assays or by metabolic activity-based assays include MTT, XTT, WST and ATP measurements. Multiple markers serving as cell proliferation antigen like Ki-67, topoisomerase IIB, phosphohistone H3 and PCNA are utilised for measuring the proliferation

(*312*). Although the above mentioned techniques provide general insight about lymphocyte proliferation, fluorescence dye based methods have been developed to monitor the proliferation of the labelled lymphocytes via progressive two-fold dilution of the dye in dividing cells (*313, 314*).



Scheme 2.2 Schematic representation of the mechanism of fluorescently labelling cells with carboxyfluorescein diacetate succinimidyl ester. Carboxyfluorescein (CF) becomes coupled to long-lived intracellular molecules (R2-NH2) to form conjugates (CFR2) that cannot escape from the cell and thus, stable fluorescent labelling of cells is achieved. Figure adapted with modifications from (*312*).

We have employed carboxyfluorescein diacetate succinimidyl ester (CFSE) labelling method to monitor the proliferation of lymphocytes. CFSE is a fluorescent dye with two acetate moieties and one succinimidyl ester functional group. The two acetate side chains

make the molecule highly permeable across the membrane. However, the acetate groups are removed by intracellular esterases and the resultant carboxyfluorescein exits from cells at a much slower rate due to reduced lipophilicity. The slow exit rate also provides ample time for the succinimidyl moiety in CFSE to covalently couple the amine groups of intracellular molecules to form highly stable amide bond. These long-lived conjugates provide stable labeling of cells with CFSE to allow monitoring of lymphocytes over a period of time. Cell division results in sequential halving of fluorescence in daughter cells, and up to 8 divisions can be monitored before the fluorescence is decreased to the background fluorescence of unstained cells. Therefore the ratio of the proportion of cells in each division peak to the expected progeny provides the number of cells that have entered division. This gives a precursor frequency estimate of responding cells in the cultures. CFSE staining was used for assessment of lymphocyte proliferation in vitro. Lymphocytes (100 X 10^6) were suspended in RPMI 1640 (500µl) were mixed with equal volume of 20µM CFSE. The density of cells was kept very high to buffer any toxic effects of CFSE. Cells were incubated at 37°C for 5min. Cells were washed three times with ice cold RPMI 1640 medium supplemented with 10 % FCS to remove excess dye.

2.1.18 Estimation of T cell proliferation:

Measurement of T cell proliferation is commonly used to evaluate the overall immuno-competence of an animal. Plethora of agents can act as mitogen to induce T cell activation, resulting in cytokine production, receptor expression and proliferation of the activated T cells. Two million lymphocytes were treated with NQ (0.5µM to 5µM, 2h) or H_2O_2 (50µM, 10min) or t-BHQ (20µM, 2h) and were stimulated with concanavalin A (Con A, 5µg/ml) or plate coated anti-CD3 and soluble anti-CD28 antibody (1µg/ml) for 72h at 37°C in 2ml RPMI with 10% FCS 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.

2.1.19 Measurement of cytokine secretion:

Cytokine production by stimulated lymphocytes was measured by sandwich ELISA technique. In this method, the cytokine serves as an antigen which is captured between two antibodies specific for two different epitopes on the same cytokine. Sandwich ELISA is 2 to 5 times more sensitive than solid phase antigen detection method due to involvement of two antibodies. The concentration of IL-2, IL-4, IL- 6 and IFN-γ in the supernatant of control unstimulated lymphocytes and lymphocytes stimulated with Con A or anti-CD3/CD28 mAb in presence or absence of NQ for 24h or concentration of IL1β, TNF-α and IL-6 in RAW cells stimulated with LPS in presence or absence of NQ was estimated using cytokine ELISA sets (BD Pharmingen, USA).

Capture antibody for each cytokine was dissolved in bicarbonate buffer and used for coating the wells in a 96 well microtitre plate (1:250 dilution, 100µl/well). The plates were sealed with parafilm to avoid evaporation and incubated at 4°C overnight. Unbound antibody was aspirated and wells were washed three times with wash buffer (PBS with 0.05% Tween 20). Wells were blocked with blocking buffer (PBS with 10% FCS) for 1h at room temperature and washed three times with wash buffer (PBS with 0.05% Tween 20). Test solutions containing antigen (100µl) was added to three wells in each group and incubated at room temperature for 2h. To another set of wells, cytokine standards IL-2 or IL-4 or IL- 6 or IL1β or TNF-α or IFN-γ were prepared (as per manufacturer's protocol) and added in triplicates. The wells were aspirated and washed four times to remove any unbound cytokines. Streptavidin conjugated detection antibody and biotin conjugated Horse Radish Peroxidase (HRP) enzyme were diluted in blocking buffer and 100µl of freshly prepared

mixture was added to each well. Plates were incubated at room temperature for 1h. The wells were aspirated and washed five times to remove any unbound antibody. 100µl tetramethyl benzidine (TMB) substrate was added to each well and incubated at room temperature for 15min in dark. Stop solution (1N HCl, 50µl) was added to inhibit further reaction and absorbance was taken at 450nm using an ELISA plate reader. Standard curves were generated from mean±S.E.M. of OD readings of known cytokine standards. The amount of cytokine in each well was estimated from the standard curve of respective cytokines.

2.1.20 Measurement of nitric oxide in culture supernatants using Griess reagent:

The concentration of nitric oxide (nitrite concentration) in the supernatant of RAW 264.7 cells pre-treated with NQ and cultured for 24h at 37°C in the presence of LPS was measured by using Griess reagent as described earlier (*315, 316*). Culture supernatant (100µl) was incubated with 100µl of Griess reagent (1% sulfanilamide, 0.1% NEDD, 2.5% phosphoric acid in distilled water). The absorbance was measured at 550nm using an ELISA plate reader (Bio-Tek Instruments). Amount of NO in each sample was calculated from a standard curve generated with known dilutions of sodium nitrite.

2.1.21 Estimation of nuclear Nrf2 levels by confocal microscopy:

Two million lymphocytes were treated with NQ (5µM) for different time intervals. Plate was centrifuged to pellet down cells onto coverslip. The cells on the coverslip were fixed in 4% paraformaldehyde (500µl) for 10min at 4°C. Excess paraformaldehyde was aspirated and the cells were permeabilized with 1X PBS containing 0.02% Tween-20 twice and then blocked with 5% BSA at 37°C for 1h. Anti-Nrf2 antibody was added at a dilution of 1:100 in 1% BSA and incubated overnight at 4°C. Cells were then washed with 1X PBS four times before incubating in the dark with FITC labeled secondary antibody at dilution of 1:200 in 1% BSA for 2h. Cells were then washed and incubated in the dark with Hoechst33342 solution for

10min at room temperature for nuclear staining. Excess Hoechst stain was removed by washing twice with PBS and coverslips were mounted on glass slides with an antifade solution DABCO. Slides were examined using a LSM510 scanning module (Carl Zeiss Microscopy, Jena GmbH, Germany) with a Krypton–Argon and Helium-Neon laser, coupled to an Orthoplan Zeiss photomicroscope. Overlay images were recorded by superimposing simultaneous images from each channel.

2.1.22 Estimation of mRNA expression by quantitative Real Time PCR:

Real time PCR is combined with reverse transcription to quantify messenger RNA in cells. Real time PCR enables both detection and quantification of one or more specific sequences in a DNA sample. The quantity can be either an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing genes. Its key feature is that the amplified DNA is detected as the reaction progresses in real time. For detection of products fluorescent dyes like SYBR Green I are used which intercalate with double-stranded DNA. An increase in concentration of DNA product during PCR therefore leads to an increase in fluorescence intensity and is measured at each cycle, thus allowing DNA concentrations to be quantified.

Jejunum cells were isolated from NQ (2mg/kg bw, four doses) administered mice at indicated time intervals and were processed for RNA isolation. For in vitro studies, lymphocytes (Twenty million) were treated with or without NQ (5µM) for indicated time at 37°C. Vehicle treated cells served as control. Total RNA was isolated from the samples using Trizol reagent (Sigma) following the manufacturer's instructions and was dissolved in 30µl deionised DEPC treated water. RNA was quantified and 1µg was converted to cDNA by reverse transcription (cDNA synthesis kit, Roche) following the manufacturer‘s instruction. qPCR was carried out using the Rotor Gene 3000 (Corbett Research) machine. The PCR was

setup by mixing 10x SYBR green PCR mix (Roche) with 5µl cDNA, 10 picomoles each of forward and reverse gene specific primers and PCR-grade water in 20µl reaction system. The gene specific primers were designed using IDT SciTools and sequences are listed below (Table 2.1).

Gene	Sequence
HO-1	Forward: AGGTACACATCCAAGCCGAGA Reverse: CCATCACCAGCTTAAAGCCTT
GCLC	Forward: CTACCACGCAGTCAAGGACC Reverse: CCTCCATTCAGTAACAACTGGAC
Nrf2	Forward: CTTTAGTCAGCGACAGAAGGAC Reverse: AGGCATCTTGTTTGGGAATGTG
MnSOD	Forward: CAGACCTGCCTTACGACTATGG Reverse: CTCGGTGGCGTTGAGATTGTT
Catalase	Forward: AGCGACCAGATGAAGCAGTG Reverse: AGGACATCAGGTCTCTGCGA
β-actin	Forward: GCGGGAAATCGTGCGTGACATT Reverse: GATGGAGTTGAAGGTAGTTTCGTG

Table 2.1 Gene specific primer sequences.

The above reaction mixtures were amplified in the following steps: step 1- denaturation at 95°C for 5 minute; step 2-denaturation at 95°C for 15 seconds ; step 3- annealing at 56°C for 15 seconds; step 4-extension at 72°C for 20 seconds ; step 5- melting curve analysis. Steps from 2 to 4 were repeated for 40 cycles. The specificity of respective amplicons was confirmed from the melting curve analysis. The amplification of each gene was carried out in triplicates for each treatment group. The threshold cycle (the cycle at which the amplification

enters into exponential phase) values (Ct value) obtained from above runs were used for calculating the expression levels of genes by REST-384 version 2 software129. The expression of genes were normalized against that of a housekeeping gene, β-actin, and plotted as relative change in the expression with respect to control.

2.1.23 Absorption spectroscopy:

Absorbance spectra of NQ with or without NAC/GSH were determined using spectrophotometer. NQ (1mM) was mixed with 1mM of NAC or 1mM of GSH in a total volume of 1ml and incubated at 37°C for 1h. Absorption spectra of the samples were recorded.

2.1.24 Electrophoretic mobility shift assay:

For in vitro studies, lymphocytes were treated with NQ (5µM, 2h) and were stimulated with Con A (5 µg/ml) for 3h at 37°C or treated with 5µM NQ for various time intervals. For ex vivo studies, lymphocytes isolated from NQ or vehicle administered mice were used for performing EMSA. Nuclear extracts were prepared from each treatment group using extraction buffer (20mM HEPES, pH 7.9, 0.4 M NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT, 1mM PMSF, 2.0 pg/ml leupeptin, 2.0 µg/ ml aprotinin, and 0.5 mg/ml benzamidine). EMSA for NF-κB (Con A stimulated samples in presence or absence of NQ) or Nrf2 (NQ treated samples) was performed by incubating 8µg of nuclear proteins with 16 fmol of ^{32}P-end-labeled, in the presence of 0.5 µg of poly dI-dC (2′- deoxyinosinic-2′-deoxycytidylic acid) in binding buffer (25mM HEPES, pH 7.9, 0.5mM EDTA, 0.5mM DTT,1% Nonidet P 40, 5% glycerol, and 50mM NaCl), or 45-mer double-stranded NF-κB oligonucleotides from the human immunodeficiency virus long terminal repeat (5'–TTGTTACAA*GGGACTTT*CCGCTG*GGGACTTT*C-CAGGGAGGCGTGG-3'; italic indicates NF-κB binding sites) or the antioxidant response element (ARE; 5'–

CGGTCACCGTTACT-CAGCACTTTG-3'), respectively for 30min at 37^0C. The DNA–protein complex was separated from free oligonucleotide on 7.6% native polyacrylamide gel and the dried gel was exposed on Molecular Dynamics phosphorImager Screen. Radioactive bands were visualized using phosphorImage scanner (Amersham Biosciences, USA).

2.1.25 Western Blotting:

For in vivo studies, mice were administered with four consecutive doses of NQ (2mg/kg bw i.p.) at an interval of 24h. Mice were sacrificed and jejunum cells were isolated at indicated time points after the last dose. To determine the levels of pERK and ERK, cytoplasmic extracts were prepared. For in vitro studies in lymphocytes, cytoplasmic extracts were prepared from NQ (5μM) treated splenic lymphocytes to determine the levels of IκBα, IKKβ and KEAP-1 as described previously (*317*). Cells were washed with ice-cold PBS and suspended in 90μl lysis buffer (10mM HEPES, pH 7.9, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM dithiothreitol, 0.5mM PMSF, 2mg/ml leupeptin, 2mg/ml aprotinin, and 0.5mg/ml benzamidine). These cells were allowed to swell in ice for 45min after which 10μl of 10% NP40 was added and tubes were vortexed thrice for 60 seconds with intermittent incubation on ice for 5min each. The supernatants were collected by centrifuging the cells at 13,000 rpm for 5min at 4°C and used as cytosolic fraction. The protein content was determined using Bradford reagent (BioRad Protein assay kit). Equal amount of protein (50μg) from each sample was resolved on 10% SDS PAGE at 80Volts. The proteins were electro-transferred onto nitrocellulose membranes. Membrane was blocked with 5% fat free milk in PBST for 30min. Further, membranes were probed with primary antibodies for pERK or ERK or IκBα or IKKβ or KEAP-1 overnight at 4°C in 2.5% fat free milk. Membranes were washed three times with PBST to remove non-specific binding with PBST buffer. HRP conjugated Immunoglobulin secondary antibody was freshly prepared in 1% fat free milk and

incubated for 2h. Specific bands were visualized on X-ray films using Enhanced Chemiluminiscence Kit (Roche, Germany). β-actin was used as a loading control.

2.1.26 Immunoprecipitation:

Lymphocytes were treated with NQ for 6h and whole cell lysates were prepared as described earlier (*317*). Immunoprecipitation was performed following manufacturer's protocol (Protein G Immunoprecipitation Kit, Sigma, USA). Briefly, volume of cell lysate was made upto 600µl 1X IP buffer provided in the kit and was pre-incubated with anti-GSH antibody at 4℃ overnight. Protein G-agarose beads were washed thrice and resuspended in 50µl volume in 1X IP buffer provided in the kit. Washed beads were transferred to the cell lysate in the spin column and incubated overnight at 4°C under constant agitation. The beads were pelleted by centrifugation at 12000 rpm for 30sec at 4°C. The supernatant was removed and the beads were resuspended in 1ml of 1X IP buffer. Beads were pelleted and washed sequentially with 1X IP buffer with 0.5 M NaCl, 1X IP buffer alone and PBS. After a final centrifugation step, beads were resuspended in 30 µl non reducing Laemelli buffer and heated to 90°C for 5min. The supernatant was separated from the beads by centrifugation in spin columns provided in kit and subjected to non reducing gel electrophoresis.

2.1.27 Statistical analysis:

The statistical analysis was done using analysis of variance with Microcal OriginPro 8.0 software followed by post hoc analysis using Schiffe's test. Statistical significance of survival was assessed using log–rank test. In radioprotection studies of NQ *$p<0.01$, as compared to vehicle treated group, #$p<0.01$, as compared to irradiated group. In immunomodulation studies of NQ **$p<0.01$, as compared to vehicle treated group, #$p<0.01$, as compared to stimulated group and @$p<0.05$ as compared to Con A stimulated cells. Data points represent mean±SEM from three replicates and two independent experiments were performed

CHAPTER 3

PROTECTION AGAINST IONIZING RADIATION INDUCED GASTRO-INTESTINAL SYNDROME

This chapter describes the prophylactic as well as therapeutic radioprotective effects of 1,4-naphthoquinone on the epithelial cells and stem cells present in the gastro-intestinal system.

3.1 RESULTS:

3.1.1 NQ administration protected mice against WBI induced GI syndrome associated mortality:

Radiation doses of 8Gy and above are known to induce GI syndrome associated mortality. To evaluate the effect of NQ on WBI induced intestinal damage, mice were administered four consecutive doses of NQ (2mg/kg bw) i.p. at an interval of 24h and were given WBI (8Gy) after 30min of the last dose. Vehicle administered mice served as control. Mice were monitored for changes in body weight and mortality for 30 days. Exposure to WBI induced 100% mortality and loss in body weight in vehicle treated mice. However, NQ administration significantly ($p<0.001$) protected mice against WBI induced mortality and weight loss (Fig: 3.1A and B).

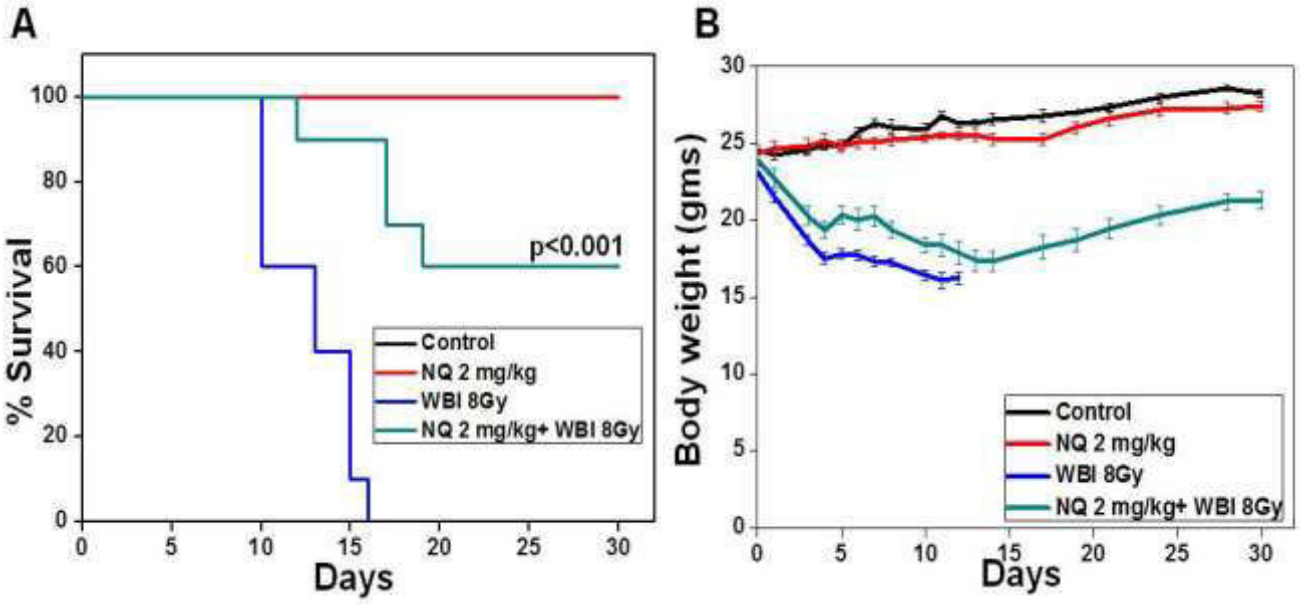


Fig. 3.1 NQ administration protected mice against WBI induced GI syndrome associated mortality: Mice were administered with NQ (2mg/kg bw) i.p. and were exposed to 8Gy WBI. The survival and body weight of mice were monitored for 30 days. Ten mice per group were taken (A) Line graph represents the percent survival. (B) Changes in the bodyweight of the mice after WBI of respective treatment groups. Data points represent mean±SEM from 10 mice. Two such independent experiments were carried out. Log rank test was used to compare mortality of WBI exposed mice administered with or without NQ.

3.1.2 NQ administration protected mice against WBI induced loss of intestinal integrity and apoptosis in intestinal crypts:

Radiation induced GI syndrome is characterized by loss in the absorptive surface in the jejunum part of small intestine due to denudation of villi and apoptosis in submucosal invaginations called crypts of Lieberkuhn resulting in vomiting, diarrhoea, anorexia, dehydration, systemic infection resulting in septic shock induced death (*318*). Experiments were carried out to investigate the potential of NQ to protect mice against WBI induced loss in integrity of intestinal lining. Mice were administered with four consecutive doses of NQ (2mg/kg bw) i.p. at an interval of 24h and were given WBI (8Gy) 30min after last dose. Vehicle administered mice served as control. Jejunum from respective groups was isolated on day 4 to evaluate the radiation induced histopathological changes and apoptosis in intestine. WBI treated group showed denudated, shortened and oedematous villi with epithelial irregularities. NQ administration prior to WBI protected mice against radiation induced intestinal damage evident from normal intact villi architecture and epithelial alignment (Fig. 3.2A). Further, paraffin embedded sections derived from isolated jejunum tissue from respective treatment group was subjected to TUNEL assay. Exposure to WBI 8Gy induced apoptosis in intestinal crypts as evident with increased FITC labelled TUNEL positive cells. Interestingly, NQ administration protected against WBI induced apoptosis in crypts further corroborating its protective role (Fig. 3.2B).

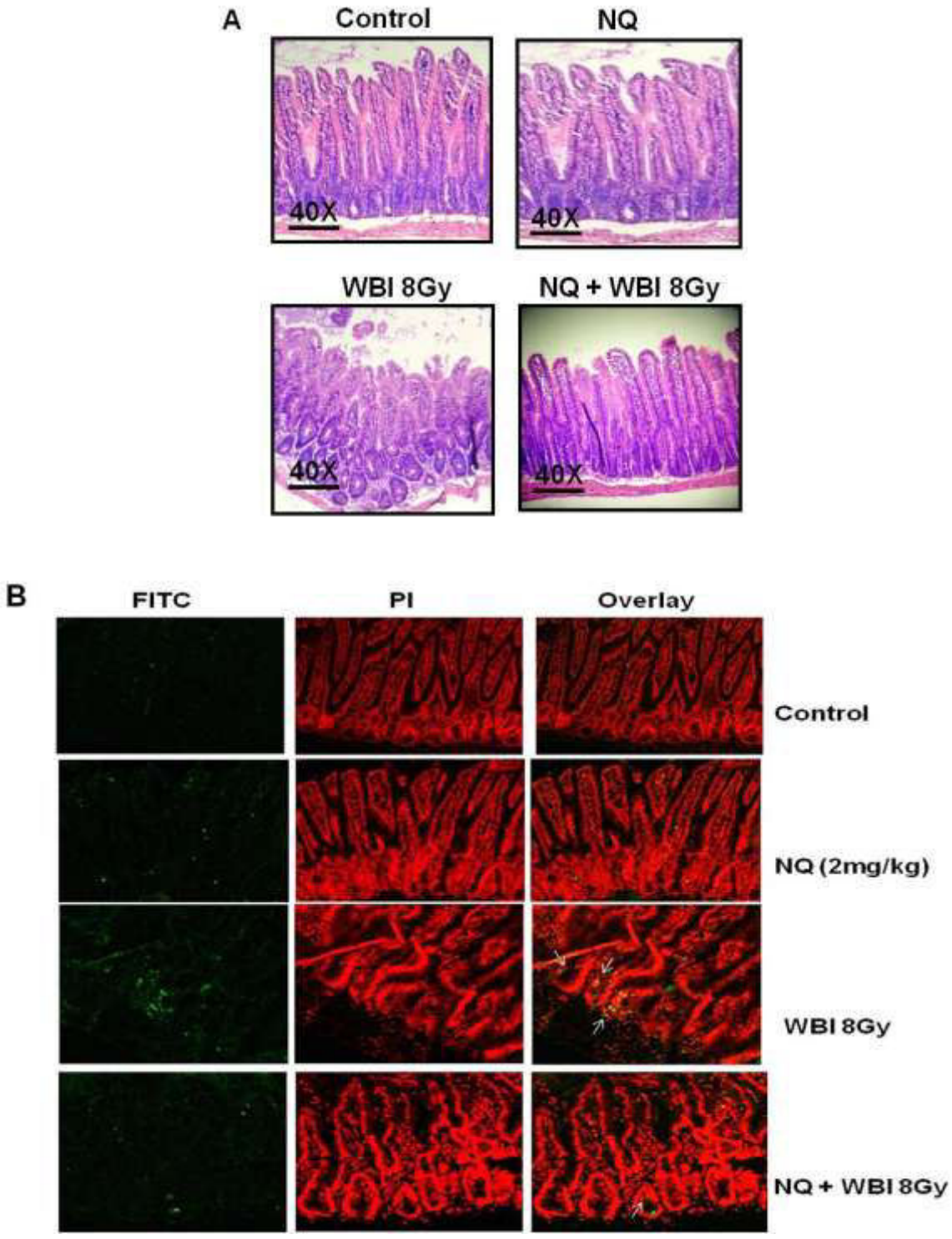
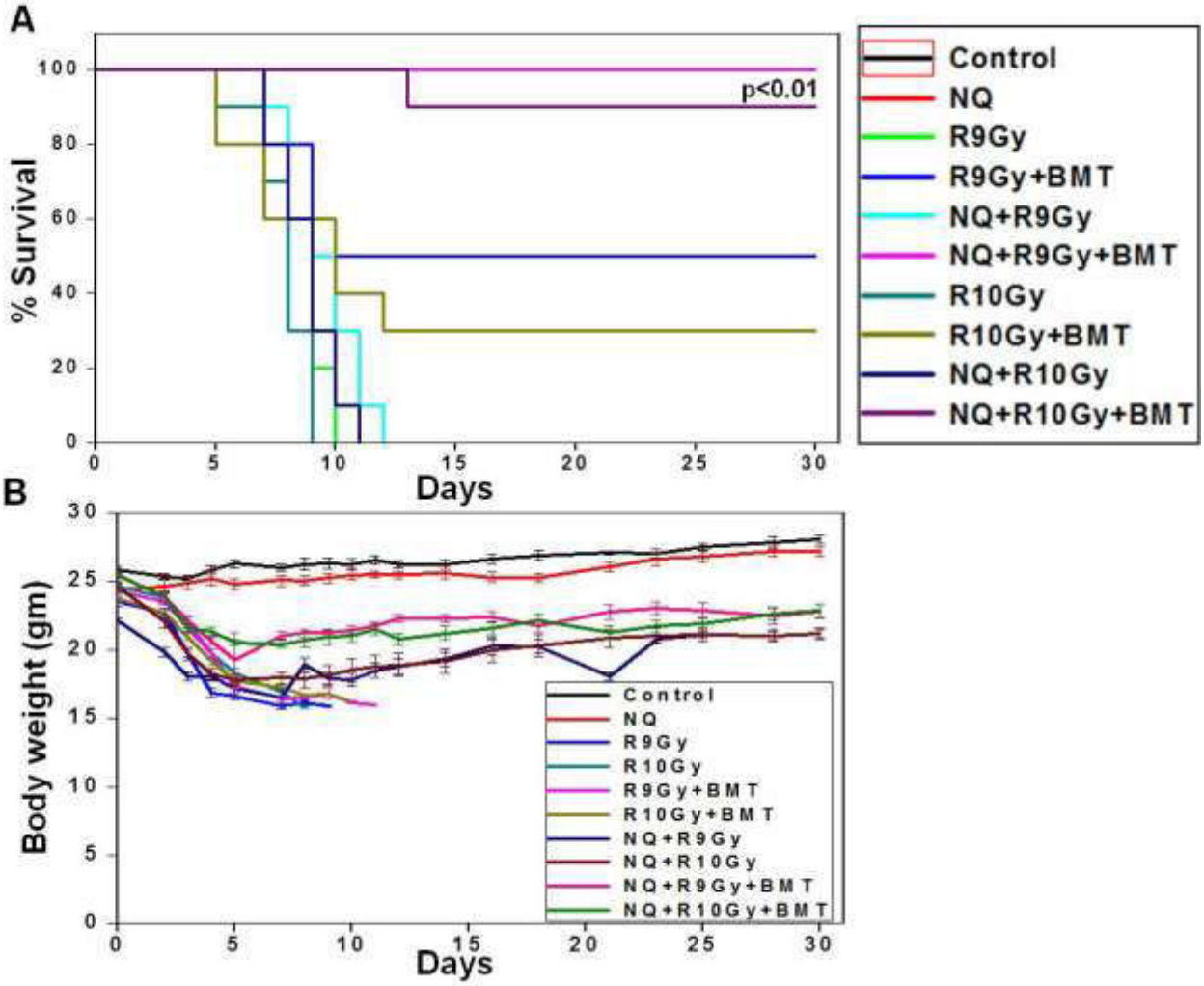


Fig. 3.2 NQ administration protected mice against WBI induced loss of intestinal integrity and apoptosis in intestinal crypts: Mice were administered with four doses of NQ (2mg/kg bw) i.p. 24 hourly and were exposed to 8Gy WBI after 30min of last dose of NQ administration. Histological changes and apoptosis in crypts were monitored on day 4. Three mice were taken in each group. (A) Sections were stained with hematoxylin and eosin. NQ treatment protected against WBI induced intestinal damage. (B) Paraffin embedded sections

were processed for TUNEL assay (FITC label) and nuclear staining using PI. Increase in FITC signals represents the TUNEL positive cells as analysed by confocal microscopy.

3.1.3 Administration of NQ ameliorated high dose WBI induced mortality in combination with BMT:

At doses >4Gy, WBI induces bone marrow aplasia and leukopenia which contributes to morbidity and mortality by increasing susceptibility to infection. Potential of NQ to protect against WBI (>8Gy) induced damage to GI tract was assessed using specific model of GI syndrome wherein autologous BMT was carried out to compensate for WBI induced bone marrow aplasia (*319*). Mice were administered with four consecutive doses of NQ (2mg/kg bw) i.p. at an interval of 24h and were given WBI (9, 10 and 12Gy) 30min after last dose of NQ. Two million bone marrow cells were injected in the lateral tail vein 24h after WBI. Vehicle administered mice reconstituted with autologous bone marrow served as control. Mice were monitored for change in body weight and mortality for 30 days. NQ administration alone was not able to exhibit protection against mortality at these doses of WBI (9, 10 and 12Gy). However, in combination with BMT, it offered complete protection against WBI 9Gy and significant protection against WBI 10Gy (50%, $p<0.01$) induced mortality and loss in body weight (Fig. 3.3 A and B). Further, at WBI 12Gy, BMT alone did not offer protection against radiation induced mortality in mice. However, NQ along with BMT offered significant protection 12Gy (40%, $p<0.05$) against WBI induced mortality and weight loss (Fig. 3.3 C and D).


A
% Survival
Days
p<0.01
Control
NQ
R9Gy
R9Gy+BMT
NQ+R9Gy
NQ+R9Gy+BMT
R10Gy
R10Gy+BMT
NQ+R10Gy
NQ+R10Gy+BMT
B
Body weight (gm)
Days
Control
NQ
R9Gy
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NQ+R9Gy+BMT
NQ+R10Gy+BMT

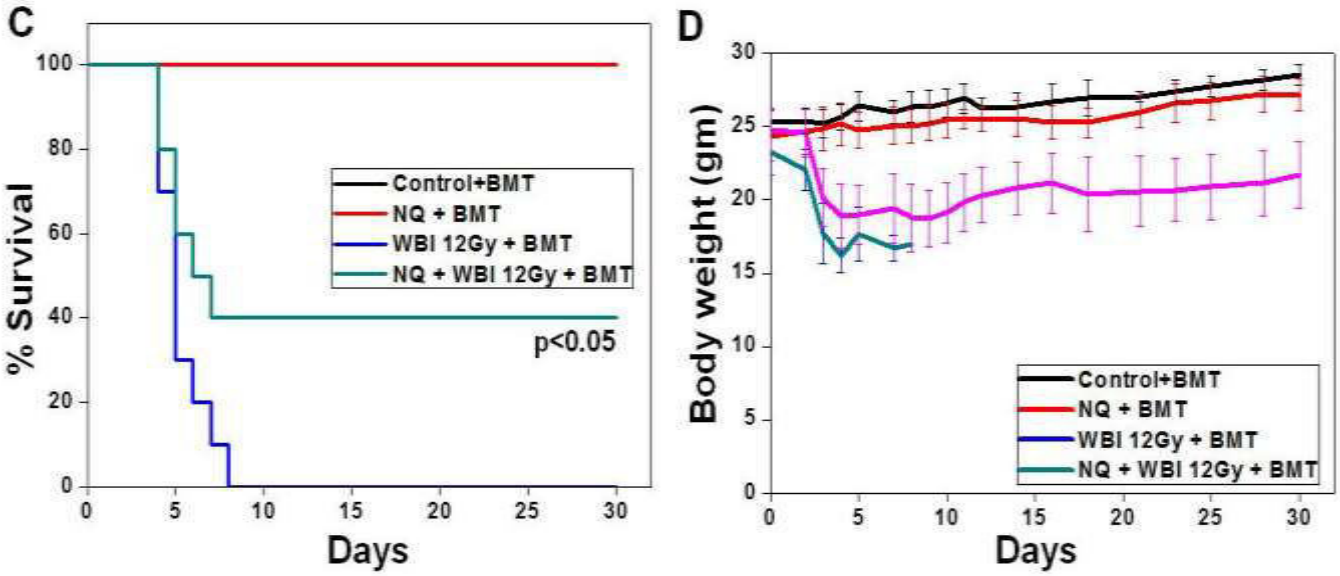

C
% Survival
Days
Control+BMT
NQ + BMT
WBI 12Gy + BMT
NQ + WBI 12Gy + BMT
p<0.05
D
Body weight (gm)
Days
Control+BMT
NQ + BMT
WBI 12Gy + BMT
NQ + WBI 12Gy + BMT

Fig. 3.3 NQ ameliorated high dose WBI induced mortality in combination with BMT: Mice were administered four consecutive doses of NQ (2mg/kg bw) i.p. and were exposed to 9-12Gy WBI 30min after last injection. Two million bone marrow cells were administered intravenously. Survival and body weight of mice were monitored for 30 days. Ten mice per group were taken (A) Graphed the percent survival values at WBI 9 and 10Gy. NQ treatment along with bone marrow transplant (BMT) significantly improved 30 day survival of mice. (B) Graph represents the body weight of the mice after exposure to 9 and 10Gy dose of WBI in different treatment groups. NQ administration protected mice against radiation induced loss in body weight. (C and D) Graph represents the percent survival and body weight of mice exposed to 12Gy dose of WBI. Data points represent mean±SEM from 10 mice. Two such independent experiments were carried out. Log rank test was used to compare mortality of WBI exposed mice administered with or without NQ.

3.1.4 NQ administration protected mice against WBI induced loss of viability and proliferation potential of crypts and villi in jejunum:

Exposure to ionizing radiation induces loss in proliferating potential of intestinal cells. To investigate the potential of NQ in protecting mice against WBI induced loss of viability in villi and crypts present in the small intestine, mice were administered 4 doses of NQ (2 mg/kg bw, i.p.) with 24h interval and were exposed to 10Gy dose of WBI 30min after last injection. BMT was done in all indicated groups 24h after WBI exposure. Mice were pulsed with BrdU (200mg/kg bw) 2h prior to sacrifice on day 4 after WBI treatment for isolation of jejunum. Paraffin embedded sectioning were subjected to immunofluorescent staining with anti-BrdU antibody. Hoechst33342 was used for staining the nucleus. NQ + WBI group showed significantly higher number of BrdU+ cells when compared with WBI treated group indicating the potential of NQ to protect against WBI induced intestinal damage. Also, Normal villous architecture, epithelial alignment and crypts were observed in NQ administered group when compared to shortened villi with epithelial irregularities in radiation treated group (Fig. 3.4A).

In another experiment, jejunum was isolated from mice given above treatment regimen and single cell suspension of jejunum cells was prepared as mentioned in section

(2.1.11). The cells were stained with anti-BrdU antibody followed by FITC-anti-mouse IgG and Hoechst staining. Then cells were acquired on a flow cytometer to enumerate the frequency of BrdU positive cells. Exposure to WBI 8Gy induced significant reduction in BrdU+ cells. However, cells isolated from NQ+WBI group showed significantly higher number of BrdU+ cells, indicating the potential of NQ to protect the proliferating ability of intestinal cells (Fig. 3.4 B and C).

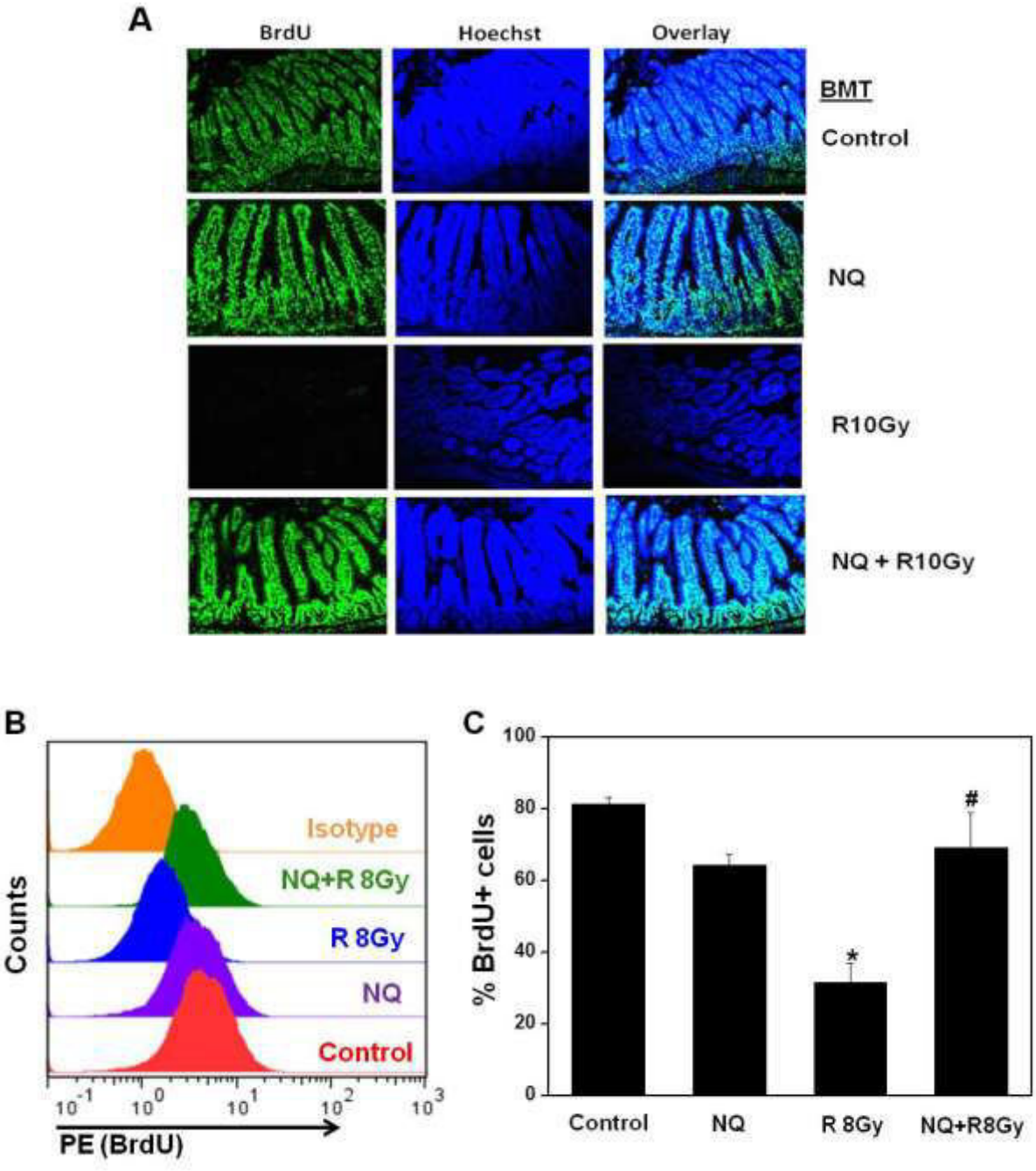


Fig.3.4 NQ administration protected mice against WBI induced loss of viability and proliferation potential of crypts and villi in jejunum: Mice were administered with four consecutive doses of NQ (2mg/kg bw) i.p. at an interval of 24h and were given WBI (8Gy) after 30min of the last dose. Vehicle administered mice served as control. Mice were pulsed with BrdU 2h prior to isolation of jejunum from respective groups on day 4. (A) Paraffin embedded sections from the respective treatment groups were stained with FITC-conjugated anti-BrdU antibody. Hoechst33342 was used as nuclear stain. Stained sections were analysed using confocal microscope. (B) Single cell suspension of jejunum cells was intracellulary stained with anti-BrdU antibody. Thirty thousand cells in each group were acquired on a flow cytometer and overlaid histograms are shown. (C) Graph represents mean±S.E.M from three replicates in each treatment group. Two such independent experiments were carried out. *$p<0.01$, as compared to vehicle treated group, #$p<0.01$, as compared to irradiated group.

3.1.5 NQ significantly protected against bacterial translocation induced due to GI damage after WBI:

WBI induced GI syndrome is characterized by denudation of villi in the jejunum of GI tract. Denudation of villi causes translocation of luminal bacteria to different organs inducing systemic inflammation. Mice were administered with four consecutive doses of NQ (2mg/kg bw) i.p. at an interval of 24h and were given WBI 10Gy 30min after last dose. BMT was performed using two million bone marrow cells in all treatment groups 24h after WBI. Mice were sacrificed on day 4, homogenates of liver, spleen and jejunum were prepared and plated on growth medium. Exposure to WBI reduced the bacterial load in jejunum and increase bacterial load in spleen and liver indicating the WBI induced loss of intestinal integrity and induced bacterial translocation. Interestingly, a significant increase in bacterial colonies obtained from jejunum of NQ + WBI treated group compared WBI group was observed (Fig. 3.5A). Also, NQ pre-treatment significantly protected against WBI induced bacterial translocation in spleen and liver (Fig. 3.5B).

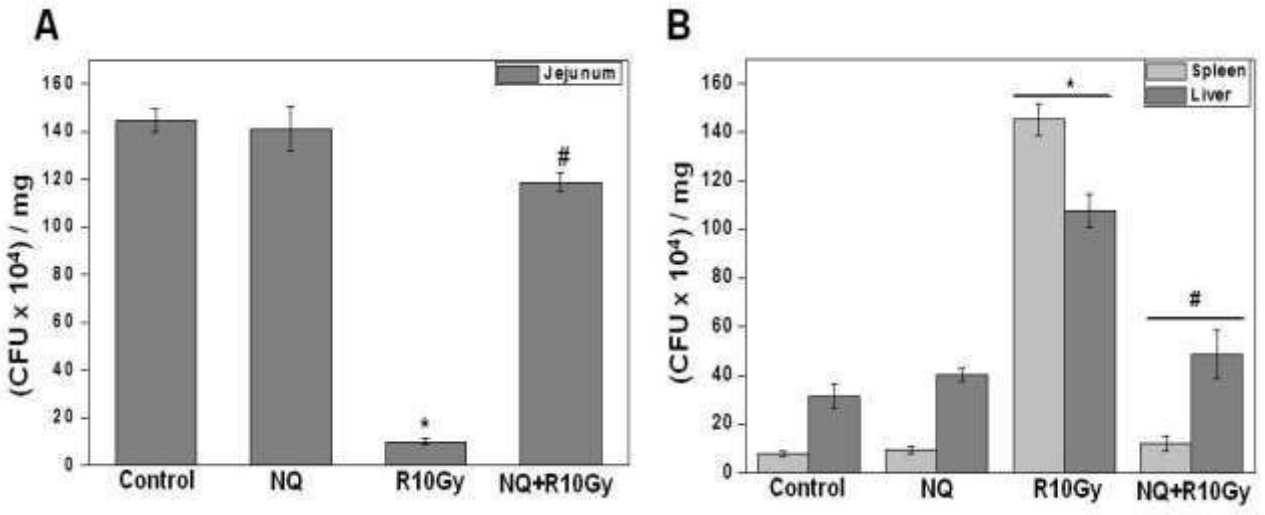


Fig. 3.5 NQ significantly protected against bacterial translocation induced due to GI damage after WBI: Jejunum, spleen and liver were isolated from the respective treatment groups on day 4 after WBI 10Gy. Homogenates of each were made using 1X PBS under sterile conditions, serially diluted and plated on Luria agar growth medium to evaluate the bacterial load. Colony forming units were enumerated after 24h of incubation at 37°C. Three animals were kept in each group. (A) Graph represents mean±S.E.M from three replicates in each treatment group in jejunum homogenates. (B) Graph represents mean±S.E.M from three replicates in each treatment group in spleen and liver homogenates. Two such independent experiments were carried out. *$p<0.01$, as compared to vehicle treated group, #$p<0.01$, as compared to irradiated group.

3.1.6 Post administration of NQ significantly protected mice against WBI induced mortality:

Therapeutic potential of NQ was investigated to mitigate the WBI induced mortality. Mice were exposed to WBI 8Gy and NQ (2mg/kg bw) was administered i.p. 1h after radiation treatment. NQ administration was given for 5 days a week at an interval of 24h for 3 weeks. 10 mice per group were taken. Mice were monitored for radiation induced changes in body weight and mortality for 30 days. Interestingly, NQ administration showed significant (40%, $p<0.01$) protection against WBI induced mortality and weight loss (Fig. 3.6 A and B).

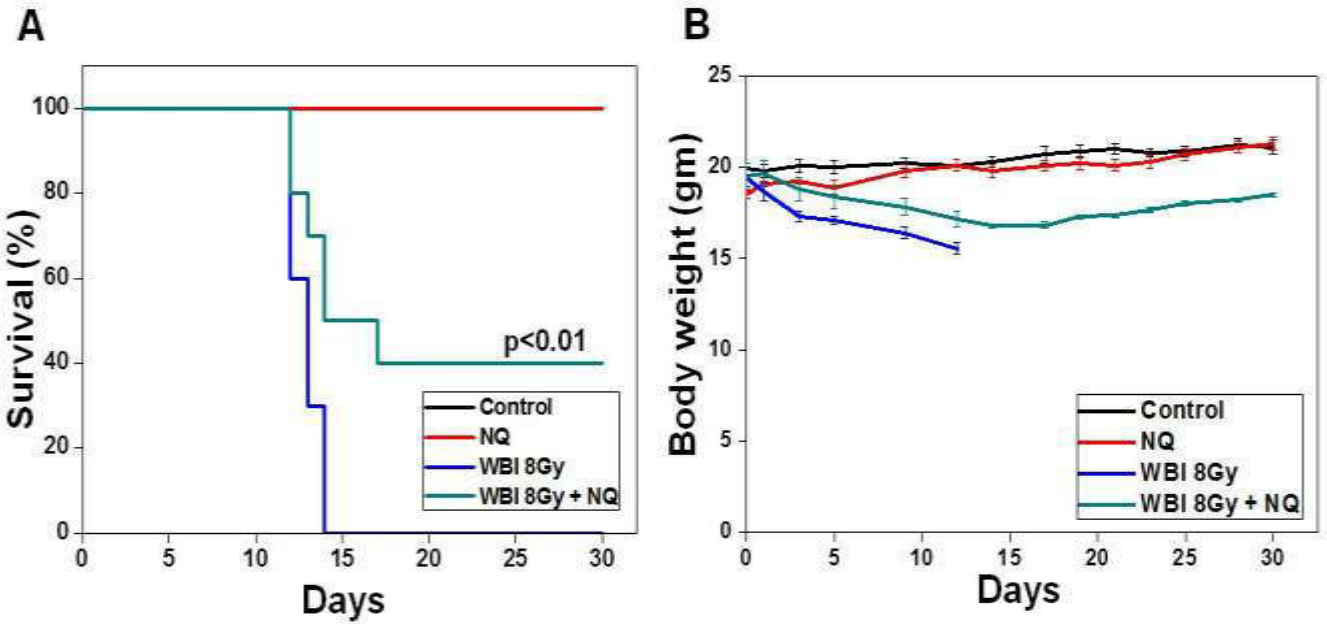
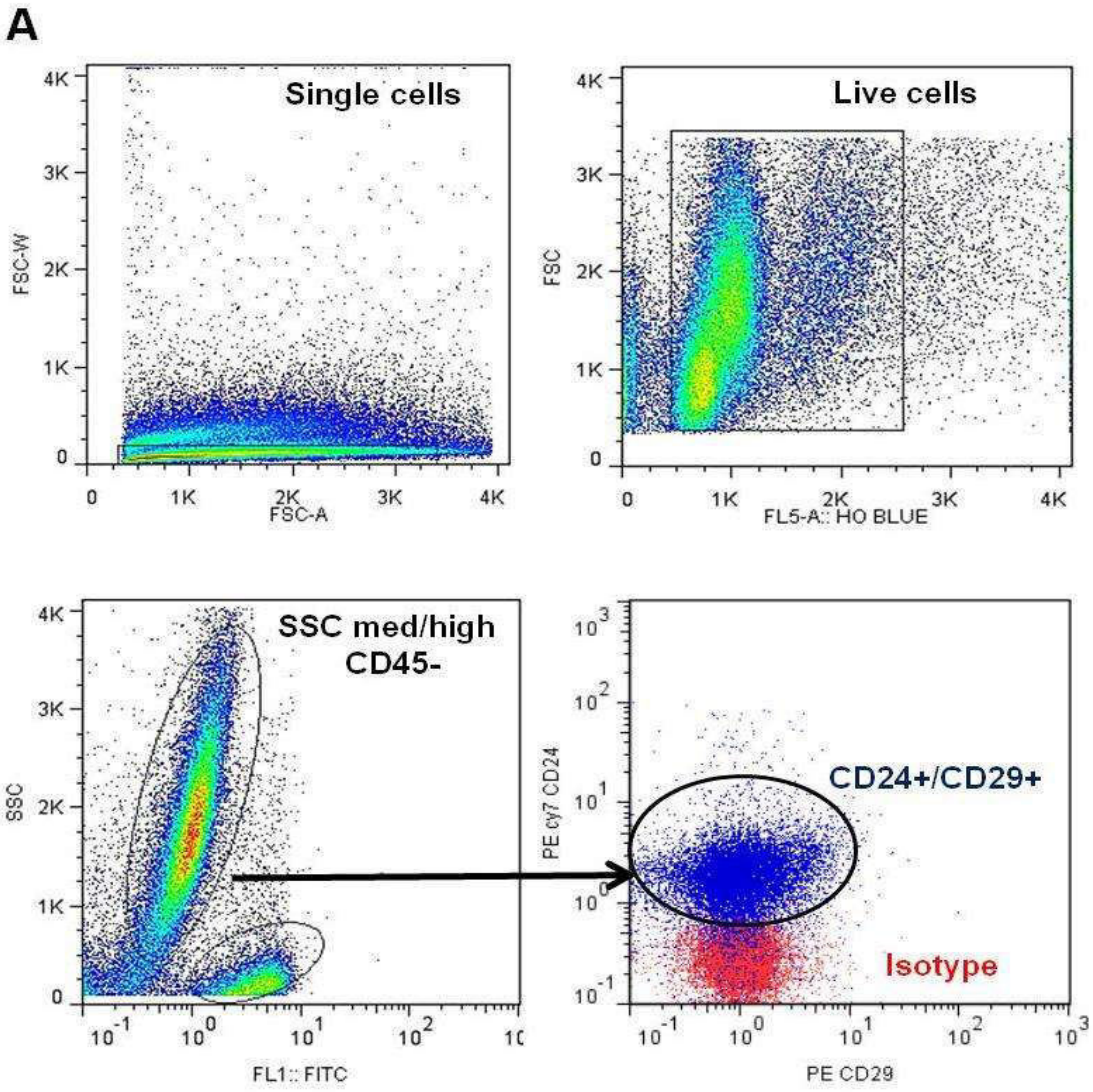


Fig. 3.6 Post administration of NQ significantly protected mice against WBI induced mortality: NQ (2mg/kg bw) was administered 1h after WBI 8Gy in mice. Survival and body weight of mice were monitored for 30 days. Ten mice per group were taken (A) Line graph represents the percent survival at WBI 8Gy. NQ treatment significantly improved 30 day survival of mice. (B) Graph represents the bodyweight of the mice after WBI 8Gy of indicated treatment groups. NQ administration protected mice against WBI induced loss in body weight. Data points represent mean±SEM from 10 mice. Two such independent experiments were carried out. Log rank test was used to compare mortality of WBI exposed mice administered with or without NQ.

3.1.7 NQ significantly protected against WBI induced cell death in intestinal epithelial cells:

To investigate the efficacy of NQ to protect intestinal epithelial cells from radiation induced cell death, mice were administered four consecutive doses of NQ (2mg/kg bw) i.p. at an interval of 24h and were given WBI 8Gy 30min after last dose. Vehicle treated mice served as control. The epithelial cells were flow cytometrically characterized as SSC med/high CD45- population in the single cell suspension derived from jejunum (Fig. 3.7A). These cells expressed CD24 and CD29 markers. Apoptotic cells and cell cycle analysis was done in all sub populations {epithelial cells (SSC med/high CD45-) with Hoechst33342 on the basis of DNA content. WBI significantly induced cell death as evident from pre-G1 population and

reduced S+G2+M population in intestinal epithelial cells. NQ administration protected against radiation induced cell death and cell cycle arrest (Fig. 3.7B-D).



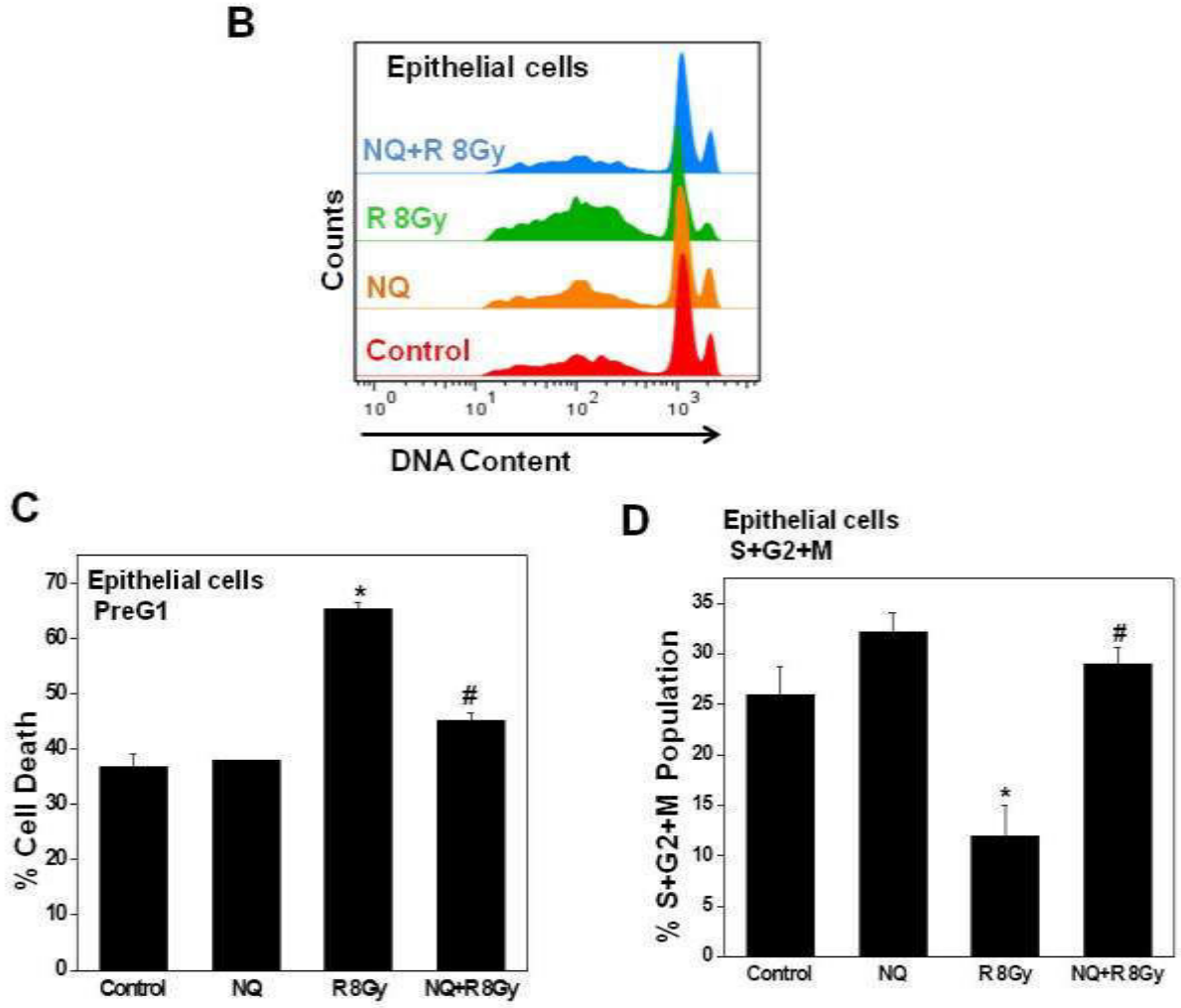
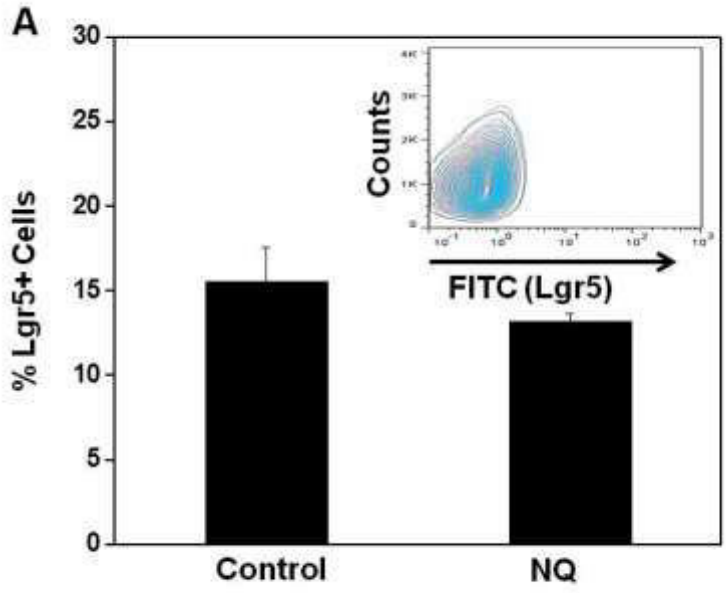


Fig. 3.7 NQ significantly protected against WBI induced cell death in intestinal epithelial cells: Mice were administered with four consecutive doses of NQ (2mg/kg bw) i.p. at an interval of 24h and were given WBI (8Gy) 30min after last dose. Vehicle administered mice served as control. (A) Single cell suspension of jejunum cells was stained with FITC conjugated anti-CD45, PE conjugated anti-CD29 and PECy7 conjugated anti-CD24 antibody and acquired on a flow cytometer. Single viable epithelial cells were gated by forward scatter, side scatter and pulse width parameter. (B) Percent cell death was calculated from sub-G1 population and percent S+G2+M cells was calculated based on >2n DNA content as shown in overlaid flow cytometric histograms. (C) Graph represents percent cell death in each treatment group. (D) Graph represents percent S+G2+M in each treatment group. Each data point represents mean±S.E.M. from 3 replicates and two such independent experiments having similar results were carried out. *$p<0.01$, as compared to vehicle treated group, #$p<0.01$, as compared to irradiated group.

3.1.8 NQ significantly protected against WBI induced cell death in intestinal stem cells:

Each crypt in jejunum contains intestinal stem cells (ISC) at the base which divide and differentiate to replace the dying enterocytes and maintain homeostasis (*320*). Exposure to ionizing radiation induces cell death in intestinal stem cells that disables the repopulation of the denudated villi. Jejunum cells isolated from all the treatment groups were stained with anti-Lgr5 antibody for identifying ISC population. Apoptotic cells and cell cycle analysis was done in all sub populations [epithelial cells (SSC med/high CD45-) and intestine stem cells (Lgr5+ fraction in SSC med/high CD45-)] with Hoechst33342 on the basis of DNA content. Intriguingly, no significant change was observed in the abundance of Lgr5+ cells with NQ administration (Fig. 3.8A). WBI significantly induced cell death as evident from pre-G1 population and reduced S+G2+M population in ISC. NQ administration protected against radiation induced cell death and cell cycle arrest (Fig. 3.8B-D).



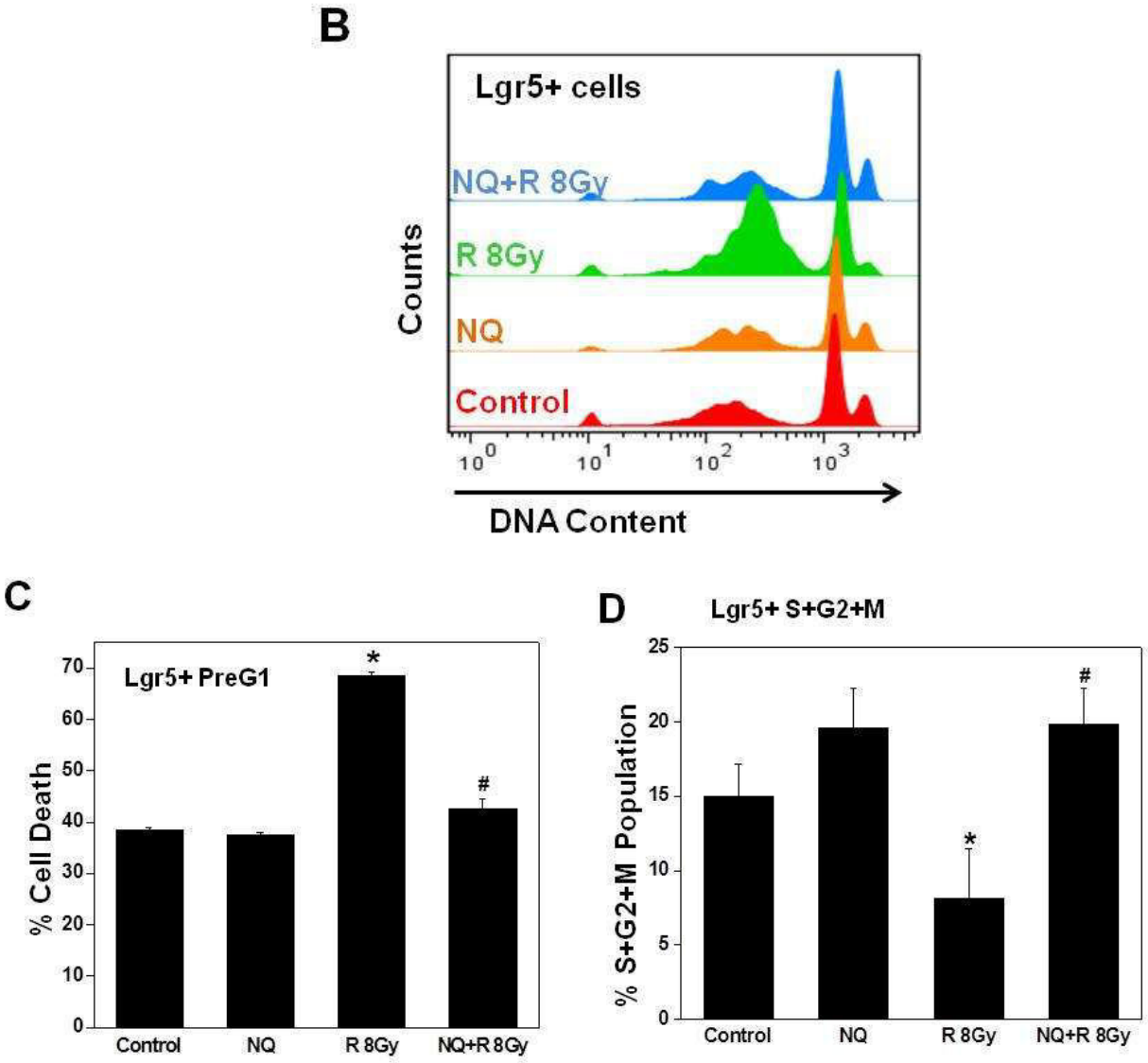


Fig. 3.8 NQ significantly protected against WBI induced cell death in intestinal stem cells: Mice were administered with four consecutive doses of NQ (2mg/kg bw) i.p. at an interval of 24h and were given WBI (8Gy) 30min after last dose. Vehicle administered mice served as control. Single viable epithelial cells were gated by forward scatter, side scatter and pulse width parameter. (A) Graph along with flow cytometric histogram in the satellite represents the changes in abundance of intestinal stem cells (% Lgr5+ cells). (B) Single cells from jejunum were stained with anti-Lgr5 antibody followed by staining with Hoechst33342. Intestinal stem cells were gated as Lgr5+ fraction in SSC med/high CD45- epithelial cells. Percent cell death was calculated from sub-G1 population and percent S+G2+M cells was calculated based on >2n DNA content as shown in overlaid flow cytometric histograms. (C) Graph represents percent cell death in each treatment group. (D) Graph represents percent S+G2+M in each treatment group. Each data point represents mean ±S.E.M. from 3 replicates and two such independent experiments having similar results were carried out. *$p<0.01$, as compared to vehicle treated group, #$p<0.01$, as compared to irradiated group.

3.1.9 NQ administration induced phosphorylation of ERK in jejunum cells:

Earlier studies using NQ have shown its ability to activate ERK/Nrf2 pathway in lymphocytes. Experiments were carried out to assess the involvement of ERK/Nrf2 pathway in NQ mediated radioprotection in vivo. Mice were administered with 4 doses of NQ (2 mg/kg bw) with 24h interval. Jejunum cells were isolated from vehicle or NQ administered mice at indicated time intervals. Changes in the pERK levels were monitored by Western blotting as well as intra-cellular antibody staining and flow cytometry. Cytoplasmic fraction of the isolated cells prepared from vehicle or NQ administered mice was used for Western blotting. Interestingly, NQ administration induced phosphorylation of ERK (Fig. 3.9A). Further to substantiate our findings, jejunum cells were isolated from vehicle or NQ administered mice 4 days after the last dose of NQ. Isolated cells were intracellulary stained with PE conjugated anti-pERK antibody followed by flow cytometric analysis. NQ administration induced a significant increase in pERK levels in intestinal epithelial cells (Fig. 3.9B and C).

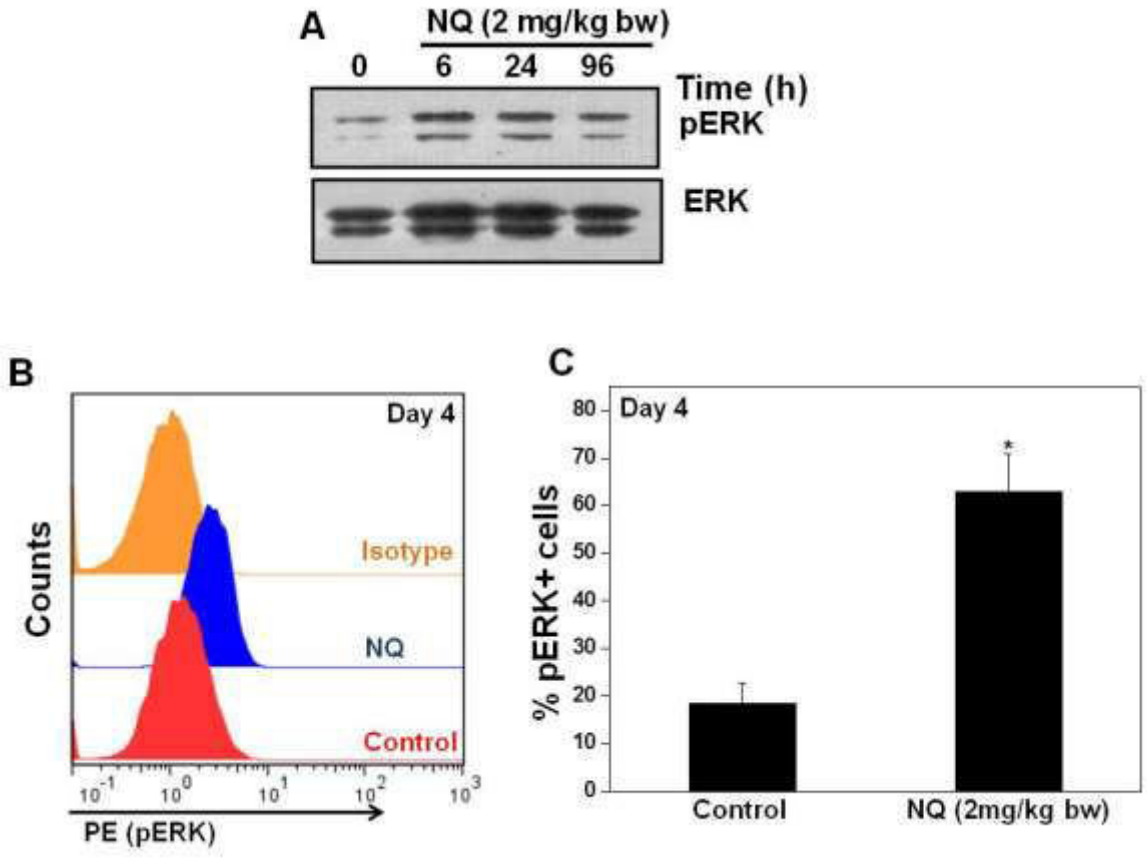
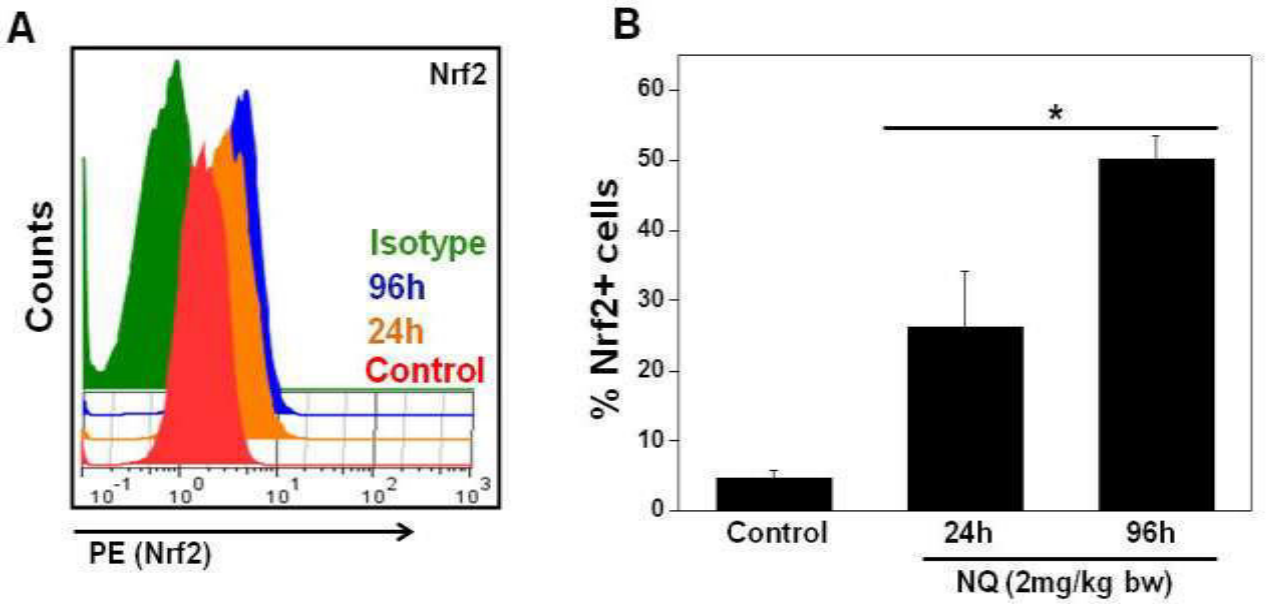


Fig. 3.9 NQ administration induced phosphorylation of ERK in jejunum cells: Mice were administered four consecutive doses of NQ (2mg/kg bw) with 24h interval. Jejunum cells were isolated from treated mice at indicated time points. Cells from vehicle administered mice served as control. (A) Cytoplasmic fraction was used to perform Western blotting to monitor the changes in phosphorylation levels of ERK using anti-pERK and anti-ERK antibodies. (B) Jejunum cells were isolated from vehicle or NQ administered mice on day 4. Cells were intracellulary stained with PE conjugated anti-pERK antibody and were acquired on a flow cytometer. Overlaid flow cytometric histograms showing pERK positive cells. (B) Graph represents the percent pERK positive cells with their respective mean±SEM from three replicates in each treatment group. Two such independent experiments were carried out. *$p<0.01$, as compared to vehicle treated cells.

3.1.10 NQ administration induced activation of Nrf2 in jejunum cells:

Figure 3.10 demonstrates the potential of NQ to activate Nrf2 in intestinal epithelial cells. Mice were administered with 4 doses of NQ (2 mg/kg bw) with 24h interval. Jejunum cells were isolated from vehicle or NQ administered mice at indicated time intervals to monitor the changes in the Nrf2 levels. Cells were stained intracellulary with anti-Nrf2 antibody and were acquired on a flow cytometer. NQ administration induced an increase in Nrf2 levels in jejunum cells as evident from an increase in Nrf2 positive population (Fig. 3.10A and B). Further, NQ treatment also led to a significant increase in mRNA copy number of Nrf2 and its dependent genes HO-1, GCLC, MnSOD and Catalase (Fig. 3.10C).



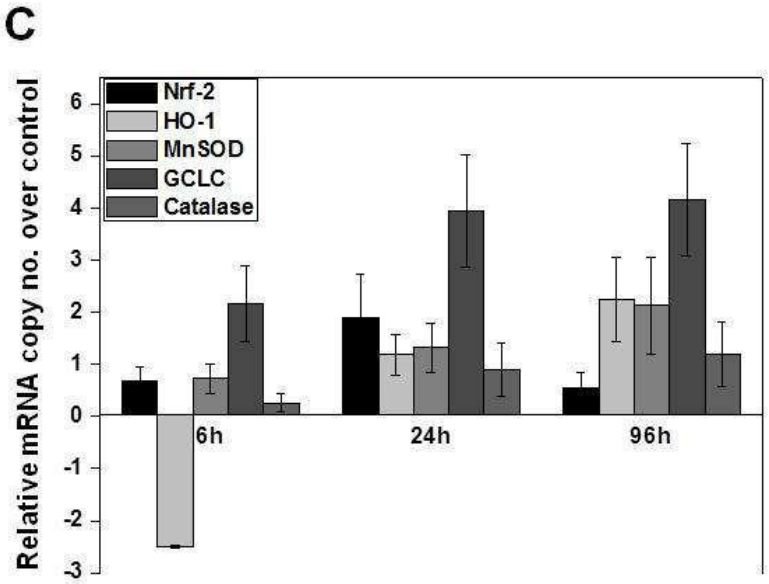


Fig. 3.10 NQ administration induced activation of Nrf2 in jejunum cells: Mice were administered with four consecutive doses of NQ (2mg/kg bw). Jejunum cells were isolated from treated mice at indicated time points. Cells from vehicle administered mice served as control. (A) Jejunum cells were isolated from vehicle or NQ administered mice on day 4. Cells were analysed for Nrf2 expression by antibody staining using a flowcytometer. Representative flow cytometric histograms show Nrf2 positive cells. (B) Graph represents the percent Nrf2 positive cells with their respective mean±SEM in each treatment group. (C) Jejunum cells were isolated from NQ administered mice at indicated time points, mRNA was isolated and used for real time RT-PCR. Graph represents relative mRNA copy number of Nrf2 and its dependent genes over control. Two such independent experiments were carried out. *$p<0.05$, as compared to vehicle treated cells.

3.1.11 Inhibition of Nrf2 pathway abrogated NQ mediated protection against WBI induced mortality in mice:

Further, to establish a casual role of Nrf2 pathway in NQ mediated radio-protection in mice, pharmacological inhibitor of Nrf2, ATRA was employed. ATRA was injected i.p biweekly to respective treatment groups. Mice were administered four doses of NQ (2mg/kg bw) at an interval of 24h and were given WBI 10Gy 30min after last dose. BMT was done 24h after WBI to mitigate the radiation induced hematopoietic depletion. ATRA administration did not offer protection against WBI induced mortality. NQ administration along with BMT showed

significantly increased survival. Interestingly, ATRA administration significantly ($p<0.001$) abrogated the NQ mediated protection against WBI induced mortality in mice (Fig. 3.11).

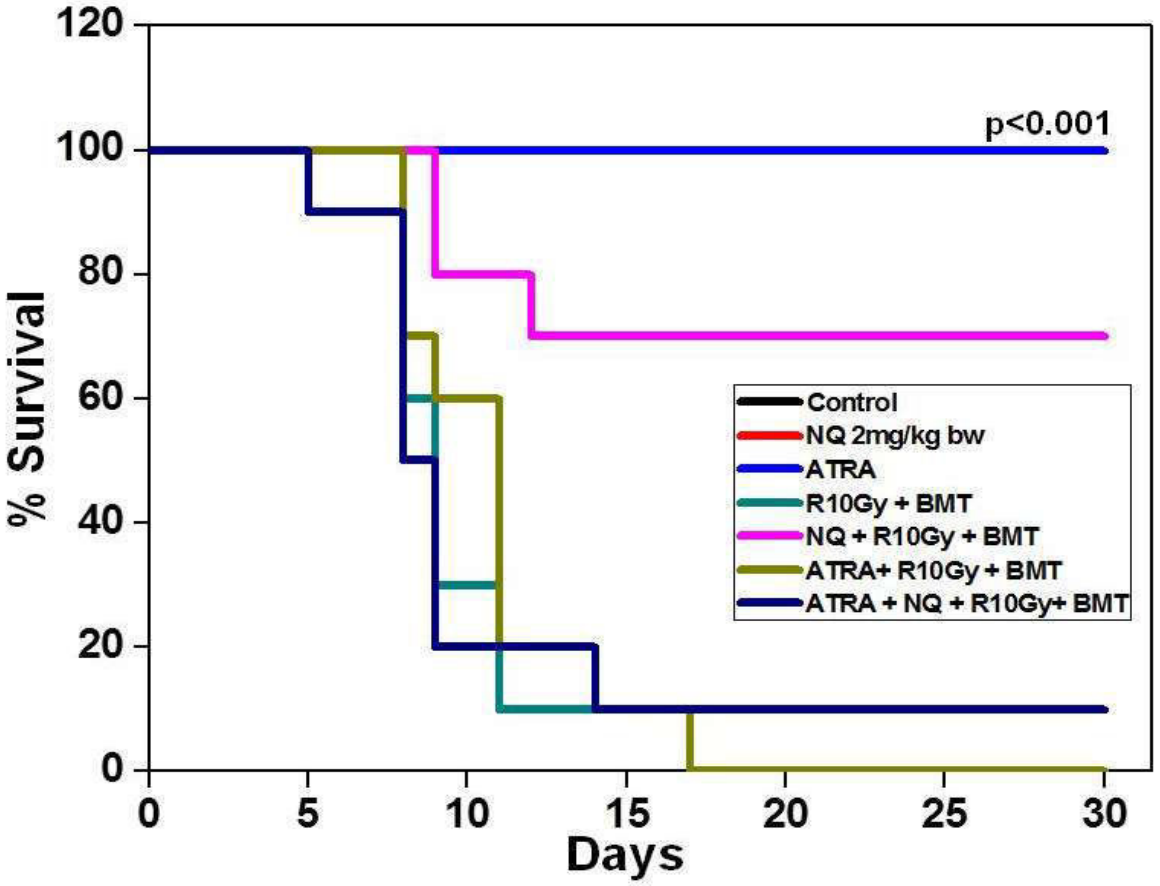


Fig. 3.11 Inhibition of Nrf2 pathway abrogated NQ mediated protection against WBI induced mortality in mice: Mice were administered with ATRA 50µg/mice twice a week i.p to respective treatment groups. Mice were administered NQ i.p. consecutively for 4 days with an interval of 24h. WBI of 10Gy was given 30min after the last dose of NQ followed by BMT 24h after WBI. Mice were monitored for radiation induced changes in body weight and mortality for 30 days. Graph represents the percent survival at WBI 10Gy. Data points represent mean±SEM from 10 mice. Two such independent experiments were carried out. Statistical significance of survival was assessed using Log-rank test.

3.2 DISCUSSION:-

Mortality at doses equal to or greater then 8Gy is associated with GI syndrome. Previous studies carried out in our laboratory have shown that NQ, a well known pro-oxidant, was able to ameliorate radiation induced hematopoietic syndrome via upregulation of ERK/Nrf2 pathway in lymphocytes (*287*). Based on these studies, we hypothesized that NQ may extend its protection against radiation induced GI syndrome by initiating adaptive responses via activation of Nrf2 pathway in the GI tract. We indeed found that, administration of NQ (2mg/kg bw, four consecutive doses) significantly protected mice against WBI 8Gy induced mortality and loss in body weight (Fig 3.1). Exposure to IR induces denudation of villi and apoptosis in crypts to manifest the onset of GI syndrome. NQ administration rescued mice from WBI induced denudation of villi, epithelial misalignment and protected against induction of apoptosis in the crypts (Fig 3.2). To the best of our knowledge, this is the first report showing the potential of a prooxidant to ameliorate radiation induced GI injury. NQ administration alone did not offer significant protection against higher doses of radiation (9 & 10Gy). Further, hematopoietic system is among the most sensitive and critical systems for early radiation-induced health affects that contribute to the mortality at high dose exposure (*321, 322*). Therefore, we adopted a syndrome specific model in combination with autologous bone marrow transplant (BMT) to compensate for the IR induced bone marrow aplasia. BMT after WBI would lead to reconstitution of damaged hematopoietic system resulting in radiation induced GI syndrome as prime contributor towards observed mortality (*319, 323*). BMT after 24h of WBI was able to repopulate the bone marrow but could not completely rescue mice against WBI induced mortality. These results suggested that GI syndrome may be the major contributor towards mortality at high doses of IR. Interestingly, NQ administration in combination with BMT showed complete protection at WBI dose of 9Gy and significant protection against 10Gy induced mortality and weight loss (Fig 3.3A and

B). BMT alone could not rescue mice at WBI 12Gy, however NQ administration along with BMT offered significant (40%) protection against mortality (Fig 3.3C and D). These results clearly demonstrated that NQ in combination with BMT can serve as a potent modality to protect the host from high doses of radiation induced death.

Due to dynamic nature of the intestinal absorptive surface, epithelial cells present in villi/crypt axis are highly proliferating and are hence more sensitive towards IR induced damage (*320, 324*). Exposure of GI tract to high doses of IR is known to induce loss of clonogenicity and proliferation capacity in intestinal epithelial cells (*40, 320*). Since NQ administration was able to prevent 8Gy induced mortality in mice, we studied the effect of NQ on proliferative potential of intestinal epithelial cells and permeability of the intestine in irradiated mice. It was found that NQ treated mice had a higher frequency of BrdU+ cells in the villi/crypt of NQ treated mice compared to vehicle treated irradiated control indicating that NQ administration alone and in combination with BMT was able to prevent radiation induced loss of proliferating ability of intestinal epithelial cells which may be responsible for the observed radioprotection (Fig 3.4). Gastrointestinal tract is inhabited by microbial flora which upon exposure to IR translocates to different organs inducing inflammation. Radiation induces the loss of intestinal integrity that results in bacterial translocation from the GI tract to different organs (*325, 326*) and is considered to be a clinically important event during radiation-induced GI syndrome (*327, 328*). NQ prevented radiation induced increase in permeability of the intestine leading to decreased bacterial load in liver and spleen with reduced loss of bacterial load in jejunum, as compared to irradiated mice demonstrating its ability to protect against radiation induced damage to GI (Fig 3.5).

Apart from finding application of NQ as a prophylactic agent during planned settings like radiotherapy, radioprotective drugs are also required during unplanned or accidental exposure scenarios. Hence, in order to use NQ in such a scenario of unplanned exposure, the

therapeutic efficacy of this agent needs to be evaluated. To investigate the therapeutic efficacy of NQ against radiation induced mortality, it was administered to mice post IR and the survival of these mice was monitored. Interestingly, post irradiation administration of NQ in mice offered significant protection against WBI 8Gy induced mortality and loss in body weight (Fig 3.6A and B). These results further highlight the potential of NQ to protect against radiation induced GI syndrome and demonstrate its efficacy to act as generalised radioprotector. Further studies were carried out to elucidate the mechanism of NQ mediated radioprotection. It is well known that the GI mucosa is a rapid turnover system, driven by mitotic activity of its clonogenic compartment consisting of self-renewing intestinal stem cells (*47, 324*). Small intestine villi and crypts receive constant supply of enterocytes from ISC present at the base of the crypt. These enterocytes differentiate to functional form and move up to the villi from crypt and are extruded from villi. Exposure to ionizing radiation induces mitotic death which is common in mammalian cell post radiation exposure due to hyper proliferative activity of ISC resulting in induction of DNA strand breaks and chromosomal aberrations (*55, 329*). Multiple elegant lineage studies have identified Lg45/Gpr49 or Lgr5, which encodes an orphan leucine-rich-repeat–containing G-protein–coupled receptor, as a marker of intestinal stem cells (*40, 54, 330*). To investigate the effect of NQ on ISC, we used an Lgr5-dependent tracing system that allows the conditional labelling of $Lgr5^{+}$ intestinal stem cells. NQ administration did not show any increase in the abundance of ISC (Fig 3.8A) indicating that NQ does not act as a growth factor. We observed that exposure of mice to WBI 8Gy induced cell death in ISC and epithelial cells and also a reduced $S+G_2+M$ population in these cells. However, administration of NQ protected epithelial cells (Fig. 3.7) and ISC (Fig 3.8B-D) from radiation induced cell death, indicating that the ability of NQ to protect the GI tract against IR injury. It

is clear that the protection may be mediated via protecting the epithelial cells and clonogenic compartment of crypt containing ISC.

Since NQ has been previously reported to protect lymphocytes against IR by upregulating the pro-survival transcription factor Nrf2, we proposed that the observed protection of ISC and epithelial cells by NQ administration may also be mediated via the Nrf2 pathway in the GI tract. Higher redox potential and electrophilic nature of quinones imparts high affinity for cellular nucleophiles like thiols of cysteine residue present in proteins and glutathione contributing to its biological activity of modulating cellular redox balance (*305, 307*). Perturbation in cellular redox status is known to activate redox sensitive pro-survival transcription factors like Nrf2. Activation of Nrf2 could be achieved by two major mechanisms: 1) KEAP-1 dependent or 2) MAPK dependent. KEAP-1 is a negative regulator of Nrf2 which contains multiple redox sensitive critical cysteine residues. Exposure of cells to oxidative, xenobiotic or electrophilic stress abrogates KEAP-1 induced degradation of Nrf2. Several studies have highlighted the role of mitogen activated protein kinases (MAPK) in inducing the activation of Nrf2. Jeong et al. studied MAPK mediated activation of phase II detoxification enzymes using multiple inducers (*227*). In hepatoma cells, sulforaphane and tBHQ induced activation of ERK, MAPK kinase and Raf-1, to mediate induction of phase II detoxification enzymes via Nrf2/ARE pathway (*229*). MAPK/ERK upon activation initiates phosphorylation cascade that modulate activity of multiple downstream transcription factors (*230*). Dithiolcarbamate was shown to activate ERK resulting in transcriptional up-regulation of Nrf2 dependent γ-glutamylcysteine synthetase (*232*). Further, NQ was shown to induce activation of ERK in lymphocytes (*287*). Based on these observations, further studies were carried out to elucidate the in vivo mechanism of NQ mediated protection. Interestingly, NQ administration induced the activation of ERK as evident by increased pERK levels in jejunum cells (Fig 3.9). Further, administration of NQ

also showed an increase in the Nrf2 levels and increased expression of Nrf2 dependent genes in jejunum cells (Fig 3.10). To further confirm our hypothesis that activation of Nrf2 pathway mediates NQ induced protection against WBI induced GI syndrome, a pharmacological inhibitor of Nrf2 (ATRA) was employed. Wang et al showed that ATRA did not block the nuclear accumulation of Nrf2 but reduced the binding of Nrf2 to the ARE enhancer as a consequence of forming a complex with retinoic acid receptor alpha RARα (*331*). We found that ATRA was able to significantly abrogate NQ mediated protection against WBI 10Gy induced mortality confirming the involvement of Nrf2 pathway (Fig 3.11). These results further confirmed the role of Nrf2 in NQ mediated protection against WBI induced GI syndrome associated mortality.

Plethora of compounds with different underlying mechanisms has been shown to protect against IR induced damage. Out of which very few have shown the potential to protect against IR induced GI syndrome. Many compounds with antioxidant activities, including neutralization of IR induced ROS, are effective radioprotectors. Ascorbic acid has been shown to protect several biological systems against ionizing radiation. However, ascorbic acid is effective at preventing cell apoptosis only at lower doses of radiation but not at lethal doses. Ascorbic acid was shown to protect mice in combination with BMT against IR induced GI syndrome by down regulating apoptosis genes in small intestine (*332*). Growth factors like insulin-like growth factor 1 (IGF-1), keratinocyte growth factor (KGF), and fibroblast growth factor-2 (FGF-2 or bFGF-2) have been shown to protect against radiation induced intestinal injury. These growth factors impaired the expression of p53 and p53 upregulated mediator of apoptosis (PUMA) in crypt cells thereby inhibiting radiation induced apoptosis in intestine (*333*). Further, other strategies focussing on employing agents that increase the stemness of ISC, R-spondin 1 and TP508, have been used to protect against radiation induced GI syndrome (*56, 334*). In the present work we have employed a strategy of

perturbing cellular redox status using a pro-oxidant, NQ, to protect against radiation induced GI syndrome. The key finding of present work is perturbation in cellular redox status induced by treatment with mild oxidative stressor (NQ, a pro-oxidant) results in activation of redox sensitive transcription factor Nrf2 in the epithelial cells of GI tract. Upregulation of these prosurvival proteins are responsible for the observed radioprotective effects of NQ against radiation induced GI syndrome.

CHAPTER 4

IMMUNOMODULATORY EFFICACY OF 1, 4 NAPHTHOQUINONE

In the previous chapter, the potential of NQ to protect mice against IR induced GI syndrome by activating Nrf2 pathway was discussed. Role of Nrf2 as an anti-inflammatory transcription factor has been well documented in the literature. Earlier studies from our laboratory employing a structural analogue of NQ, plumbagin, demonstrated potent anti-inflammatory action (*154, 293*). Since inflammatory reaction plays a major role in the pathogenesis of radiation induced damage to normal tissue, we also explored the anti-inflammatory potential of NQ. Potential of NQ to curb the inflammatory responses could address the mechanism of NQ mediated radioprotection in vivo.

This chapter describes the effects of NQ on innate and adaptive immune responses mediated by lymphocytes and macrophages respectively. The detailed mechanism of anti-inflammatory action of NQ is also described.

4.1 RESULTS:

4.1.1 Lymphocytes from NQ injected mice showed decreased responsiveness to Con A or anti-CD3/CD28mAb induced cytokine secretion:

The functional responses of lymphocytes from NQ (2mg/kg bw i.p.) treated mice were evaluated in terms of mitogen induced proliferation and cytokine production. Lymphocytes were isolated from spleen and gut associated lymphoid tissue (GALT) of vehicle and NQ administered mice 24h after administration and were stimulated with Con A (5 μg/ml) or plate bound anti-CD3/soluble anti-CD28 mAb (1 μg/ml) for monitoring cytokine (IL-2, IL-4, IL-6 and IFN-γ) secretion. Lymphocytes isolated from NQ injected mice showed reduced cytokine secretion when stimulated with anti-CD3/CD28 mAb (Fig. 4.1A and B) or Con A (Fig. 4.1C) as compared to control group.

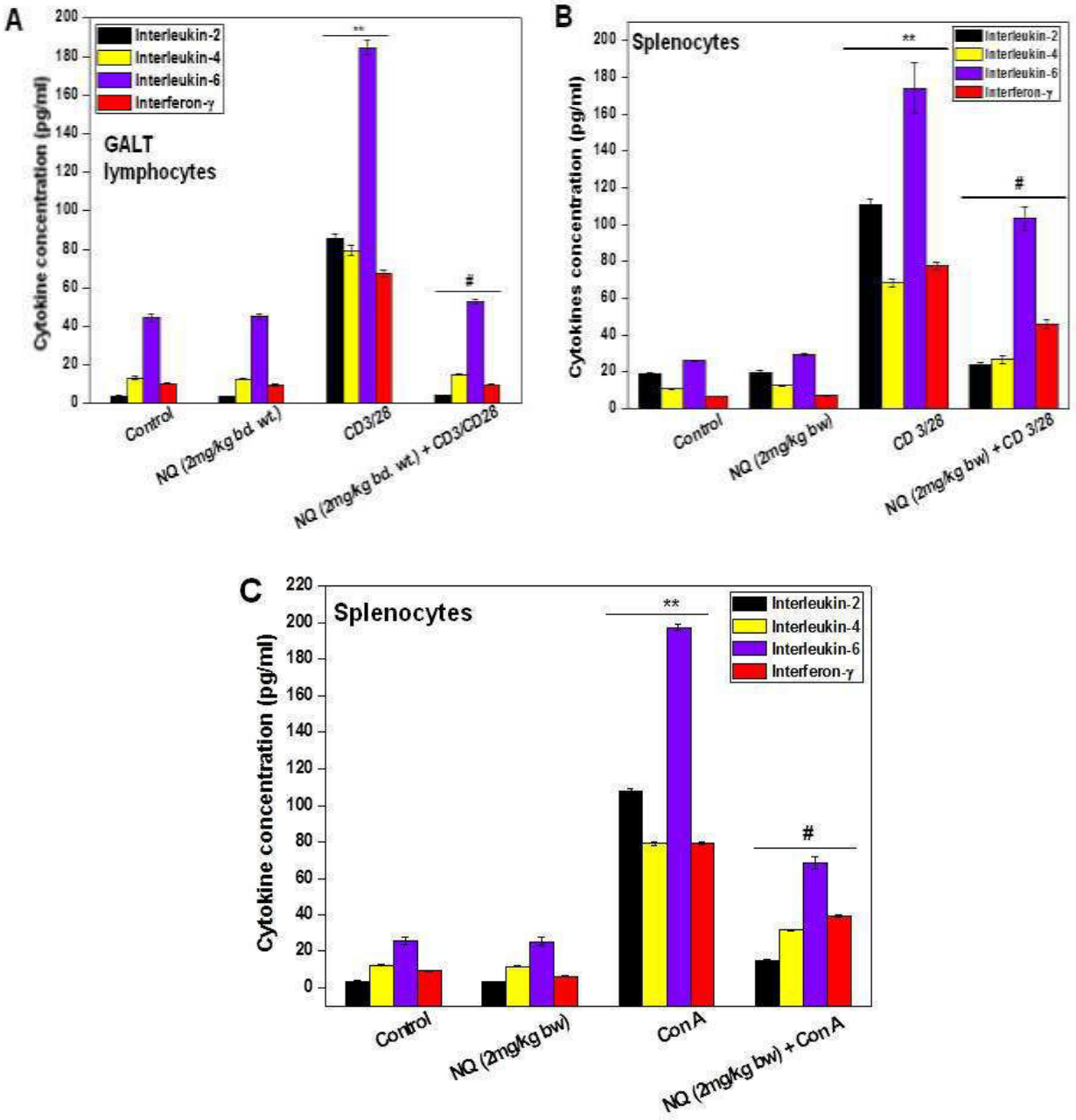


Fig. 4.1 Lymphocytes from NQ treated mice showed decreased cytokine secretion in response to anti-CD3/CD28mAb or Con A stimulation: (A-B) Mice were injected i.p. with NQ (2mg/kg body weight) or vehicle. Lymphocytes from GALT and spleen were isolated 24h after injection. Cells were stimulated with anti-CD3/anti-CD28 mAb to assess secretion of IL-2, IL-4, IL-6 and IFN-γ cytokines at 24h ex vivo. (C) Mice were injected i.p. with NQ (2mg/kg bw) or vehicle and 24h post injection, lymphocytes from spleen were isolated and stimulated with Con A for 24h. Supernatants were used to measure cytokine secretion by ELISA. Each bar represents mean±S.E.M. from three replicates. Two such independent experiments were carried out. **$p<0.01$ as compared to vehicle treated cells, #$p<0.01$ as compared to anti-CD3/anti-CD28 mAb or Con A stimulated cells.

4.1.2 Lymphocytes from NQ injected mice showed hyporesponsiveness to Con A or anti-CD3/CD28mAb induced proliferation:

Clonal expansion of lymphocytes is required for mounting antigen specific response in vivo. Proliferation of lymphocytes in response to mitogen is used as an important parameter for assessing the functional competence of immune cells. Splenic lymphocytes were isolated from mice 24h after administration of NQ and were stained with CFSE and stimulated with Con A or plate bound anti-CD3/soluble anti-CD28 mAb for inducing proliferation. Cells isolated from vehicle treated mice served as a control. Fig. 4.2A and C shows the representative flow cytometric histograms of CFSE labeled splenic lymphocytes stimulated with Con A and anti-CD3/CD28 mAb for 72h. Frequency of daughter cells increased significantly in Con A or anti-CD3/CD28 mAb stimulated lymphocytes as compared to that in unstimulated cells. The bars represent percentage of daughter cells obtained 72h after Con A (Fig. 4.2B) or anti-CD3/CD28 mAb (Fig. 4.2D) stimulation. Lymphocytes isolated from NQ injected mice showed reduced proliferation when stimulated with Con A (Fig: 4.2A and B) or anti-CD3/CD28 mAb (Fig: 4.2C and D) as compared to control group.

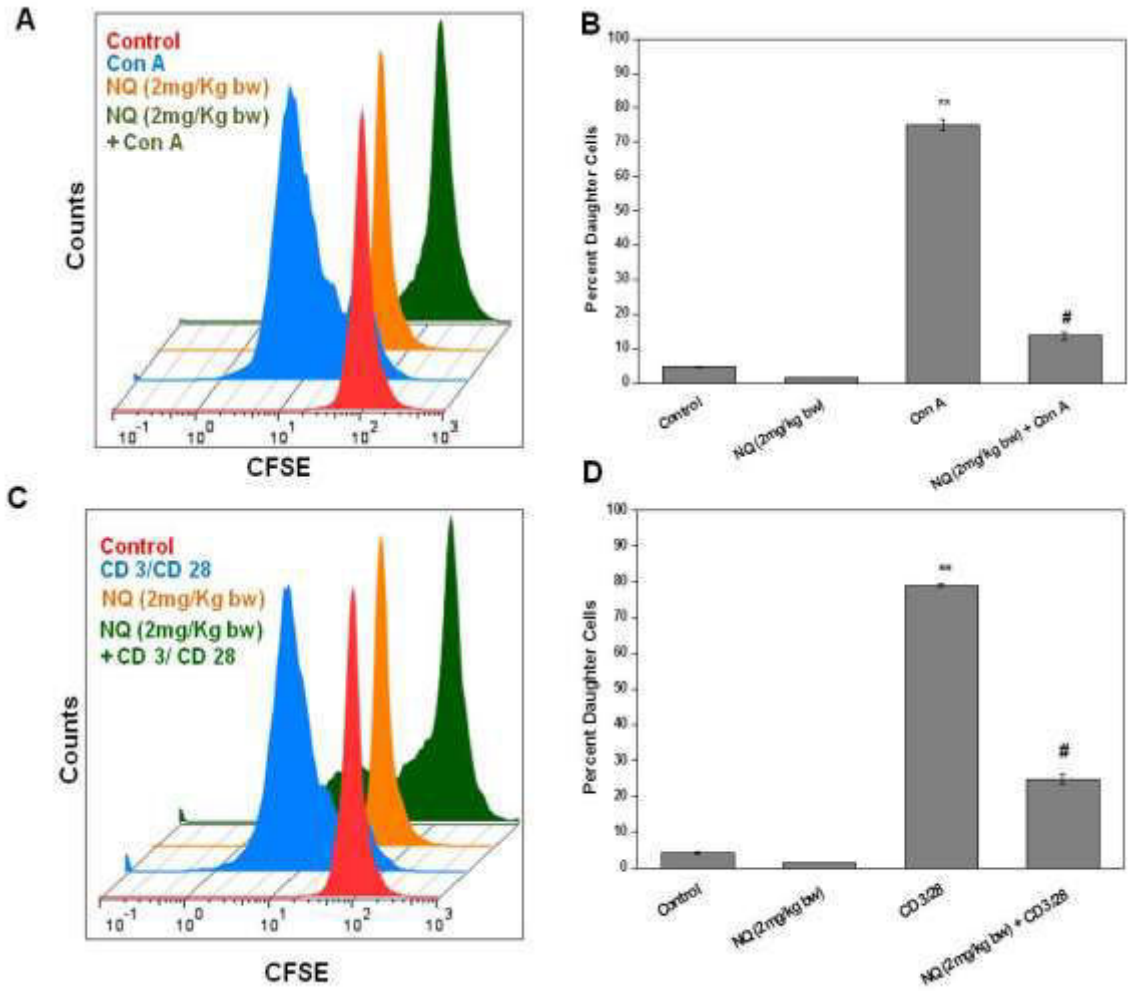


Fig. 4.2 Lymphocytes from NQ injected mice showed decreased responsiveness to Con A and anti-CD3/CD28mAb induced proliferation: (A-D) Mice were injected i.p. with NQ (2mg/kg body weight) or vehicle and splenic lymphocytes were isolated 24h after injection. Cells were stained with CFSE and stimulated with either Con A (A&B) or anti-CD3/anti-CD28 mAb (C&D) to assess proliferation at 72h ex vivo. Each bar represents mean±S.E.M. from three replicates. Two such independent experiments were carried out. **p<0.01 as compared to vehicle treated cells, #p<0.01 as compared to Con A or CD3/anti-CD28 mAb stimulated cells.

4.1.3 Lymphocytes from NQ injected mice showed suppression of nuclear levels of NF-κB and inhibited IκBα degradation:

Lymphocytes isolated from NQ administered mice or vehicle treated mice were stimulated with Con A (5µg/ml) for 4h and the cells were used for preparation of cytoplasmic extract

and nuclear extract. Levels of IκBα and NF-κB were evaluated in cytosolic and nuclear fractions respectively by Western blotting and EMSA respectively. Con A (5μg/ml) stimulated cells showed increase in NF-κB nuclear translocation and decrease in IκBα in the cytosolic fraction as compared to that in vehicle treated control cells (Fig. 4.3A and B). However, lymphocytes isolated from NQ administered mice upon stimulation with Con A did not show NF-κB activation in nuclear fraction (Fig. 4.3A) or IκBα degradation in cytosolic fraction (Fig. 4.3B).

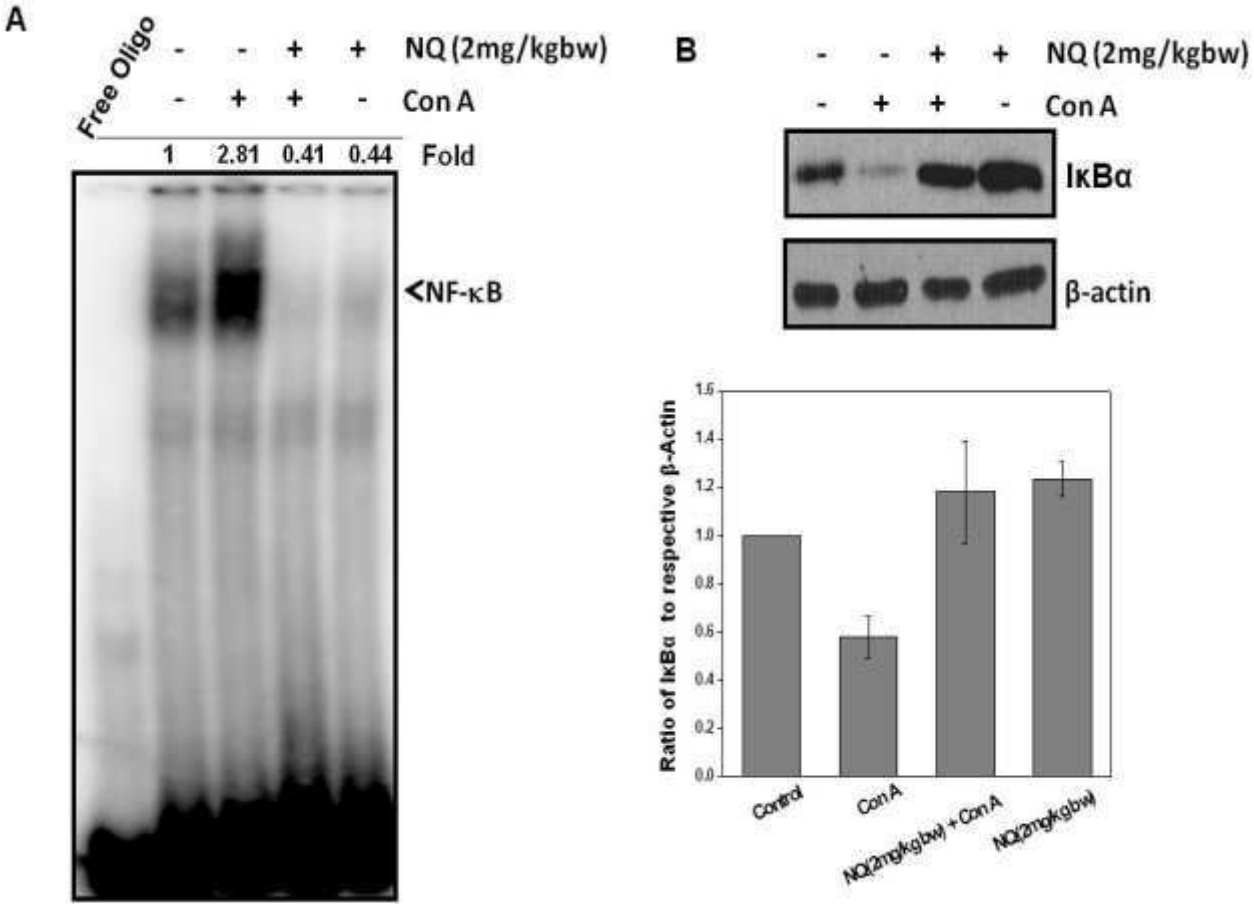


Fig. 4.3 Lymphocytes from NQ injected mice showed suppression of nuclear levels of NF-κB and inhibited IκBα degradation: (A-B) Mice were injected i.p. with NQ (2mg/kg bw) or vehicle and splenic lymphocytes were isolated 24h after injection. Isolated splenic lymphocytes were stimulated with Con A for 4h, nuclear extracts were prepared and subjected to EMSA. Cytosolic extracts were subjected to Western blot analysis with antibodies specific for IκBα and β-actin. Specific signal intensities were subsequently quantified by Syngene Gene Tools software (Cambridge, UK). Three such independent experiments were carried out.

4.1.4 NQ inhibited Con A or anti-CD3/CD28 antibody induced T cell proliferation in vitro:

Exposure to mitogen is known to induce proliferation in lymphocytes. The effect of NQ on Con A and anti-CD3/CD28 mAb induced T cell proliferation was assessed by CFSE dye dilution. Two million CFSE labeled splenocytes were treated with NQ (0.5µM to 5µM, 2h) and were stimulated with Con A (5 µg/ml) or anti-CD3/CD28 mAb (1 µg/ml) for 72h at 37 °C in 2ml RPMI with 10% FCS in a 95% air/5% CO_2 atmosphere. Vehicle treated cells served as control. Fig. 4.4A and C shows the representative flow cytometric histograms of CFSE labeled splenic lymphocytes stimulated with Con A for 72h in vitro in the presence or absence of different concentrations of NQ. Frequency of daughter cells increased significantly in Con A or anti-CD3/CD28 mAb stimulated lymphocytes as compared to that in unstimulated cells. The bars represent percentage of daughter cells obtained 72h after Con A (Fig. 4.4B) or anti-CD3/CD28 mAb (Fig. 4.4D) stimulation. Pre-treatment with different concentrations of NQ significantly inhibited mitogen induced proliferation.

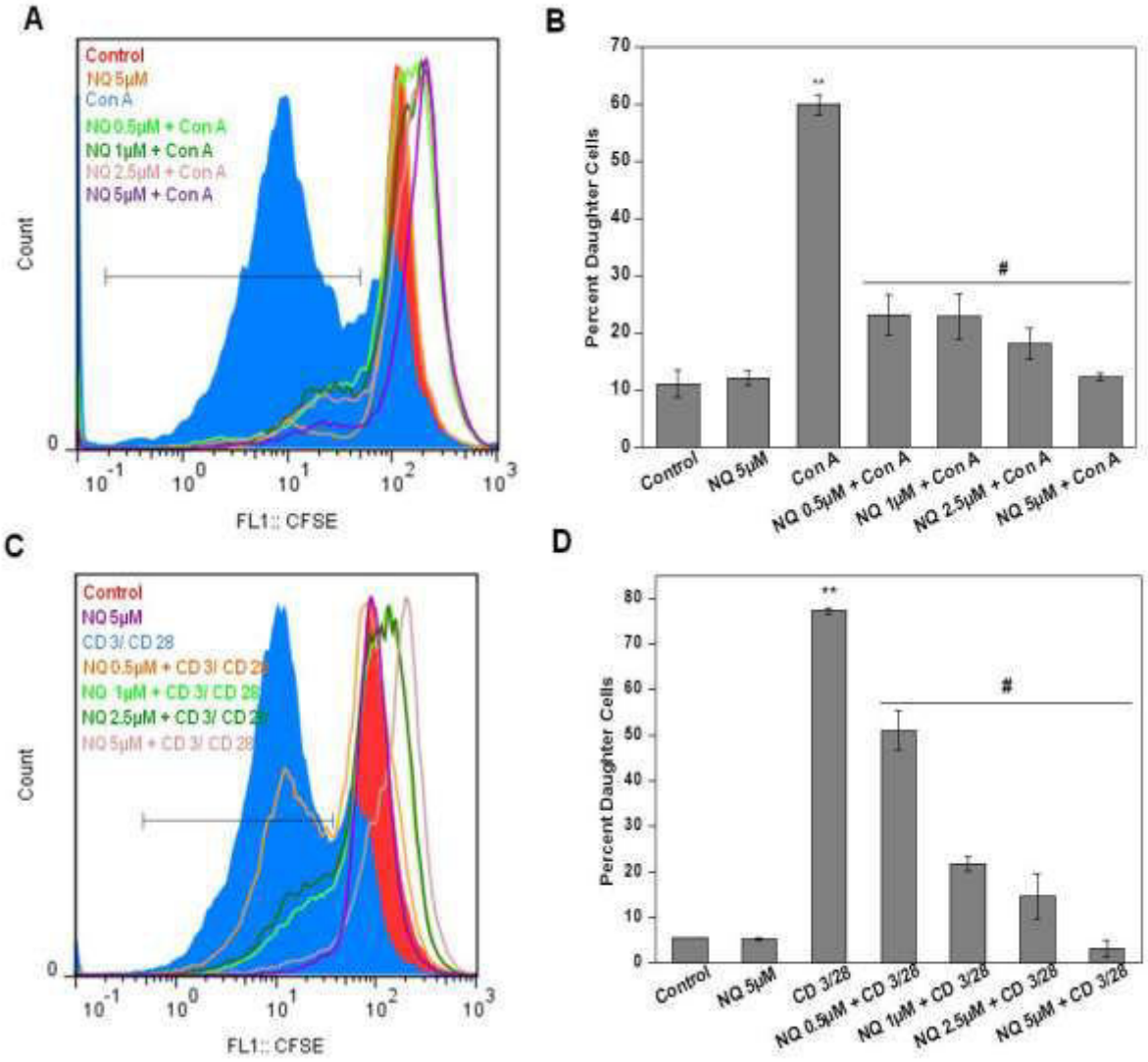


Fig. 4.4 NQ inhibited Con A or anti-CD3/CD28 antibody induced T cell proliferation: (A) CFSE labeled lymphocytes were treated with NQ (0.5- 5µM) for 2h and then stimulated with Con A at 37°C for 72h. Thirty thousand cells in each group were acquired in a flow cytometer. Vehicle treated cells served as control. Percent daughter cells were calculated from decrease in CFSE fluorescence as shown in overlaid flow cytometric histograms. (B) Graph represents mean±S.E.M from three replicates in each treatment group. (C) CFSE labeled lymphocytes were treated with NQ (0.5-5µM) for 2h and then stimulated with plate bound anti-CD3 mAb (1µg/ml) and soluble anti-CD28 mAb (1µg/ml) at 37°C for 72h. Thirty thousand cells in each group were acquired in a flow cytometer. (D) Graph represents mean±S.E.M from three replicates in each treatment group.

4.1.5 Pro-oxidants abrogated mitogen induced lymphocyte proliferation in vitro:

CFSE stained lymphocytes were pre-treated with H_2O_2 (50µM, 10min exposure followed by washing with RPMI media) or t-BHQ (20µM, 2h) or NQ (5µM, 2h) prior to stimulation with Con A. Effect of these pro-oxidants on mitogen induced increase in percent daughter cells was assessed by CFSE dye dilution using a flow cytometer. Mitogen stimulated group showed a significant increase in percentage of daughter cells. Pre-treatment of cells with H_2O_2 or t-BHQ or NQ significantly inhibited Con A induced proliferation (Fig. 4.5A and B).

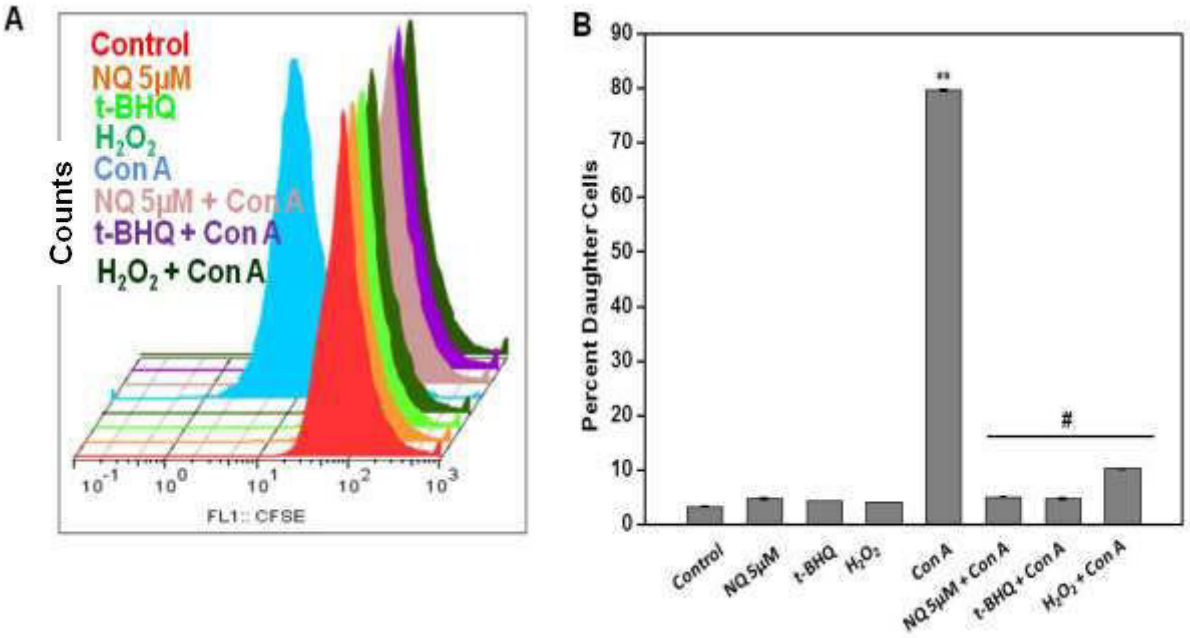
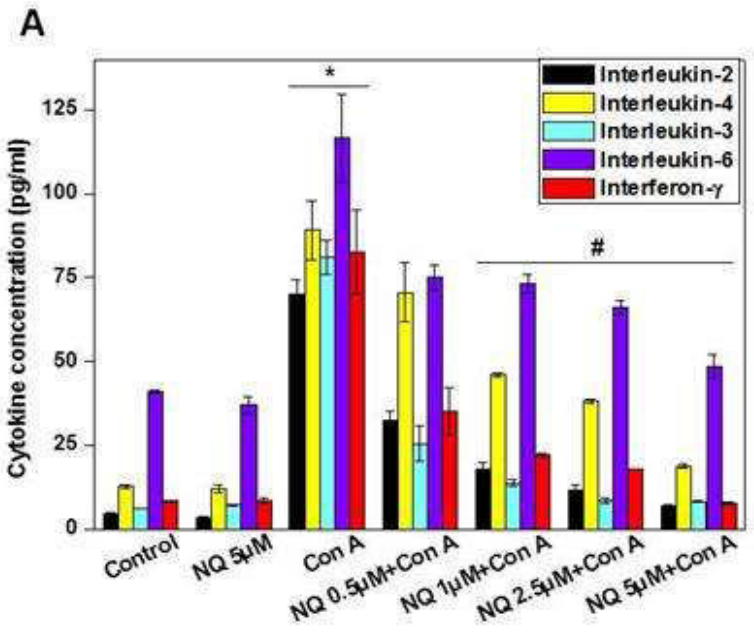


Fig. 4.5 Pro-oxidants abrogated mitogen induced lymphocyte proliferation in vitro: (A) CFSE labeled lymphocytes were treated with NQ (5µM, 2h) or t-BHQ (20µM, 2h) or H_2O_2 (50µM, 10min) and then stimulated with Con A at 37°C for 72h. Cells were treated with H_2O_2 for 10min and then washed before adding Con A. Thirty thousand cells in each group were acquired in a flow cytometer. Vehicle treated cells served as control. (B) Percent daughter cells were calculated from decrease in mean fluorescence intensity as shown in overlaid flow cytometric histograms and each bar represents mean±S.E.M from three replicates in each treatment group. Two such independent experiments were carried out. **p<0.01 as compared to vehicle treated cells, #p<0.01 as compared to Con A stimulated cells.

4.1.6 NQ inhibited Con A or anti-CD3/CD28 antibody induced cytokine secretion by lymphocytes in vitro:

Fig. 4.6 shows the concentration of cytokines in the supernatants from NQ (0.5µM to 5µM) treated cells stimulated with Con A (5µg/ml) or anti-CD3/CD28 mAb (1µg/ml) as compared to that in the control cells stimulated with Con A or anti-CD3/CD28. Con A or anti-CD3/CD28 activated cells showed significantly higher secretion of IL-2, IL-3, IL- 4, IL-6 and IFN-γ as compared to that in unstimulated cells (Fig. 4.6A and B). NQ significantly suppressed mitogen induced increase in cytokine secretion with complete suppression of IL-2, IL-3, IL-4, IL-6 and IFN-γ production observed at 5µM NQ (Fig. 4.6A and B).



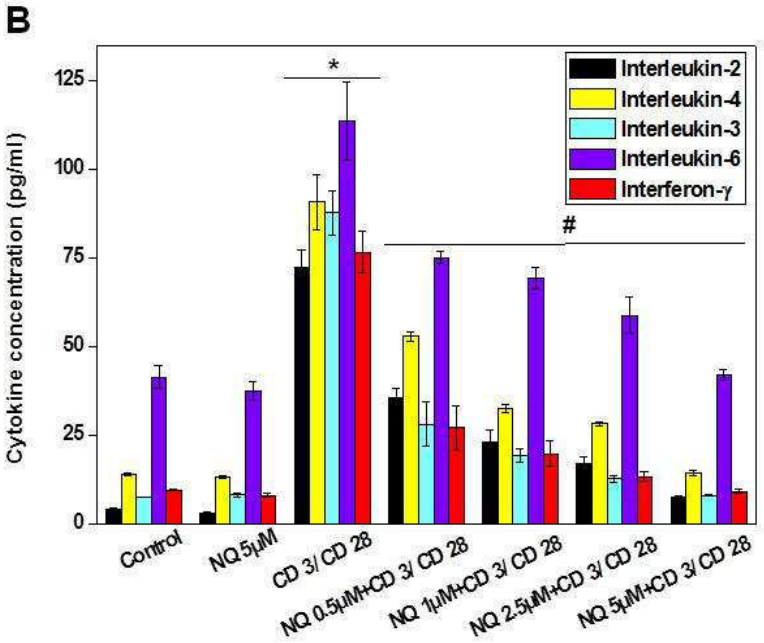


Fig. 4.6 NQ inhibited Con A and anti-CD3/CD28 antibody induced cytokine production by lymphocytes: (A-B) Lymphocytes were treated with NQ (0.5- 5μM) for 2h and then stimulated with either Con A (5μg/ml) (A) or anti-CD3/CD28mAb (B) at 37°C for 24h. Supernatants were harvested and cytokine concentration was estimated by ELISA. Graphs represent mean±S.E.M of IL-2, IL-3, IL-4, IL-6 and IFN-γ cytokines. **$p<0.01$, as compared to vehicle treated cells and $^{\#}p<0.01$, as compared to Con A or anti-CD3/CD28 stimulated cells.

4.1.7 NQ did not induce cell death in lymphocytes:

To estimate NQ induced cell death in naïve splenocytes, they were incubated with NQ (1-5μM) for 24h. These cells were harvested, washed and stained with Live and Dead fixable far red fluorescent dye and acquired using a flow cytometer. Lymphocytes treated with NQ did not show any increase in cell death at 24h as compared to vehicle treated control cells (Figure 4.7).

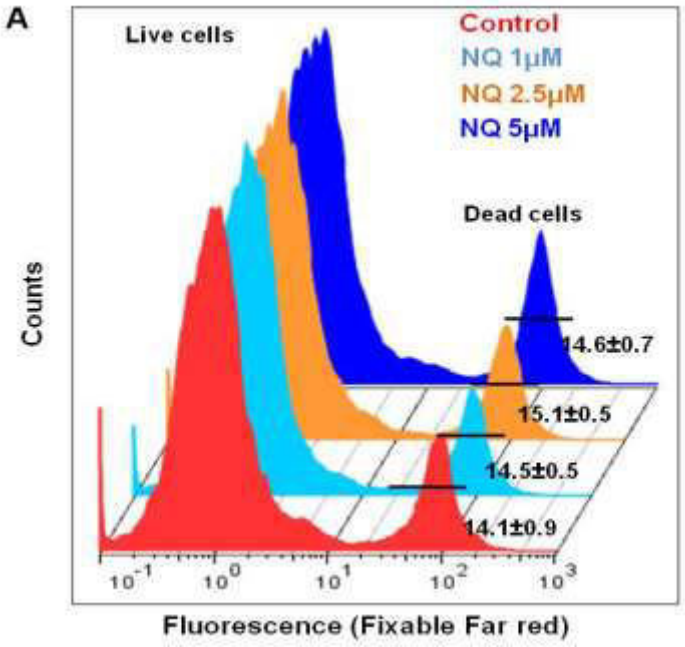
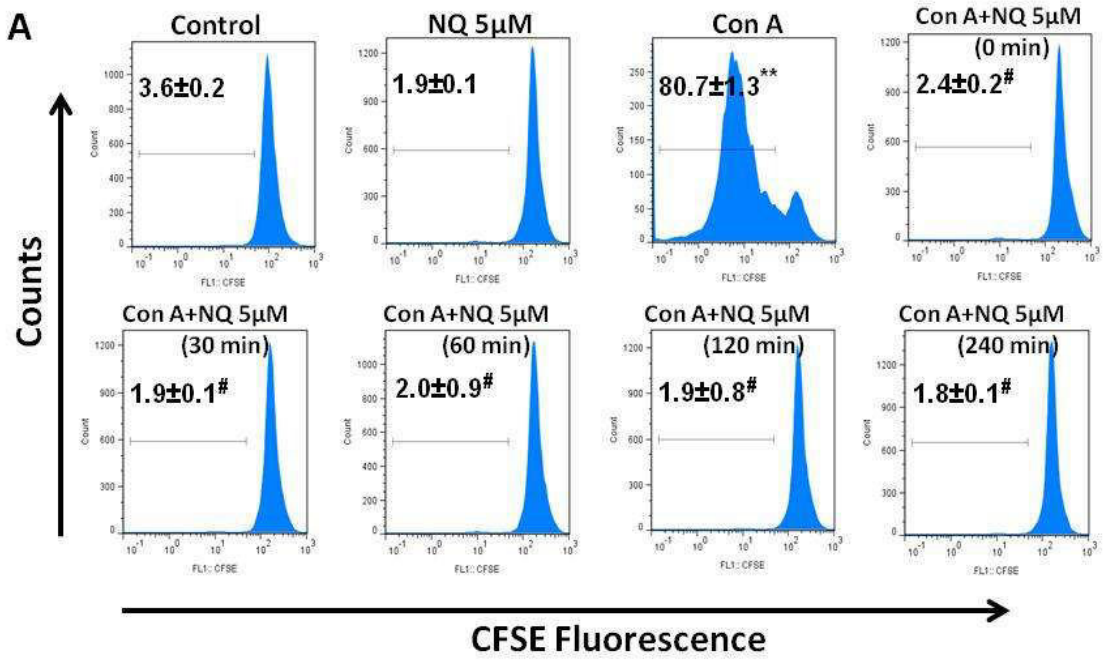


Fig. 4.7 NQ did not induce cell death in lymphocytes: Lymphocytes were treated with NQ (1-5µM) for 24h and stained with Live/Dead assay kit as described in Materials and Methods section. Thirty thousand cells in each group were acquired in a flow cytometer. Vehicle treated cells served as control. Percent cell death was calculated from increase in fluorescence.

4.1.8 NQ inhibited the proliferation and cytokine secretion in activated lymphocytes:

Experiments were carried out using activated lymphocytes to determine the therapeutic potential of NQ as an immunomodulatory agent. Lymphocytes were stimulated with Con A and then treated with 5µM of NQ at different time points. NQ completely inhibited Con A induced proliferation and cytokines secretion (IL-2, IL-4, IL-6 and IFN-γ) even when it was added up to 4h after mitogen (Fig. 4.8A and B).



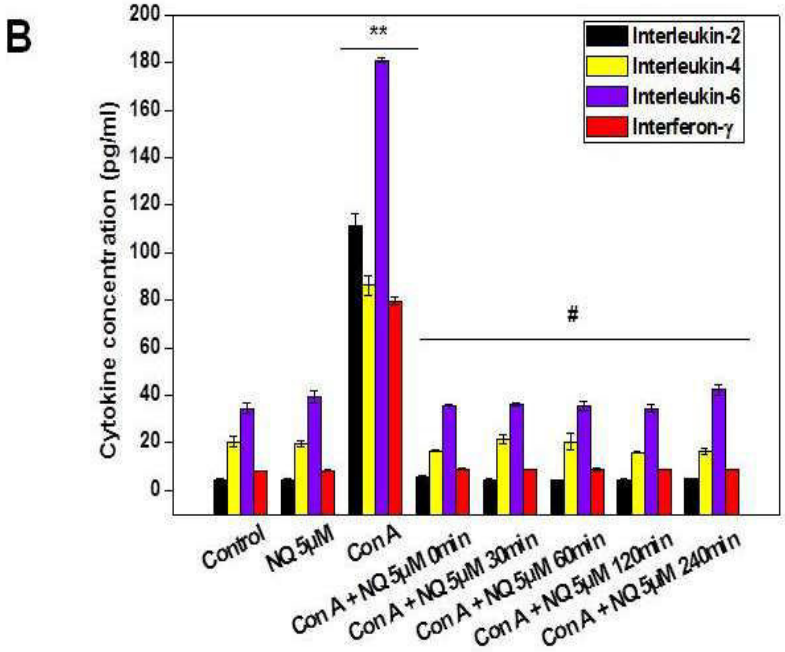


Fig. 4.8 NQ inhibited the proliferation and cytokine secretion in activated lymphocytes: (A) CFSE labeled lymphocytes were stimulated with Con A (5µg/ml) and then treated with NQ (5µM) at indicated time intervals and cultured at 37°C for 72h. Thirty thousand cells in each group were acquired in a flow cytometer. Vehicle treated cells served as control. Percentage daughter cells were calculated from CFSE dye dilution as shown in representative histograms. (B) Cytokine concentration was measured in supernatants 24h after treatment with Con A by ELISA. Each bar represents mean±S.E.M. from three replicates and two such independent experiments were carried out.**$p<0.01$, as compared to vehicle treated cells and $^{\#}p<0.01$, as compared to Con A stimulated cells.

4.1.9 NQ inhibited LPS induced nitric oxide release and production of TNF-α, IL-6 and IL-1β by RAW cells:

Macrophages play a pivotal role in both innate immunity as well as in adaptive immunity. Their functions include clearance of invading pathogen and cellular debris by phagocytosis, alert the neighbouring cells and also to stimulate lymphocytes to respond to pathogens. A mouse macrophage cell line (RAW264.7 cells) was used to assess the effect of NQ on activated macrophages. RAW 264.7 cells were stimulated with LPS in the presence or absence of NQ and cultured for 6h or 24h. Pre-treatment of cells with NQ significantly inhibited LPS induced secretion of IL-1β, IL-6 and TNF-α (Fig. 4.9A and B). Further, NQ significantly inhibited LPS induced nitric oxide release and Cox-2 levels in RAW 264.7 cells (Fig. 4.9C and D).

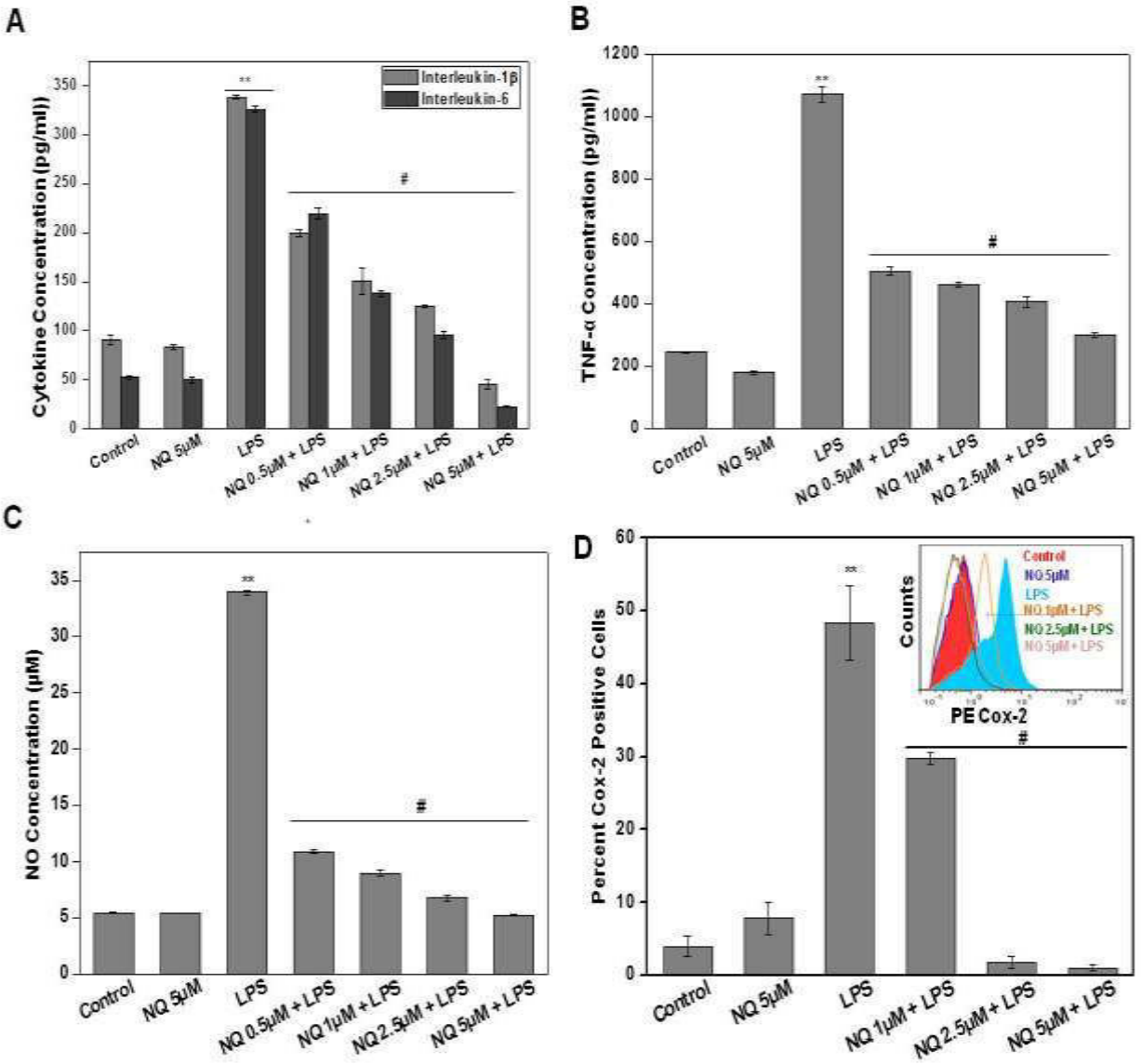
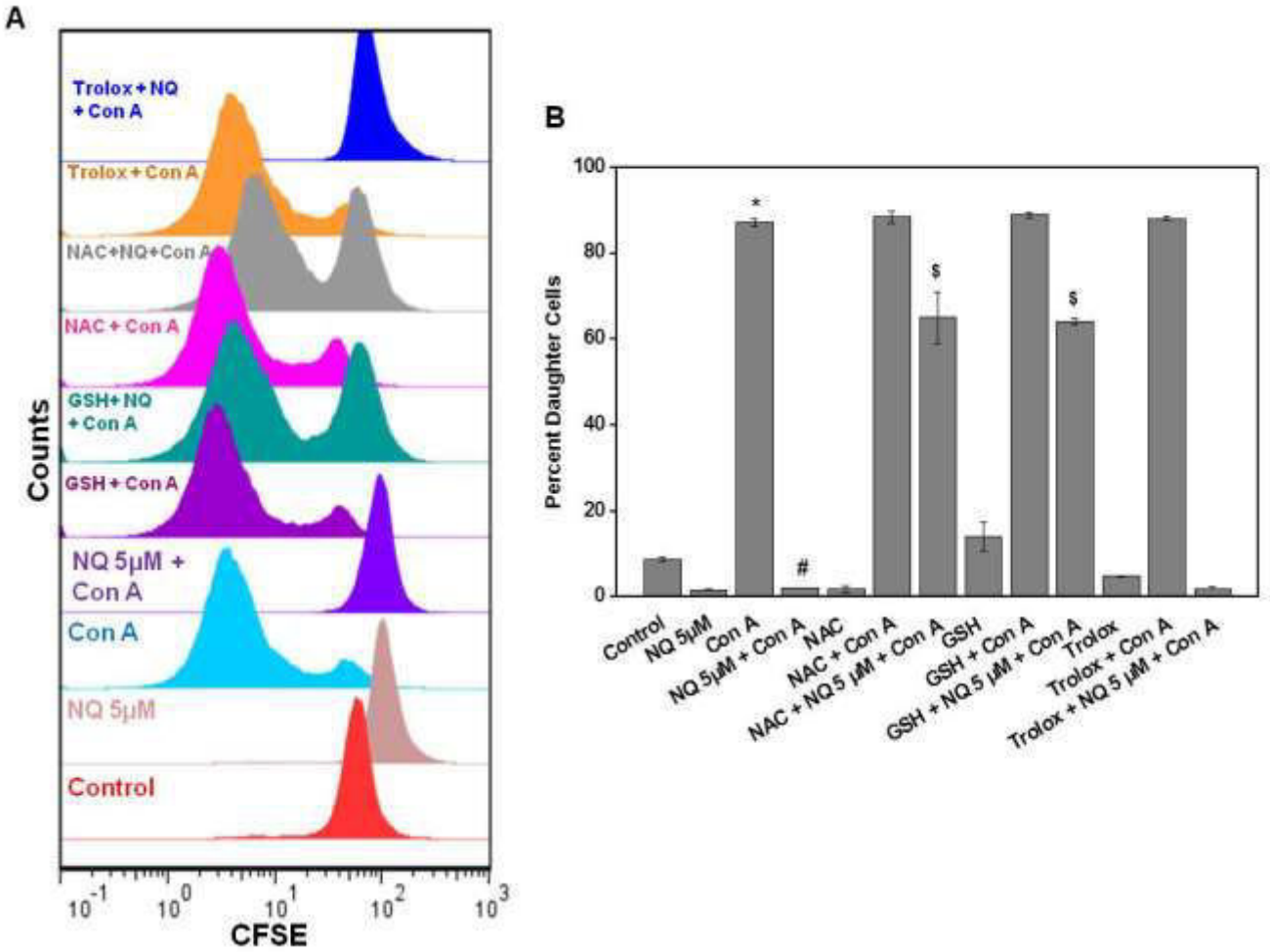


Fig. 4.9 NQ inhibited LPS induced nitric oxide release and production of TNF-α, IL-6 and IL-1β by RAW cells: RAW 264.7 cells were treated with NQ (0.5-5μM) prior to stimulation with LPS (1μg/ml) and cultured for 6h or 24h. (A) Cytokine (IL-1β and IL-6) concentration was measured in supernatant after 24h and (B) TNF-α concentration was measured after 6h of LPS treatment by ELISA. (C) Nitrite concentration was measured in culture supernatants collected 6h after stimulation with LPS using Griess reagent. (D) RAW 264.7 cells were treated with NQ (1-5μM) prior to stimulation with LPS (1μg/ml) and cultured for 24h. Cells were harvested, fixed, permeabilized and stained with PE-labeled anti-Cox-2 antibody and were acquired on a flow cytometer. Graph represents the percent Cox-2 positive cells and representative flow cytometric histograms are shown in the insert. Each bar represents mean±S.E.M. from three replicates and two such independent experiments were carried out. **$p<0.01$, as compared to vehicle treated cells and $^{\#}p<0.01$, as compared to LPS stimulated cells.

4.1.10 Anti-inflammatory action of NQ was abrogated by thiol antioxidants:

Fig. 4.10 shows the effect of different antioxidants on anti-inflammatory action of NQ. To confirm the role of cellular redox in the observed anti-inflammatory activity of NQ, different thiol antioxidants (NAC, GSH) and non-thiol antioxidant (Trolox) were used. Both thiol and non thiol antioxidants per se did not show any effect on proliferation in Con A stimulated cells. Thiol antioxidants, NAC and GSH, were able to abrogate the NQ mediated suppression of mitogen induced proliferation and cytokine secretion (Fig: 4.10 A-E), whereas, non thiol antioxidant failed to inhibit the activity of NQ.



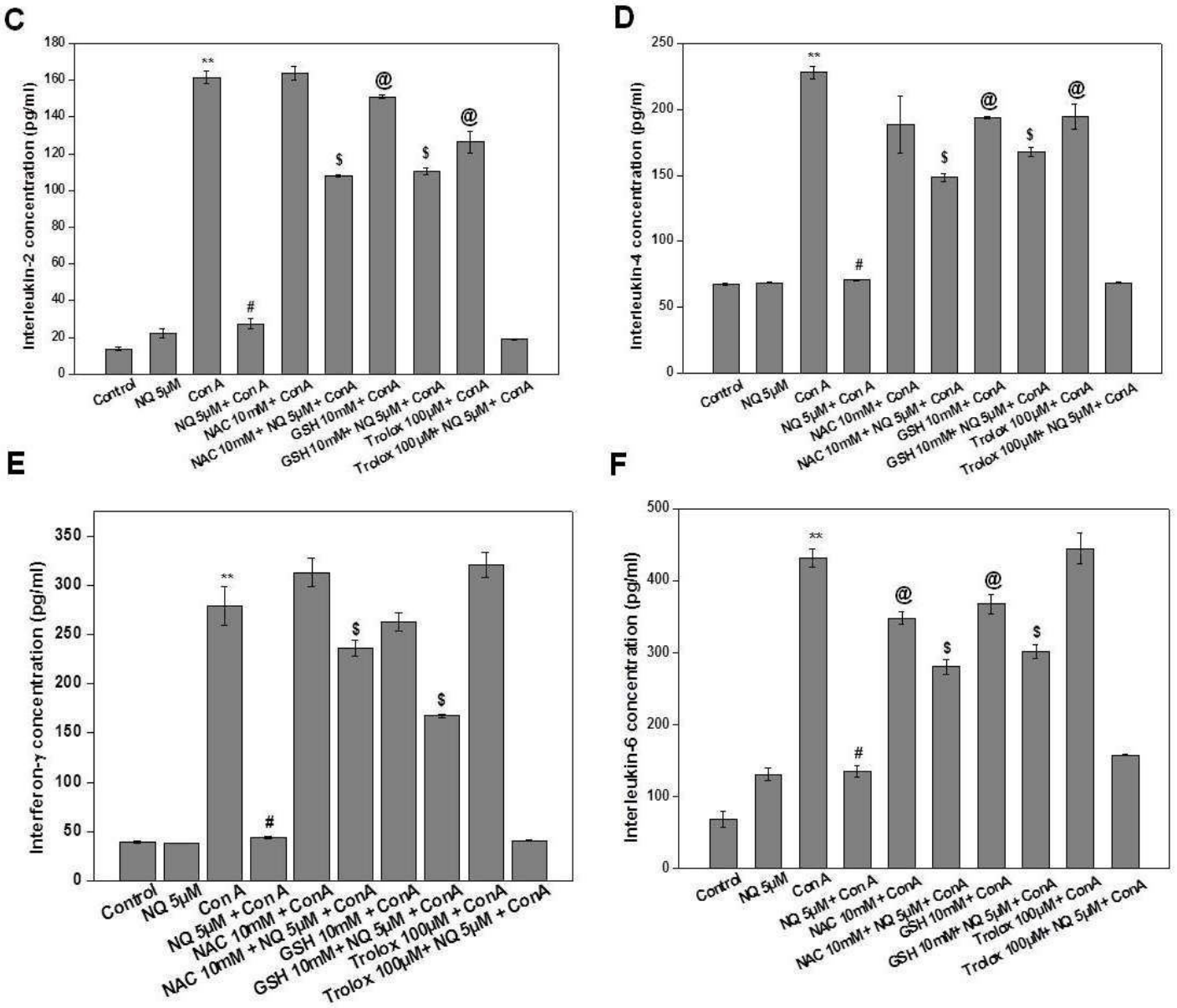


Fig. 4.10 Anti-inflammatory action of NQ was abrogated by thiol antioxidants: CFSE labeled lymphocytes were treated with NAC (10mM) or GSH (10mM) or Trolox (100μM) for 2h followed by treatment with NQ (5μM) for 2h and then stimulated with the Con A at 37°C for 72h. Thirty thousand cells in each group were acquired in a flow cytometer. (A) Representative flow cytometric histograms showing daughter cells. (B) Graph represents the percent daughter cells each treatment group. (C-F) Supernatants were harvested 24h after stimulation and cytokine (IL-2, IL-4, IL-6 and IFN-γ) concentrations were estimated by ELISA. Each bar represents mean ± S.E.M. from three replicates and two such independent experiments were carried out.**$p<0.01$, as compared to vehicle treated cells, $^{\#}p<0.01$, as compared to Con A stimulated group, $^{\$}p<0.01$, as compared to NQ treated and Con A stimulated group and $^{@}p<0.05$ as compared to Con A stimulated cells.

4.1.11 NQ interacted with thiol antioxidants:

Since anti-inflammatory effects of NQ were sensitive to presence of thiol antioxidants, experiments were carried out to determine whether NQ can physically interact with thiol groups. Interaction of NQ with NAC or GSH was studied using absorption spectroscopy. Significant changes in absorption spectra of NQ were observed in presence of both the thiol antioxidants as compared to NQ alone indicating a possible interaction of NQ with NAC and GSH (Fig. 4.11).

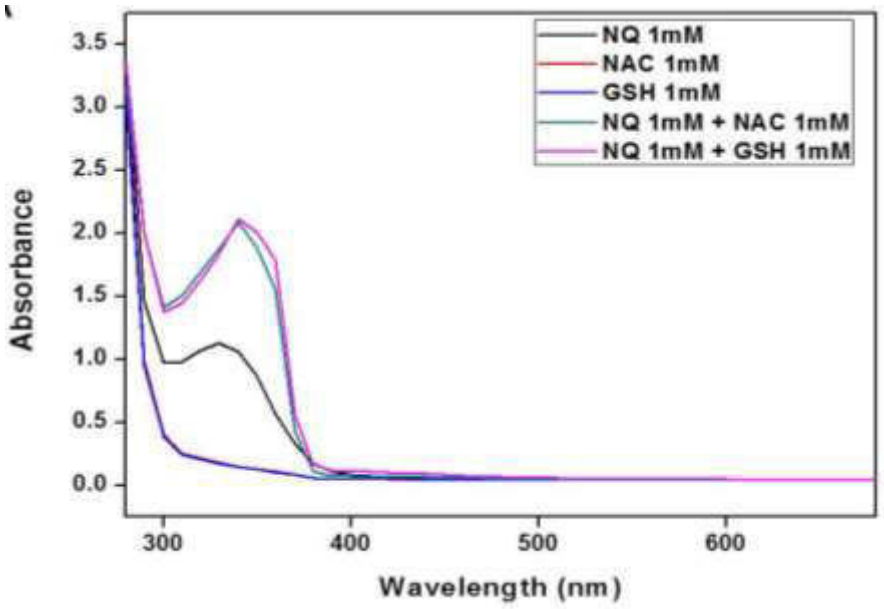
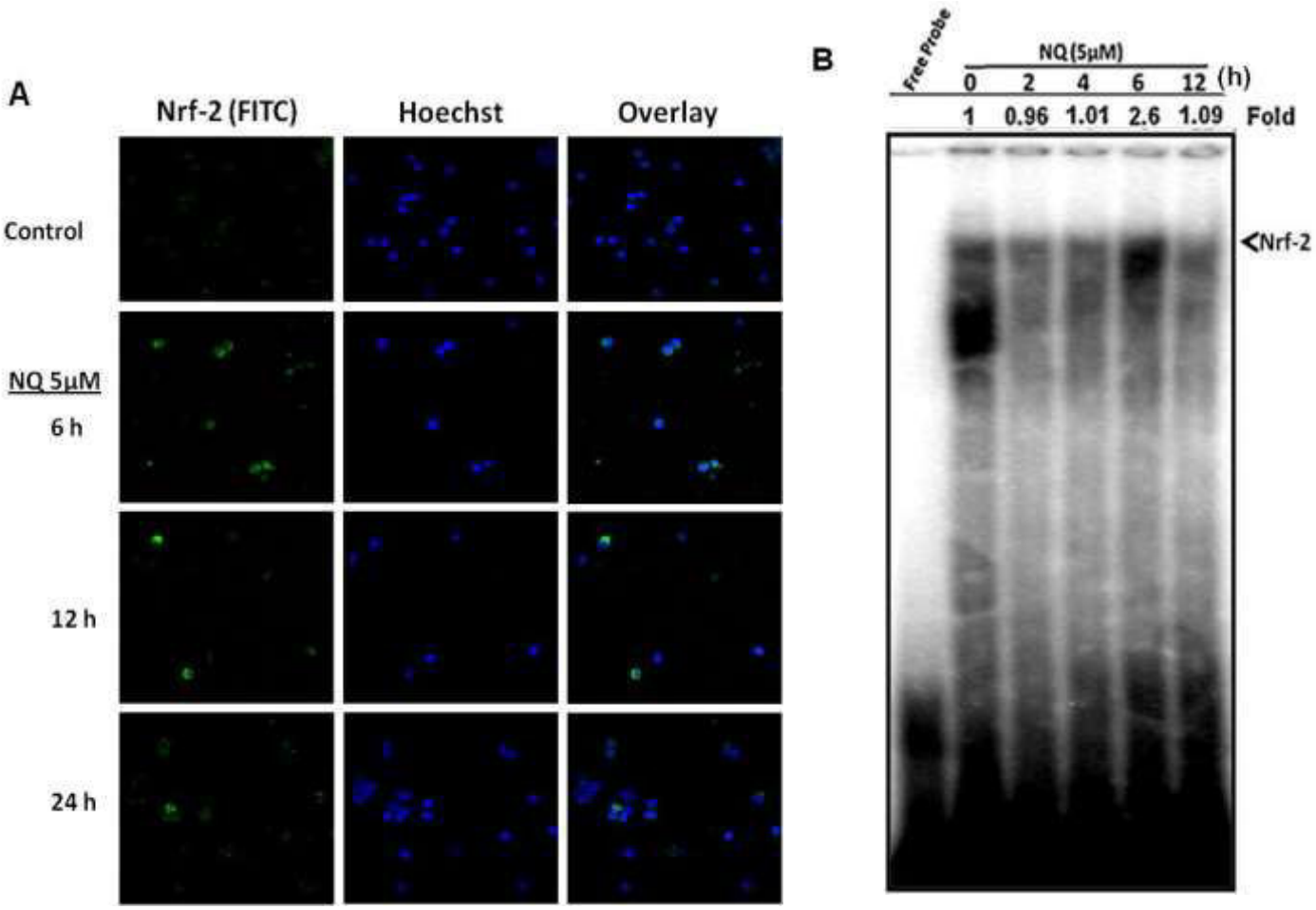


Fig. 4.11 NQ showed direct interaction with thiol antioxidants: The changes in the absorption spectra of NQ in presence of NAC or GSH were monitored by mixing the respective concentrations of NQ (1mM) with NAC (1mM) or GSH (1mM) for 1h.

4.1.12 NQ induced activation of Nrf2 in lymphocytes:

Modulation of cellular redox status is known to affect the redox sensitive transcription factors and other stress related regulatory proteins including Nrf2 [34]. Thus experiments were performed to investigate the effect of NQ on activation of immunoregulatory transcription factor Nrf2. NQ treatment significantly increased the nuclear levels of Nrf2 as seen by confocal microscopy and EMSA (Fig 4.12A and B). Significant accumulation of Nrf2 in the nucleus was seen 6h after addition of NQ to lymphocytes. Further, NQ treatment also led to a significant increase in mRNA copy number of Nrf2 and its dependent genes HO-1 and GCLC (Fig. 4.12C).



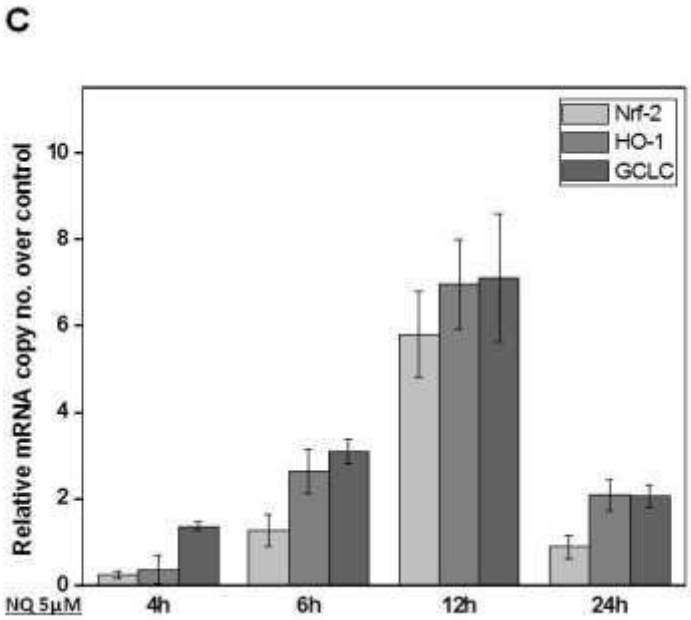
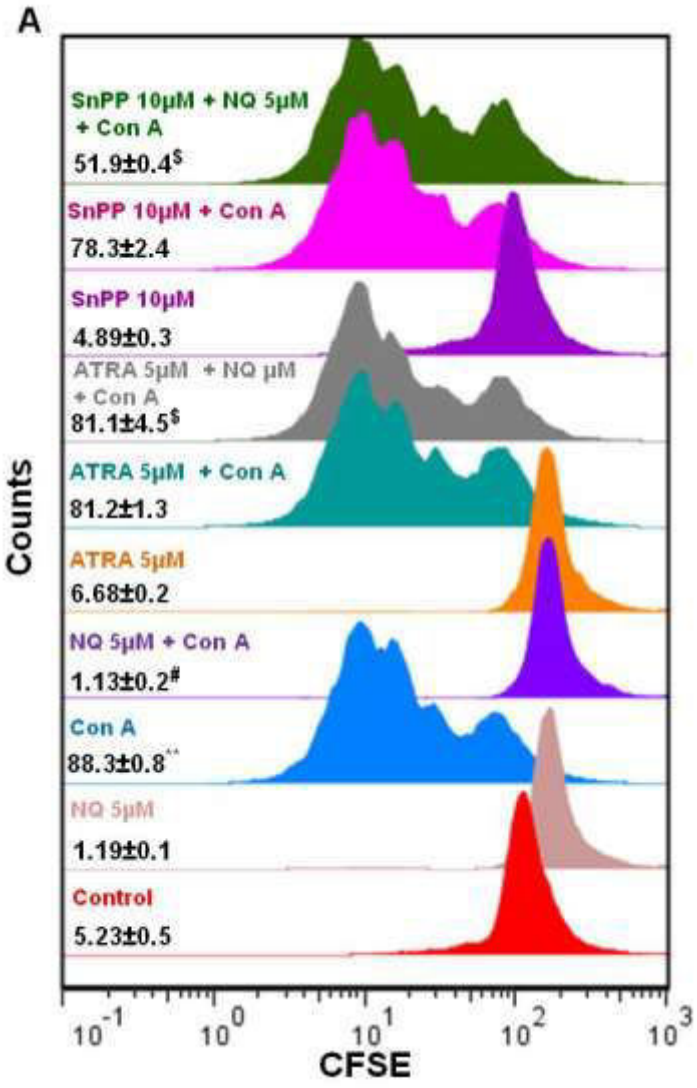


Fig. 4.12 NQ induced activation of Nrf2 in lymphocytes: (A) Lymphocytes were treated with NQ (5µM) for 6 or 12 or 24h, stained with FITC labelled anti-Nrf2 antibody and Hoechst33342. FITC (left) / Hoechst (mid) and overlay (right) is shown. (B) Lymphocytes were treated with NQ (5µM) for 2-12h and EMSA was performed using nuclear extracts and specific signal intensities were subsequently quantified by Syngene Gene Tools software (Cambridge, UK) (C) Lymphocytes were treated with NQ for 4-24h, mRNA was isolated and used for real time RT-PCR. Bar diagram shows relative mRNA copy number of Nrf2, HO-1 and GCLC over control. Each bar represents mean±S.E.M. from three replicates and two such independent experiments were carried out.

4.1.13 Anti-inflammatory and anti-proliferative effects of NQ were reverted by inhibition of Nrf2/HO-1:

To confirm the role of Nrf2 and its dependent genes in the observed anti-inflammatory effects of NQ, experiments were performed using pharmacological inhibitors of Nrf2/HO-1 pathway. CFSE labelled lymphocytes were treated with 5µM ATRA (Nrf2 inhibitor) or 10µM SnPP (HO-1 inhibitor) for 2h. These cells were then incubated with 5µM of NQ for 2h prior to stimulation with Con A and were further monitored for cell proliferation and cytokine secretion. Pre-treatment with ATRA or SnPP significantly abrogated NQ mediated suppression of Con A induced proliferation (Fig. 4.13A) and cytokine secretion (Fig. 4.13 B-E).



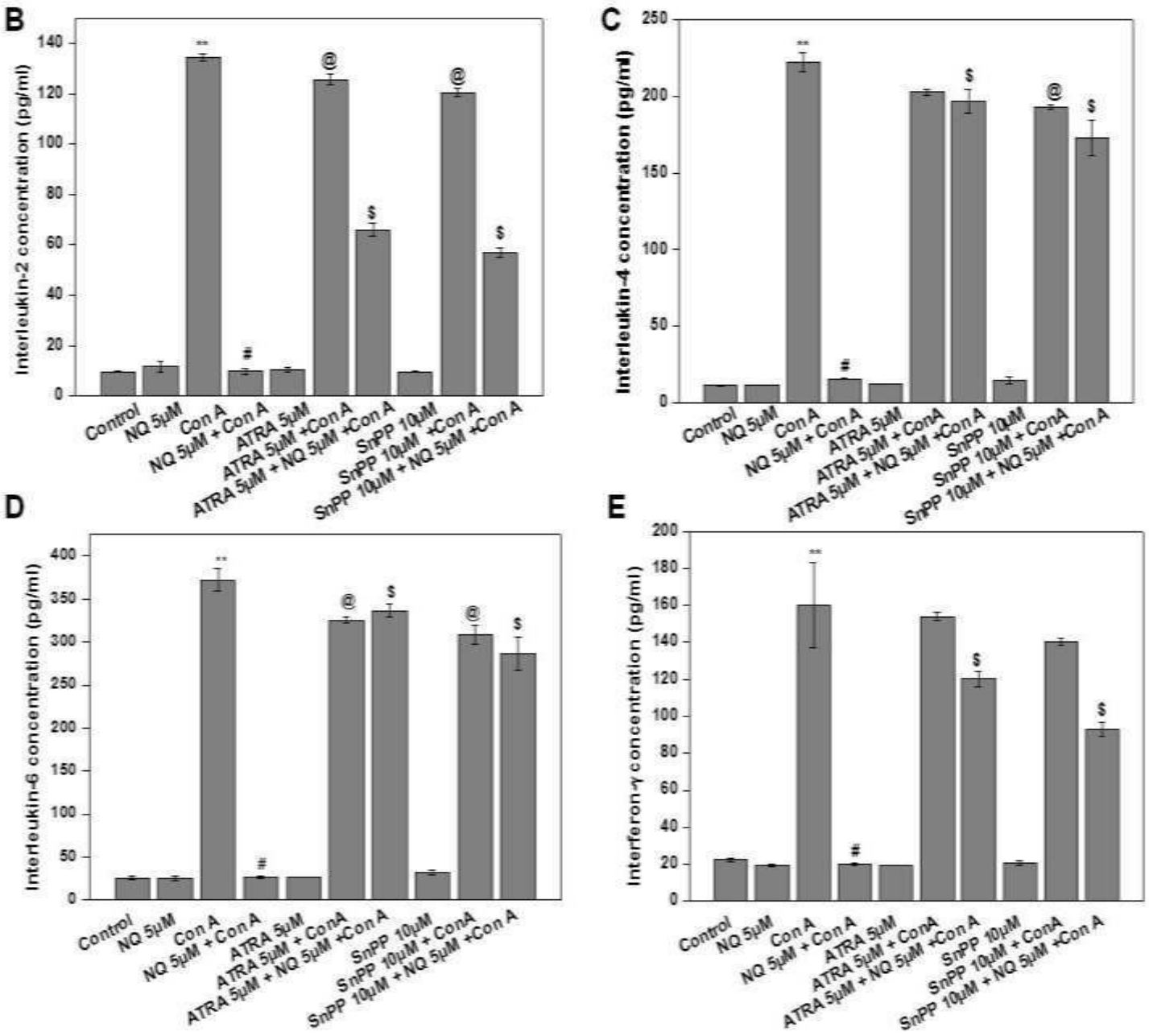


Fig. 4.13 Anti-inflammatory and anti-proliferative effects of NQ were reverted by inhibition of Nrf2/HO-1: (A) CFSE labeled lymphocytes were treated with ATRA (5μM) or SnPP (10μM) for 2h prior to incubation with NQ (5μM, 2h) and stimulated with Con A for 72h at 37^0C. Thirty thousand cells in each group were acquired in a flow cytometer. Representative flow cytometric histograms and mean±SEM percent daughter cells in each group are shown. (B-E) Cytokine levels were measured in supernatant 24h after respective treatments by ELISA. Each bar represents mean±S.E.M. from three replicates. Two such independent experiments were carried out. **p<0.01, as compared to vehicle treated cells, #p<0.01, as compared to Con A stimulated cells, $p<0.01, as compared to NQ treated and Con A stimulated cells and @p<0.05 as compared to Con A stimulated cells.

4.1.14 NQ induced glutathionylation of KEAP-1 in lymphocytes:

KEAP-1 contains multiple critical redox sensitive cysteine residues. Changes in redox status have been shown to induce post translational modifications of KEAP-1 thereby activating Nrf2 and phase 2 detoxification enzymes (*189, 335*). To gain an insight on NQ mediated induction of Nrf2, experiments were performed to investigate the effect of NQ treatment on cellular glutathionylation of KEAP-1 protein. Immuno-precipitation using anti-GSH antibody followed by Western blot analysis with anti-KEAP-1 antibody revealed that NQ treatment to lymphocytes induced glutathionylation of KEAP-1protein (Fig. 4.14A).

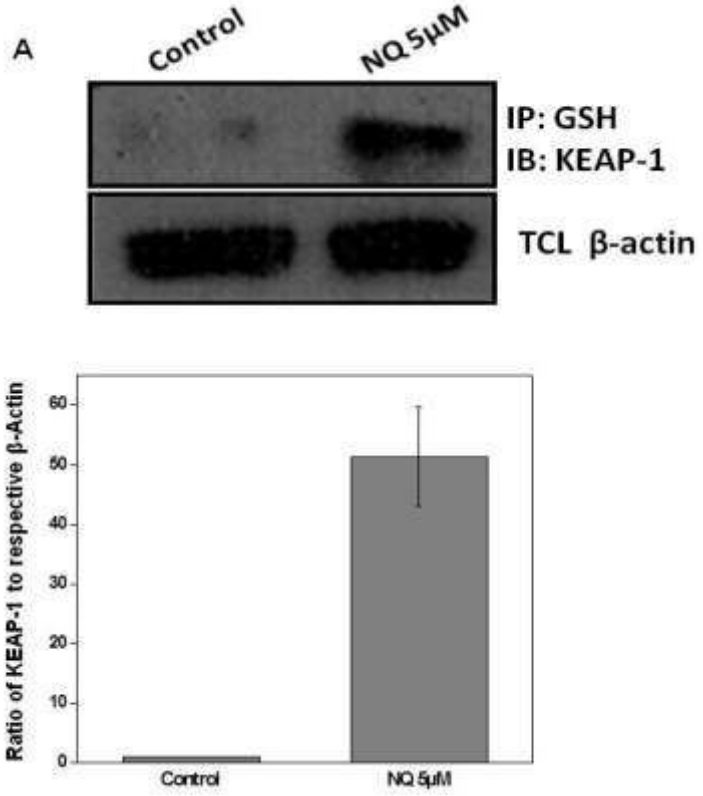
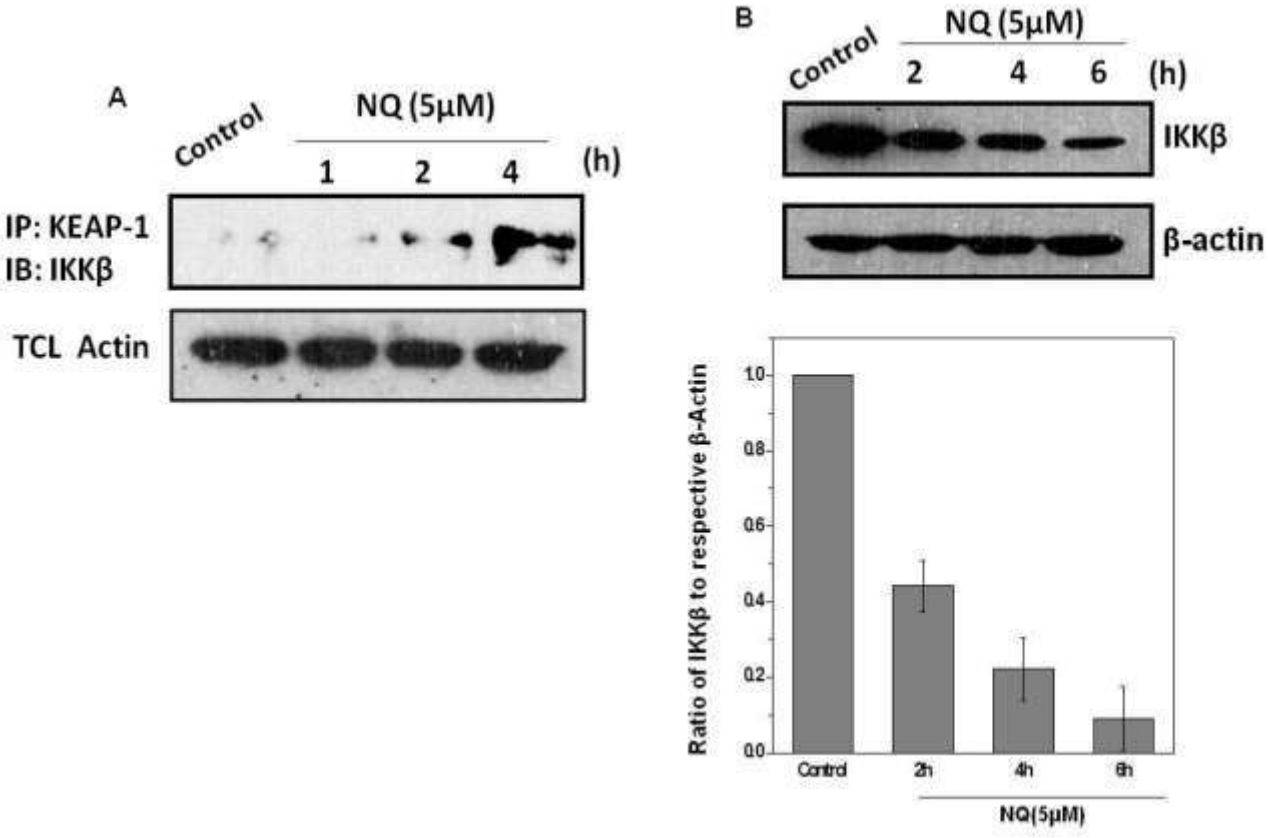


Fig. 4.14 NQ induced glutathionylation of KEAP-1 in lymphocytes: (A) Lymphocytes were treated with NQ (5µM) for 4h. Glutathionylated proteins were immuno-precipitated from whole cell lysates using anti-GSH antibody followed by immunoblotting with anti-KEAP-1 antibody. Three such independent experiments were carried out and specific signal intensities were subsequently quantified by Syngene Gene Tools software (Cambridge, UK).

4.1.15 NQ treatment induced KEAP-1 mediated degradation of IKKβ and abrogated mitogen induced activation of NF-κB pathway:

There are several studies in literature have highlighted the role of cross talk between Nrf2 and NF-κB. Since up-regulation of Nrf2 is known to down-regulate NF-κB activation pathway via IKKβ degradation, experiments were performed to investigate the effect of NQ on mitogen induced activation of NF-κB pathway. Immuno-precipitation studies revealed that NQ treatment induced binding of KEAP-1 to IKKβ (Fig. 4.15A). Further, treatment of lymphocytes with NQ resulted in a time dependent decrease in the levels of IKKβ (Fig. 4.15B) indicating the involvement of KEAP-1 mediated degradation. Pre-treatment of cells with NQ for 2h abrogated Con A induced NF-κB nuclear translocation and IκBα degradation (Fig. 4.15C and E). Anti-p65 antibody shifted the band to a higher molecular weight (Fig. 4.15D) confirming the specificity of NF-κB.



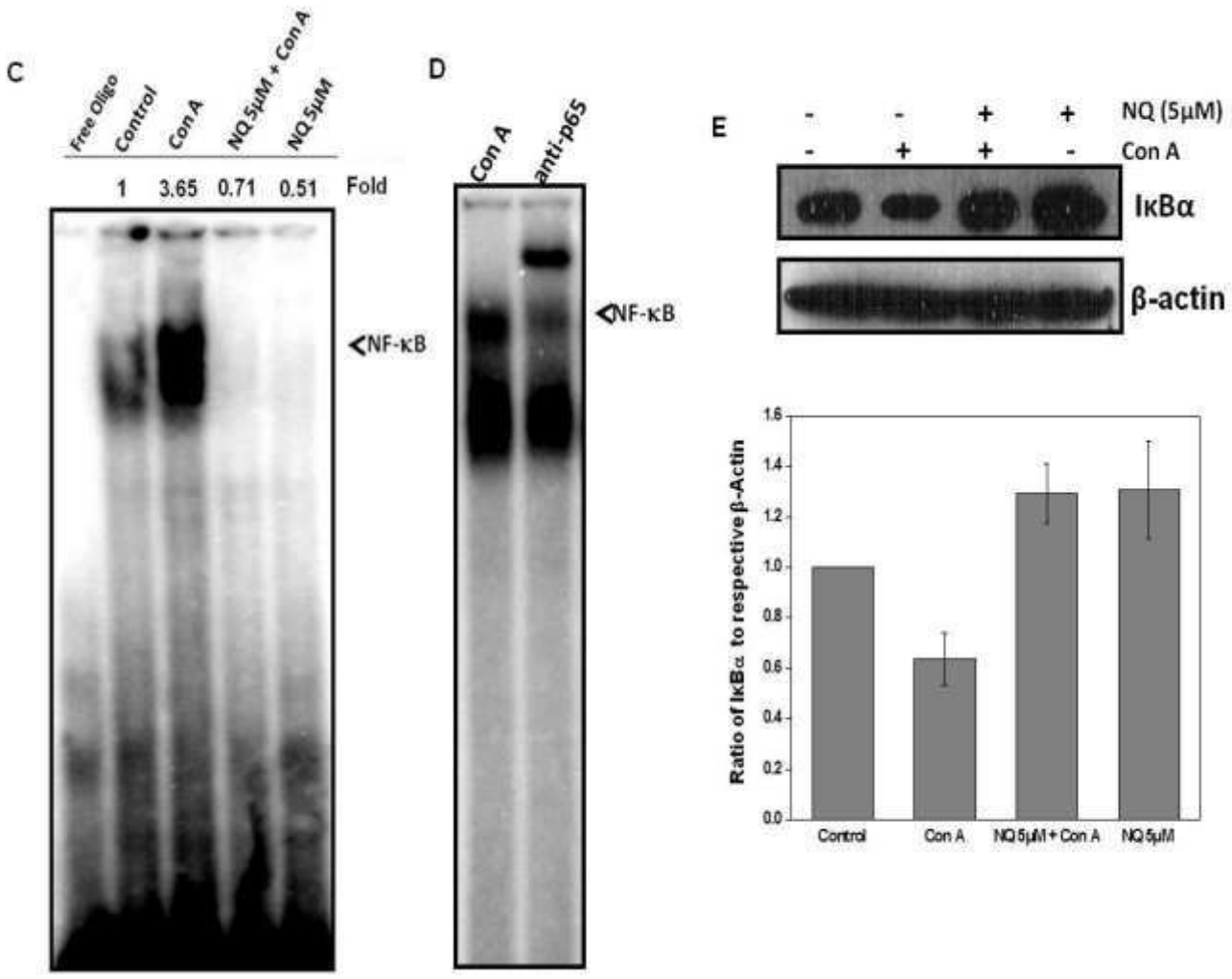


Fig. 4.15 NQ treatment induced KEAP-1 mediated degradation of IKKβ and abrogated mitogen induced activation of NF-κB pathway: (A) Lymphocytes were treated with NQ (5μM,) for indicated time points. Whole cell fractions were immuno-precipitated with anti-KEAP-1 antibody followed by immunoblotting with IKKβ. (B) Lymphocytes were incubated NQ (5μM) for 2, 4 and 6h followed by Western blot analysis with cytosolic extract using antibodies against IKKβ and β-actin. (C) Lymphocytes were treated with NQ (5μM, 2h) and stimulated with Con A (5μg/ml) for 4h. EMSA was performed using nuclear extracts to assay for NF-κB nuclear translocation. (D) Nuclear extracts prepared from Con A treated lymphocytes were incubated for 15min with anti-p65 antibody and used for super shift assay. (E) Lymphocytes were incubated with NQ (5μM, 2h) and stimulated with Con A for 4h. Western blot analysis was performed with cytosolic extracts using antibodies specific for IκBα and β-actin. Specific signal intensities were quantified by Syngene Gene Tools software (Cambridge, UK). Three such independent experiments were carried out.

4.2 DISCUSSION:

Under conditions of IR exposure, elevated production of ROS, apoptosis and increased levels of several pro-inflammatory cytokines by recruited immune cells at the site of injury underlines the initiation of radiation induced inflammation. Several studies have reiterated the role of inflammatory reaction and endogenous cytokine production in the pathogenesis of radiation-induced damage to normal tissue (*336-338*). Pro-inflammatory cytokines like IL-1, IL-6, TNF-α and IL-8 released by phagocytes during an inflammatory response promote cellular infiltration and damage to tissue (*339, 340*). In this study it was proposed that developing a radioprotective agent with anti-inflammatory action may be a promising strategy for ameliorating radiation injury.

T cell activation plays a central role in the regulation of immune responses, and hence its pharmacologic inhibition has provided a powerful tool in developing anti-inflammatory agents. Pharmacologic interventions to suppress the subsequent clonal expansion and acquisition of T cell effector functions by inhibiting the secretion of cytokines (e.g. IL-2 or IL-4) have proven to be successful to achieve immunosuppression (*341, 342*). Any perturbation in the intracellular or extracellular redox state can disturb the normal course of immune response (*343*) (*344, 345*). Modulation of immune responses by altering the cellular redox is an upcoming thrust area for identification and development of novel anti-inflammatory and immune-suppressive drugs.

Based on these observations, it was hypothesised that NQ may show anti-inflammatory activity by perturbing cellular redox and activating Nrf2. Interestingly, it was observed that lymphocytes isolated from GALT and spleen of NQ treated mice showed reduced responsiveness for mitogen induced cytokine secretion (Fig 4.1). Further, studies were done using lymphocytes isolated from spleen as model system. It was observed that NQ treated mice showed hypo-responsiveness for mitogen induced proliferation as compared to

control mice (Fig 4.2). Further, the role of nuclear factor-kappa B (NF-κB) in regulating the immune response has been well documented in literature (*250, 346, 347*). In particular, NF-κB family members control the transcription of cytokines and antimicrobial effectors as well as genes that regulate cellular differentiation, survival and proliferation (*346, 348*). Hence, studies were carried to investigate the effect of NQ on mitogen induced NF-κB activation and we found that lymphocytes isolated from NQ administered mice showed lower nuclear levels of NF-κB (Fig 4.3). Further studies were carried out to demonstrate the anti-inflammatory action of NQ and decipher its underlying mechanism. It was observed that NQ inhibited Con A and anti-CD3/CD28 mAb induced proliferation by lymphocytes in a dose dependent manner (Fig 4.4). We also employed other known pro-oxidants like H_2O_2 and t-BHQ to study the role of increased oxidative stress in immunosuppression. A direct immuno-suppressive action of oxidative stress was further supported by the results showing that pro-oxidants like H_2O_2 and t-BHQ also inhibited mitogen induced proliferation of splenic lymphocytes (Fig 4.5).

Cytokines secreted by different cells participating in the immune response are known to play a critical role in the manifestation of successful pathogen clearance. Th1 cytokines (IL-2, TNF-α, and IFN-γ) activate macrophages and promote cell-mediated immune responses. Th2 cytokines (IL-4, IL-5, IL-6, IL-10, and IL-14) promote humoral immune responses against extracellular pathogens (*349*). IL-2 induces clonal expansion of activated T cells and progression of activated T cells from G1 to S/G2/M phase of the cell cycle (*350*). IFN-γ induces the upregulation of the genes involved in pathogen recognition, antigen processing and presentation required for pathogen clearance (*351, 352*). IL-4 is also an effector cytokine needed for the differentiation of naïve T helper cells into Th2 effector cells and plays a central role in the pathogenesis of allergic inflammation (*353*). NQ suppressed both Th1 and Th2 cytokines secreted by activated lymphocytes in response to Con A or

CD3/CD28 mAb in vitro (Fig 4.6). We found that NQ treatment did not lead to a significant increase in the percent dead cells in lymphocytes suggesting that the observed anti-inflammatory effects are not due to its cytotoxicity (Fig 4.7). Further, the therapeutic anti-inflammatory efficacy of NQ was also studied in vitro and it was observed that NQ was able to inhibit Con A induced proliferation and cytokine secretion in murine lymphocytes even when added up to 4h post-mitogenic stimulation (Fig 4.8) and hence can be used as potential therapeutic agent. The potent anti-inflammatory activity of NQ was also evident from its ability to suppress LPS induced cytokine secretion, nitric oxide release and Cox-2 expression in macrophages (RAW 264.7) (Fig 4.9). These results suggested that NQ act on cells involved in both adaptive and innate immune responses in exhibiting its anti-inflammatory activity.

Further, since NQ is a known electrophile and pro-oxidant, the role of cellular redox in the anti-inflammatory effects of NQ was investigated. NQ possess high affinity towards cellular nucleophiles like thiols and thereby it may perturb cellular redox status (*305*). To discern the contribution of cellular redox in the observed anti-inflammatory activity of NQ, thiol (NAC and GSH) and non-thiol (Trolox) anti-oxidants were employed. Abrogation of NQ mediated immunosuppression was only seen with thiol antioxidants confirming the crucial role of thiol depletion in the anti-inflammatory activity of NQ (Fig 4.10A-F). Further, absorption spectroscopy showed that NQ was interacting with NAC and GSH (Fig 4.11) suggesting that interaction of NQ with intracellular free thiols may be contributing to the observed biological activity. Since changes in cellular thiols and redox status were observed to play a pivotal role in NQ mediated anti-inflammatory action, experiments were conducted to elucidate its underlying mechanism. Since modulation of cellular redox status is known to induce the redox sensitive anti-inflammatory transcription factor Nrf2, effect of NQ treatment on Nrf2 activation was investigated in lymphocytes. NQ induced an increase in nuclear

translocation of Nrf2 and its binding to target DNA (Fig 4.12 A and B). NQ treatment also resulted in increased transcription of Nrf2 and its dependent genes, hemoxygenase-1 and glutamate cysteine ligase catalytic subunit (GCLC) (Fig 4.12C). Since NQ modulated the cellular redox levels and activated Nrf2 pathway, studies were undertaken to elucidate the role of Nrf2 in NQ mediated anti-inflammatory action. For this purpose, pharmacological inhibitors, ATRA (Nrf2 inhibitor) or SnPP (HO-1 inhibitor) were employed. Both ATRA and HO-1 were able to significantly abrogate NQ mediated suppression of mitogen induced proliferation (Fig 4.13A) and cytokine secretion (Fig 4.13 B-E) confirming the involvement of Nrf2/HO-1 pathway.

Electrophiles are known to induce glutathionylation by forming thiyl radical and interacting with the sulfhydryl residues of proteins to modulate the signaling cascade (*354*). Reversible or irreversible oxidative modification of cys thiols in susceptible proteins is one of the mechanisms through which inflammatory responses can be modulated (*355*). The disulfide coupling of a GSH moiety to cys residues is known as protein glutathionylation and is prevalent S-thiolation reaction in biological systems (*356*). Glutathionylation is often considered to be a process that protects sensitive cysteinyl residues from irreversible oxidation (*357, 358*). Several proteins are known to be the targets of oxidative stress induced glutathionylation including transcription factors (Jun, NF-κB, Nrf2), enzymes (creatine kinase, human immunodeficiency virus-1 protease) and cytoskeletal proteins (actin, tubulin) all of which regulate critical pathways in growth, differentiation and metabolism of cells (*359*). Taken together, these studies propose that glutathionylation is a physiologically relevant mechanism for controlling the activation of key signaling pathways. A recent report by Zhang Y et al showed that S-glutathionylation of KEAP-1 is an important event involved in its regulation of Nrf2 induction by electrophiles and thiol-depleting agents (*190, 360*). KEAP-1, a negative regulator of Nrf2, acts as redox sensor due to presence of cysteine

residues and thiol modification of these residues changes KEAP-1 conformation causing disruption in binding with Nrf2 (*361*). Cys468 residue present in the DC domain of KEAP-1 directly associates with Nrf2 and is known to get modified during Nrf2 activation (*214, 360-362*). Interestingly, NQ treatment induced glutathionylation of KEAP-1 in lymphocytes (Fig 4.14A). These results suggested that modulating cellular redox causes glutathionylation of KEAP-1 resulting in activation of Nrf2.

Cross talk between Nrf2 and NF-κB is an important target for developing novel anti-inflammatory agents. Several Nrf2 activators like dithilethione, sulforaphane and 15-dPGJ$_2$ were shown to inhibit NF-κB activation (*363, 364*). Activation of Nrf2 has been shown to inhibit the mitogen induced expression of NF-κB and its dependent pro-inflammatory genes (*254*). There are reports showing the inhibition of NF-κB pathway by Nrf2 via KEAP-1 induced IKKβ ubiquitination (*365, 366*). KEAP-1 functions as IKKβ E4 ubiquitin ligase by directly interacting with E(T/S)GE motif of IKKβ. Depletion in KEAP-1 leads to accumulation and stabilization of IKKβ and up-regulation of NF-κB (*253, 258, 365*). Since Nrf2 has been demonstrated to suppress NF-κB, our results highlighted the possibility that NQ mediated activation of Nrf2 may inhibit NF-κB activation leading to abrogation of inflammatory responses. Our results for the first time show that addition of NQ to lymphocytes induced binding of KEAP-1 to IKKβ and led to its degradation in a time dependent manner (Fig 4.15 A and B). It is well known that IKKβ plays an important role in immune responses by regulating the activation of NF-κB pathway (*367-369*). The NF-κB pathway is known to play a pivotal role in eliciting the expression of pro-inflammatory responses against pathogenic stimuli. NQ treatment inhibited mitogen induced degradation of IκBα and activation of NF-κB in lymphocytes (Fig 4.15C-E).

Based on these results, it was concluded that KEAP-1 mediated IKKβ degradation in response to NQ might be responsible for the suppression of NF-κB pathway. In conclusion,

the present study demonstrated that pro-oxidant, NQ, suppressed inflammation by inducing oxidative stress which lead to KEAP-1 protein modification and disruption of KEAP-1/Nrf-2 interaction resulting in activation of Nrf-2 pathway. Our results also demonstrate that induction of mild oxidative stress in lymphocytes by NQ leads to IKKβ degradation and suppression of NF-kB pathway. Further, these results also highlight the potential of redox modifiers as promising anti-inflammatory and immune-suppressive agents.

CHAPTER 5

SUMMARY

AND

CONCLUSIONS

5.1 SUMMARY:

Under normal physiological conditions, cells are exposed to multiple exogenous and endogenous oxidative stressors and maintenance of cellular redox homeostasis is very important to maintain cell viability and normal physiological responses (*161, 165, 168*). To maintain redox homeostasis and to counter oxidative stress, cells are equipped with network of antioxidant enzymes that plays a pivotal role in detoxification of ROS (*165, 370*). ROS are known to play dual role depending upon the magnitude of generation. It is well accepted that high levels of ROS induce activation of apoptotic pathway whereas low levels of ROS initiate the induction of cytoprotective responses (*117, 371*). The functional status of cellular antioxidant systems and the redox-sensitive survival signaling pathways can significantly influence the outcome of the cell-fate against deleterious stimuli. Therefore, perturbation of cellular redox status by inducing mild oxidative stress can lead to activation of redox sensitive pro-survival pathways which trigger the cytoprotective responses. Induction of mild oxidative stress may thus serve one of the amenable strategies to develop novel redox based therapeutics/preventive agents.

Exposure to IR induces oxidative stress in cells and triggers a cascade of events leading to cell death. IR mediated generation of ROS and cellular damage is mainly through radiolysis of water and also by direct deposition of energy in critical biomolecules like DNA, proteins and lipids. The interaction of free radicals formed by IR with the cellular biomolecules can lead to protein oxidation, lipid peroxidation, DNA damage and activation of cellular signaling machinery to repair the damage. (*372, 373*). Exposure of cells to IR induces oxidative stress and triggers a cascade of events which may lead to cell cycle arrest or cell death. Rapidly proliferating cells like hematopoietic cell, intestinal stem cells, skin epithelial cells etc. are more sensitive towards IR induced cell death. High dose of IR (>1Gy) can induce massive cell death in radiosensitive tissues which underlines the onset of dose

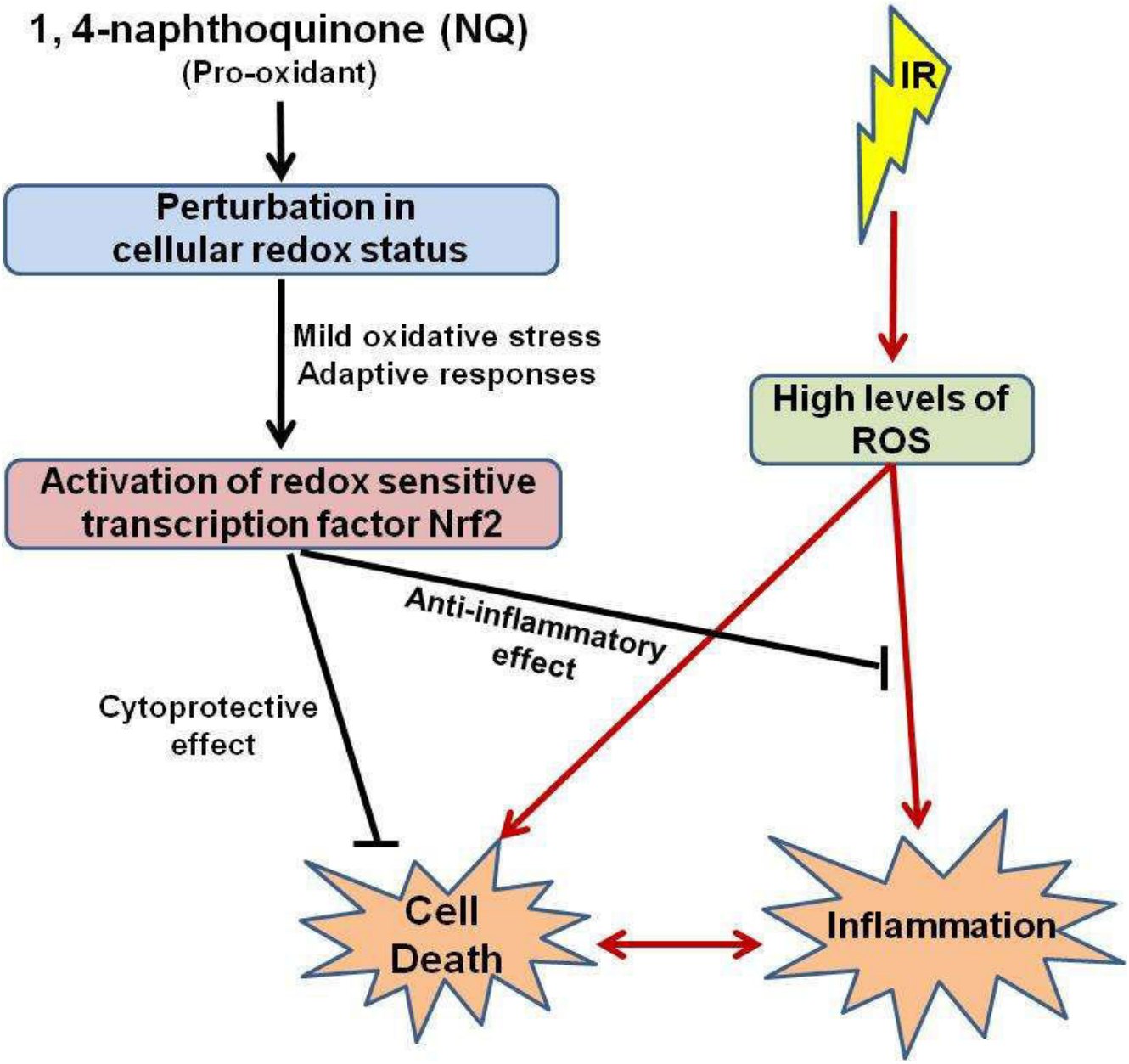
dependent acute radiation syndromes (ARS) (*260*). The hematopoietic syndrome is observed at doses >1Gy in which exposure to IR induces cell death in mitotically active hematopoietic progenitor cells. This further delimits the regeneration of lymphoid system and it may eventually lead to sepsis, shock and multiple organ failure (*374*). Doses above 8Gy induce damage to both hematopoietic system as well as GI system and can cause death within 2 weeks. At these higher doses of radiation, apoptosis and reproductive death of ISC, intestinal crypt cells and endothelial cells leads to impaired regeneration of villi and compromised epithelial integrity of the entire GI tract (*27, 34, 39, 55*). The damage to villi causes loss in absorptive surface area leading to pain, nausea, vomiting, diarrhoea, infection and electrolyte imbalance (*375*). Moreover, the loss of epithelial integrity can promote the direct access of enteric pathogens and flora into the bloodstream which can lead to systemic inflammation, sepsis and death (*373*). These potentially lethal gastrointestinal symptoms after radiation exposure are collectively referred as the radiation-induced gastrointestinal syndrome (RIGS).

IR induced GI injury is the major limiting factor for abdominal and pelvic radiotherapy. More than 200,000 patients per year receive abdominal or pelvic radiation therapy and the estimated number of cancer survivors with post radiation intestinal dysfunction is 1.5-2 million (*376*). Protection against radiation-induced GI injury either due to planned exposure or unplanned exposure is of utmost importance (*377, 378*). Thus, it is very important to understand the underlying mechanism of IR induced GI damage, role of inflammation in RIGS and develop effective modalities to mitigate IR induced GI damage.

Multiple strategies have been employed by different investigators to identify the novel targets and agents with potential to ameliorate RIGS (*281-284*). As radiation induced toxicity is mediated by generation of ROS, employing a potent antioxidant as an agent to act as radioprotector seems to be the most rational strategy. Though most of these agents investigated thus far have not yielded expected results and none of them are approved by

FDA to be used as radioprotector (*379-381*). Amifostine has been approved by FDA for a specific indication to be used in Head & Neck cancer patients undergoing radiotherapy. However, it is not approved as a general radioprotector as it is also associated with the induction of multiple side effects including nausea, vomiting, sneezing and hypertension (*259, 382-384*). Other strategies which have been used to develop novel radioprotective agents include mimetics of endogenous antioxidants, Non-steroidal Anti-inflammatory Drugs (NSAIDs), cell cycle modulators, cytokines and growth factors (*333, 385-387*). Though multiple agents have been shown to effectively mitigate radiation induced hematopoietic syndrome, very few agents have been investigated for their potential to protect against radiation induced GI syndrome (*259, 386, 388*).

In the present thesis work, a novel strategy of using an agent that can curb radiation induced inflammation as well as induce cytoprotective pathways was employed for mitigation of radiation induced GI injury. Perturbation of cellular redox status can elicit mild oxidative stress which can enhance cell survival upon subsequent radiation exposure. Apart from triggering the pro-survival response, oxidative stress can also modulate cell survival through direct oxidative modifications of the redox sensitive signaling molecules (*389, 390*). Exposure to IR induced increased oxidative stress, apoptosis and increased secretion of multiple pro-inflammatory cytokines leads to inflammatory responses. The final outcome in terms of cell survival, senescence, mitotic arrest or death in response to radiation exposure is decided by the integration of cellular signaling, extent of damage and DNA repair and is finally dependent on the cellular redox status.



Scheme 5.1: Hypothesis of pro-oxidants activating Nrf2 thereby leading to radioprotection.

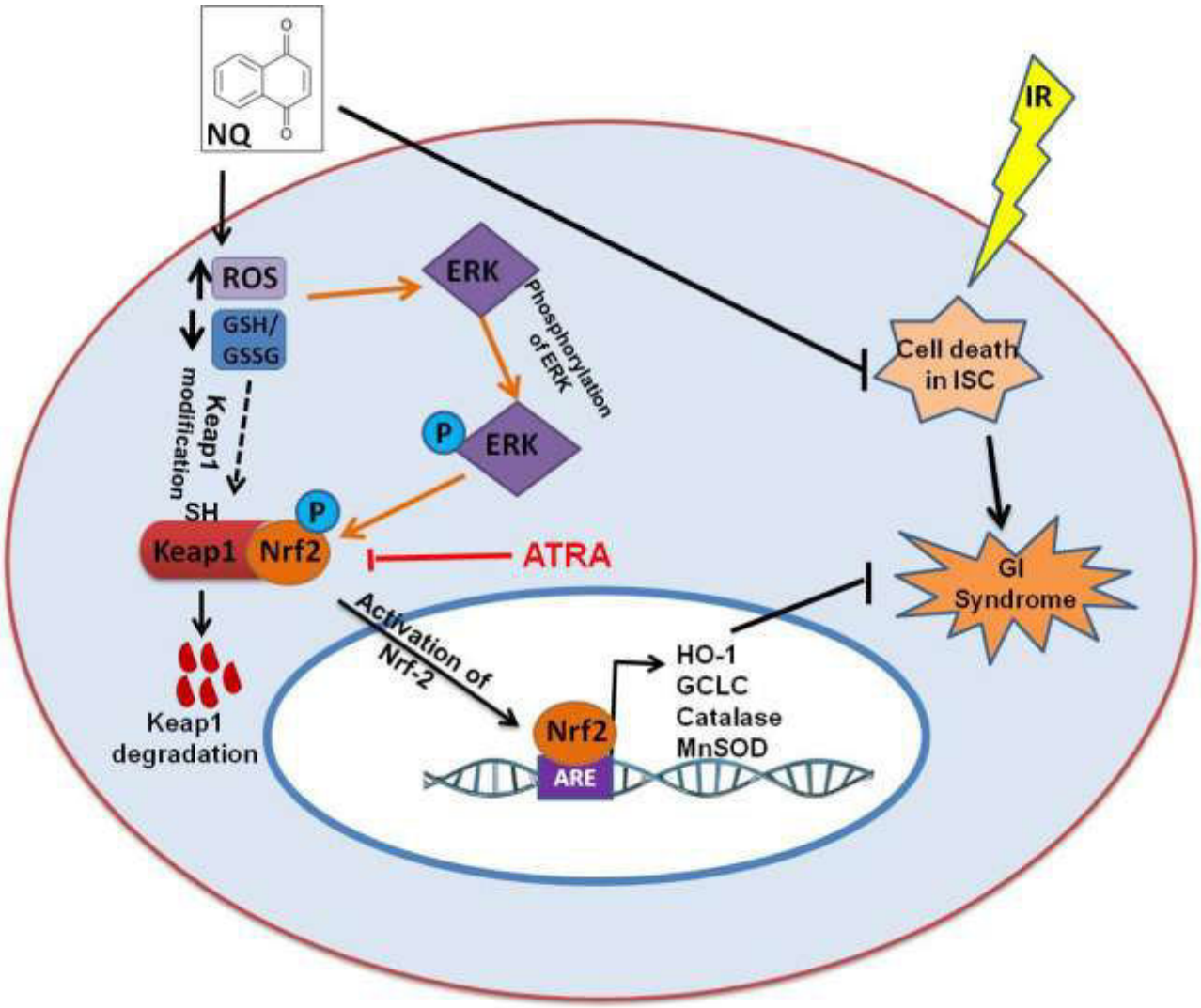
Previous studies from our laboratory have shown that NQ, a well known pro-oxidant, ameliorated radiation induced hematopoietic syndrome via upregulation of ERK/Nrf2 pathway (*287*). NQ being an electrophile and a pro-oxidant induces mild oxidative stress that may further lead to activation of cytoprotective signaling molecules. Our earlier work on structural analogue of 1,4-naphthoquinone, plumbagin, has been reported to exhibit potent

anti-inflammatory activity both in vitro and in vivo. Based on these studies from our laboratory, we hypothesized that NQ may protect against radiation induced GI syndrome by initiating adaptive responses via activation of Nrf2 pathway and also by curbing inflammation. We indeed observed that administration of NQ prior to radiation significantly protected against WBI 8Gy induced mortality and morbidity in mice. GI tract consists of rapidly proliferating cells required to maintain homeostatic conditions. Under normal conditions, there is a dynamic equilibrium in the intestinal region owing to the continuous shedding and replacement of epithelial cells from the tip of villi (*36, 50, 324, 330*). The radioprotective effects of NQ were found to be mediated via its ability to protect against WBI induced denudation of villi, epithelial misalignment, apoptosis in the crypts and bacterial translocation in mice. Since, the hematopoietic system is among the most sensitive organ to radiation-induced toxicity that may also contribute to the mortality at high dose exposure. Therefore, we standardized an experimental model of autologous bone marrow transplant (BMT) to mitigate the WBI (9-12Gy) induced bone marrow aplasia leaving the mice specifically exhibiting GI syndrome. We found that NQ administration in combination with BMT protected mice against WBI induced mortality and morbidity in mice even at high doses of IR (9-12Gy). These results clearly demonstrate the radioprotective potential of NQ can be extended in combination with BMT and this could be a novel strategy to protect against high doses of IR.

Further, studies were carried out to elucidate the underlying mechanism of NQ mediated protection against GI syndrome. We studied the effect of NQ on intestinal stem cells (ISC) that are present at the base of the crypts called as columnar base cells, and are characterised by the expression of Lgr5. These cells undergo continuous self-renewal and differentiation to replenish the villi surface and could play a role in the observed radioprotective effects of NQ. We observed that exposure of mice to IR induced apoptosis in

the ISC which abolishes the regeneration of the denudated villi and marks the rapid onset of GI syndrome. Further, administration of NQ to these mice protected the intestinal epithelial cells and Lgr5+ intestinal stem cells against WBI induced cell death suggesting that this could be the underlying mechanism of NQ mediated radioprotection at high doses of IR.

Earlier study from our laboratory implicated the ERK /Nrf2 axis as the mechanism of NQ mediated protection against radiation induced hematopoietic syndrome (*287*). In the present work, studies were carried out to investigate the potential of NQ to activate Nrf2 in intestinal epithelial cells and the role of ERK/Nrf2 axis in NQ mediated protection against radiation induced GI syndrome was also elucidated. Nrf2 is a pro-survival transcription factor which regulates the expression of cytoprotective and antioxidant proteins. Thus, activation of Nrf2 leads to the elevated levels of cellular cytoprotective genes which may enhance the survival and protect the host cell from radiation induced damage. Recently Kim et al demonstrated the potential of Nrf2 to protect colonic epithelial cells from IR induced cell death (*391*). Nrf2 has been implicated in regulating the inducible expression of intestinal detoxification, glutathione biosynthetic enzymes and regulation of proliferation in ISC (*392, 393*). Similar to our previous observations, we found that NQ administration to mice led to an increase in phosphorylation of ERK and activation of Nrf2 in intestinal cells. Further, to substantiate the role of Nrf2 in NQ mediated protection, an inhibitor of Nrf2 was employed (ATRA). We found that ablation of Nrf2 using ATRA resulted in abrogation of NQ mediated protection against WBI induced mortality. These results clearly demonstrated that alteration in cellular redox status by employing a mild oxidative stressor like NQ can offer protection against radiation induced GI syndrome by inducing Nrf2 pathway in combination with autologous BMT.



Scheme 5.2: Proposed model of radioprotective effects of NQ: NQ induced activation of a redox sensitive transcription factor Nrf2 via activation of ERK pathway and inhibited radiation induced GI syndrome. NQ might also activate Nrf2 by destabilizing Nfr2-KEAP-1 interaction by binding with cysteine residue of KEAP-1.

Since, radiation induced inflammation can also amplify the damage manifested, the role of NQ to curb anti-inflammatory reactions was also explored. Further, cellular redox balance is known to play a crucial role in regulating immune responses, the effect of NQ (a pro-oxidant) on innate and adaptive immune responses was studied. Inflammation is the reaction of vascularised tissue to local injury, which results from an immune response to allergic or chemical irritation, injury and/or infections. Main objective of inflammation is to

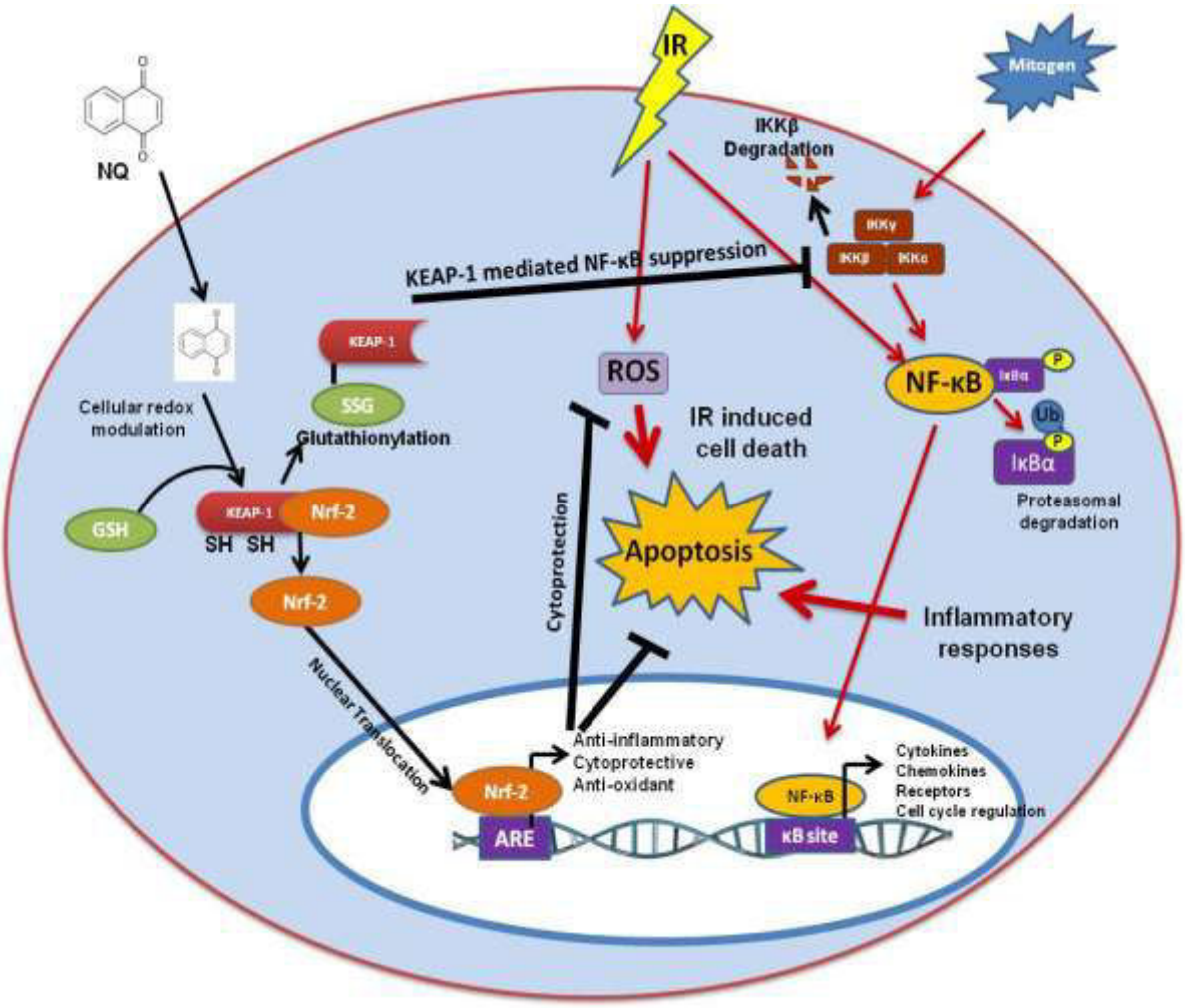
elicit the immune response against pathogenic stimuli to protect the integrity of the host or to resolve the tissue injury (*394, 395*). However inflammation persisting for weeks, months or even years despite resolving the infection/injury leads to deleterious responses. The persistent inflammation and oxidative stress is associated with etiology of the degenerative diseases including rheumatoid arthritis, atherosclerosis, Alzheimer, asthma, acquired immunodeficiency syndrome, cancer, congestive heart failure, multiple sclerosis, diabetes, gout and inflammatory bowel disease (*396, 397*). Unregulated release of inflammatory mediators from host leucocytes including prostaglandins, leukotrienes, cytokines and reactive oxygen species cause damage to host tissues by amplifying the inflammatory process. Several classes of drugs, such as corticosteroids and NSAIDs are used to treat the inflammatory disorders. However, associated side effects like hypertension, hyperglycemia, muscular weakness, increased susceptibility to infection etc provide an urge to develop anti-inflammatory regimens for the treatment of inflammatory disorders (*59, 398-401*). Ionizing radiation can stimulate pro-inflammatory responses due to local tissue injury (radiotherapy) or whole body injury (accidental exposure), and normal tissue response to radiation exposure is immediate and endures with time. IR induced ROS and tissue damage leads to increased secretion of soluble inflammatory mediators (*402*). Thus, employing a radioprotective agent with potential to suppress inflammatory responses and up-regulate pro-survival factors could be a wise strategy.

The second part of the thesis highlights the potential of NQ to suppress inflammatory response using lymphocytes as a model system. Lymphocytes isolated from spleen and gut associated lymphoid tissue of NQ injected mice were hyporesponsive towards mitogen induced proliferation, cytokine secretion and NF-κB activation. NQ inhibited Con A and anti-CD3/CD28 antibody induced proliferation and cytokine (IL-2, IL-4, IL-6 and IFN-γ) secretion by murine lymphocytes. NQ also inhibited LPS induced cytokine (TNF-α, IL-1β

and IL-6) secretion, nitric oxide and cyclooxygenase-2 expression by macrophages. Balance between intracellular redox couple like GSH/GSSG, cysteine/cystine, thioredoxin reduced/oxidized maintains the cellular redox status requisite for generation of efficient immune response against pathogenic invasion (*403-405*). Further, it is well known that any perturbation in the intracellular or extracellular redox state can disturb the normal course of immune response (*343-345*). NQ modulated cellular redox status in lymphocytes and only thiol antioxidants like NAC and GSH could abrogate the anti-inflammatory activity of NQ. These results indicated a critical role of cellular redox modulation behind NQ mediated anti-inflammatory action. Further, alteration in cellular redox status is known to induce the activation of redox sensitive anti-inflammatory transcription factor Nrf2. Several reports have shown that depending on the extent of modulation of cellular redox levels, different redox sensitive transcription factors including Nrf2 may be activated or suppressed (*403, 406-408*). An indispensable role of Nrf2 in the regulation of inflammatory responses via regulation of cytokines and pro-inflammatory mediators has been highlighted in the literature showing that its deficiency can increase susceptibility to inflammatory and autoimmune disorders via hyperactivation of T cells (*197, 237, 244-246, 248*). Among the cytoprotective genes up-regulated by Nrf2, HO-1 has been identified as a potent anti-inflammatory enzyme due to production of carbon monoxide (CO) and bilirubin during heme degradation. Increased expression of HO-1 was shown to protect against airway inflammation (*243, 246*), brain infections (*245, 247*), skin inflammation (*247, 362*) and gastro-intestinal inflammation (*214, 244*). NQ was found to induce the activation of Nrf2 and its dependent genes (HO-1 and GCLC) in lymphocytes. Increase in oxidative stress by an electrophile is known to increase the glutathionylation of cellular proteins. KEAP-1, a negative regulator of Nrf2, acts as redox sensor due to presence of cysteine residues which under oxidative stress conditions can get glutathionylated and cause disruption in KEAP-1:Nrf2 interaction. S-glutathionylation of

KEAP-1 plays a pivotal role in regulation of Nrf2 induction by electrophiles and thiol-depleting agents (*360*). NQ induced glutathionylation of KEAP-1 leading to activation of Nrf2.

Cross talk between Nrf2 and NF-κB pathway has been implicated as an important target to develop potent anti-inflammatory regimens. Activation of Nrf2 has been shown to suppress NF-κB activity (*364, 366, 409*). NQ inhibited mitogen induced degradation of IκBα and activation of NF-κB in murine lymphocytes. KEAP-1 is known to interact with E(T/S)GE motif of IKKβ and functions as E3 ubiquitin ligase to mediate its degradation. Based on these observations, we proposed that NQ may induce KEAP-1 mediated degradation of IKKβ thus leading to suppression of NF-κB pathway. Indeed, we observed that NQ induced binding of KEAP-1 to IKKβ and also led to its subsequent degradation. Thus, KEAP-1 mediated IKKβ degradation in response to NQ might be responsible for the suppression of NF-κB pathway. Further, to confirm the role of activation of Nrf2 pathway in NQ mediated anti-inflammatory action, ATRA (Nrf2 inhibitor) and SnPP (HO-1 inhibitor) were employed. Inhibitors of Nrf2 and HO-1 significantly abrogated NQ mediated suppression of mitogen induced proliferation corroborating the impervious role of Nrf2/HO-1 pathway in NQ mediated anti-inflammatory effects. The present study demonstrates that other pro-oxidants (H_2O_2 and tBHQ) also inhibit immune responses. Further, we also highlight the potential of redox modifiers as promising radioprotective, anti-inflammatory and immune-suppressive agents.



Scheme 5.3: Proposed model of NQ mediated radioprotection and anti-inflammatory activity: NQ induced S-glutathionylation of KEAP-1 resulting in Nrf2 activation. Activation of Nrf2 leads to expression of cytoprotective proteins and antioxidant machinery that confers protection against radiation induced cell death. NQ induced KEAP-1 mediated degradation of IKKβ resulting in suppression of mitogen induced activation of NF-κB, thereby suppressing inflammatory responses and associated cell death.

5.2 CONCLUSIONS:

The results presented in this thesis demonstrate the potential of NQ to protect against radiation induced GI syndrome and exhibit the potent anti-inflammatory activity of NQ by activating a redox sensitive Nrf2 pathway. The present study exemplifies the concept of modulating cellular redox status as a novel strategy to protect against radiation injury and inflammatory responses. These results for the first time illustrates the salutary effect of a pro-oxidant in the perspective of radiation induced GI syndrome where it played a protective role by inhibiting radiation induced cell death in ISC and activating Nrf2 pathway. NQ induced activation of Nrf2 led to an increased expression of antioxidant enzymes and cytoprotective proteins that confer protection against radiation induced damage. ROS and tissue damage due to IR exposure leads to generation of inflammatory response which further amplifies the radiation induced damage. NQ induced activation of Nrf2 curbed the inflammatory responses by inhibiting the activation of NF-κB pathway. Thus, NQ induced activation of Nrf2 pathway plays a central role in conferring protection against GI syndrome by inhibiting radiation induced cell death and inflammation. Since, NQ has a profound anti cancer activity; its application in protecting intestinal cells during chemo-radiation therapy may increase the therapeutic ratio of abdominal irradiation in GI malignancies. Since, radiation induced injuries are also manifested by the induction of inflammation, anti-inflammatory effects of NQ further contribute its application as radioprotective and immunosuppressive agent. The present findings also provide further insights into the cross talk between Nrf2 and NF-κB pathways as novel targets for developing radioprotective and anti-inflammatory agents.

The major conclusions drawn from this study are:

1. NQ, a pro-oxidant, the parent molecule of many clinically approved anticancer drugs can be potential radioprotector.
2. NQ administration to mice protected against radiation induced GI syndrome up to 8Gy.
3. NQ in conjunction with bone marrow transplant protected against high dose (9, 10 & 12Gy) IR induced mortality.
4. Administration of NQ protected intestinal stem cells against radiation induced cell death.
5. Administration of NQ induced activation of ERK/Nrf2 pathway in jejunum cells in vivo.
6. Inhibition of Nrf2 abrogated NQ mediated protection against WBI induced mortality in mice.
7. Lymphocytes isolated from NQ treated mice were hyporesponsive towards mitogenic stimulation.
8. NQ inhibited mitogen induced proliferation and cytokine secretion by T cells.
9. NQ inhibited LPS induced nitric oxide production and cytokine secretion by macrophages (RAW 264.7 cells).
10. Suppressive effects of NQ on mitogen induced proliferation and cytokine secretion were sensitive to thiol antioxidants.
11. NQ induced glutathionylation of KEAP-1 and activation of Nrf2 pathway in lymphocytes.
12. NQ induced KEAP-1 mediated degradation of IKKβ and inhibited mitogen induced activation of NF-κB in lymphocytes.
13. Inhibiting Nrf2 pathway abrogated anti-inflammatory action of NQ.

14. Cross talk between Nrf2 and NF-κB pathway may serve as a novel target for developing radioprotective and anti-inflammatory agents.

CHAPTER 6

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ANNEXURE

1. Trypan blue dye solution:

0.5% Trypan Blue+ 0.9% NaCl in 1X PBS, For100ml:

Trypan Blue 0.5gm + NaCl 0.9gm + 1X PBS 100ml

2. Mitogen dilutions:

Concanavalin A: Stock solution 25mg/ml in RPMI.

For working solution of 200µg/ml, add 40µl of stock solution and 4.960ml RPMI.

Lipopolysaccharide: Stock solution 5mg/ml in RPMI.

3. Propidium Iodide (PI) Staining Solution:

50µg/ml PI in 0.1 % Na-citrate + 0.1 % Triton X-100, For 100 ml: Na Citrate

0.1g+Triton X-100, 0.1 ml+ PI 5mg+ 1X PBS 100ml

4. Phosphate Buffer Saline (PBS):

10mM, pH 7.4, For 100ml: NaCl 0.9gm + Na_2HPO_4 0.126 gm + NaH_2PO_4 0.0451gm + Distilled Water 100ml

5. Tris–Borate EDTA (5X):

TRIZMA base 54gm + Boric acid 27.5gm +0.5M EDTA 20ml (pH: 8.0)+ DW

1000 ml

6. Gel Loading Dye:

Bromophenol Blue 0.25 % W/V+ Sucrose 40 % W/V in Distilled Water

7. Griess Reagent:

Sulphanilamide 500mg+ Naphthylethylene diamine dihydrochloride (NEDDH) 50mg + H_3PO_4 1.25ml+ Distilled Water 50ml

8. Neutral Buffered Formalin For Tissue Fixation:

NaH_2PO_4 4gm + Na_2HPO_4 6.5gm + 40% Formaldehyde 100ml+ DW 900ml

9. Coating Buffer for ELISA:

(0.1 M Sodium Carbonate): $NaHCO_3$ 8.4gm + Na2Co3 3.56gm + Distilled Water 1000ml and adjust pH to 9.5

10. Assay Diluent for ELISA:

1X PBS + 10% FCS

11. Wash Buffer for ELISA:

1X PBS + 0.05% Tween 20

12. Stop solution for ELISA:

0.1N HCl

13. 1.5M Tris Cl pH 8.8:

121.14 gm Tris Cl in 100 ml - 1 M

18.117 gm Tris Cl in 100 ml - 1.5M

Dissolve 18.117 gm tris base in some amount of D/W.

Adjust pH to 8.8 with conc. HCl and make up final volume to 100 ml with D/W.

14. 0.5M Tris Cl pH 6.8:

121.14 gm Tris Cl in 100 ml - 1 M

6.057 gm Tris Cl in 100 ml - 0.5 M

15. Acrylamide Solution:

Acrylamide - 29.2 gm

Bisacrylamide - 0.8 gm

Dissolve in some amount of D/W. Make up the final volume to 100 ml with D/W. Filter and store at 4°C.

16. 10% SDS:

Dissolve 10 gm SDS in DW. Make up vol. to 100 ml and store at RT.

17. 10% Ammonium persulphate(APS):

Dissolve 0.05 gm APS in 500 µl DW. Prepare fresh.

18. 2X Sample buffer/Loading buffer:

0.5 M Tris Cl pH 6.8 - 2.5 ml

Glycerol - 2.0 ml

10% SDS - 4.0 ml

2-Mercaptoethanol - 1.0 ml

0.05% Bromophenol blue - 0.5 ml

Add everything except 2-ME to get 9 ml vol. Make 9ml aliquots of it. Add 100 µl 2ME freshly prepared. Store at – 200C.

19. 0.5 M EDTA pH 8.0:

Dissolve 9.305 gm EDTA in DW. Adjust pH 8.0 with NaOH. Make up vol. to 50 ml.

20. Tank Buffer (5X-1000 ml):

Tris - 15 gm

Glycine- 72 gm

SDS - 5 gm

Dissolve in DW and make up the vol. to 1 litre. Store at RT.

21. Western Blot Transfer Buffer (5X-1000 ml):

Tris - 15 gm

Glycine- 72 gm

Dissolve in DW and make up the Vol. to 1 L. Store at 4°C.

1X transfer buffer for Western blot-2000ml

20% v/v methanol in DW:400 ml

DW:1200 ml.

22. 10X TBS : 250 ml

Tris -15.125 gm

NaCl - 21.9 gm

Dissolve in 150 ml DW. Adjust pH to 7.5 with HCl and make up vol. to 250 ml with DW.

23. 1X TBST:

1X TBS solution, 0.5% Tween 20. Store at 40C.

24. 100mM PMSF stock:

Dissolve 17.42 gm of PMSF (phenyl methyl sulphonyl fluoride) in 1ml isopropanol.

Divide it in 10 aliquots of 100 µl each.

25. Whole Cell Lysis Buffer:

DW - 1.2 ml 1M HEPES- 30 µl

10% NP40 - 150 µl 0.1M EGTA- 75 µl

5M NaCl - 75 µl 0.5M EDTA- 6 µl

Store at 4°C..

26. Protease Inhibitors:

0.2M NaVanadate - 10 µl

1 µg/ml Leupeptin - 2 µl

1 µg/ml Aprotinin - 2 µl

0.1M PMSF - 5 µl

Add just before use to the lysis buffer.

27. CFSE dye (20 µM):

5mM CFSE stock dissolved in DMSO – 4 µl stored as aliquots at -20°C.

Add it to a final vol. of 1ml RPMI medium.

28. Antibody Labeling Buffer:

1X PBS - 100 ml

0.1% sodium azide - 0.1 gm

1% FCS - 1 ml

29. Antigen Retrieval Solution:

10 mM Sodium Citrate Buffer:

2.94 g sodium citrate trisodium salt dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$)

1 L dH_2O. Adjust pH to 6.0.

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1,4-Naphthoquinone, a pro-oxidant, suppresses immune responses via KEAP-1 glutathionylation

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Cytokines

ABSTRACT

Low levels of oxidative stress have been shown to activate Nrf-2, an important anti-inflammatory transcription factor, by us and also by several other investigators. Earlier we showed that pro-oxidants protect normal lymphocytes against radiation injury by activating Nrf-2. In the present study, we have investigated the effect of oxidative stress on immune responses and delineated the underlying mechanism. Hydrogen peroxide, *tert*-butylhydroquinone and 1,4-naphthoquinone (NQ) inhibited mitogen induced proliferation of lymphocytes. NQ also inhibited mitogen (Concanavalin A) induced cytokine secretion by murine T cells and lipopolysaccharide induced release of cytokines, nitric oxide and cyclooxygenase-2 expression by macrophages. NQ modulated cellular redox by decreasing GSH/GSSG ratio and the immunosuppressive effects of NQ were significantly abrogated by thiol containing antioxidants and not by non-thiol antioxidants. This redox perturbation led to activation of Nrf-2 pathway and inhibition of NF-κB. NQ treatment increased total protein *S*-thiolation, induced glutathionylation of KEAP-1 protein and decreased IKKβ levels in lymphocytes. Molecular docking studies revealed that NQ can disrupt KEAP-1/Nrf-2 interaction by directly blocking the binding site of Nrf-2 in the KEAP-1 protein. Further, inhibitors of Nrf-2 and HO-1 abrogated the anti-inflammatory effects of NQ. T cells isolated from spleen and gut associated lymphoid tissue of NQ administered mice also showed suppression of NF-κB activation and were hyporesponsive to mitogenic stimulation. These results demonstrate that pro-oxidants modulate inflammatory and immune responses via oxidative stress mediated KEAP-1 glutathionylation and IKKβ degradation.

1. Introduction

Nuclear factor erythroid 2-related factor 2 (Nrf-2) is a redox sensitive transcription factor which belongs to basic leucine zipper containing Cap 'N' collar family of proteins [1]. Upon activation, Nrf-2 translocates into the nucleus and binds to antioxidant response element (ARE) present in the promoter region upstream to its dependent genes that code for phase II detoxifying enzymes, anti-oxidant enzymes and stress responsive proteins. Nrf-2 knockout mice showed prolonged inflammation during cutaneous wound healing [2], high susceptibility for lupus like autoimmune syndrome, enhanced lymphocyte proliferation, impaired redox status [3], higher secretion of Th2 cytokines [4], higher mortality in response to lipopolysaccharide (LPS) induced septic shock [5], leukocyte migration, adhesion [6–8] and nitric oxide (NO) production in murine macrophages [9]. KEAP-1 (Kelch-like ECH-associated protein-1), a negative regulator of Nrf-2 is known to be sensitive towards redox alterations due to the presence of multiple, highly reactive and functionally important cysteine residues [10]. Modification of cysteine residues on KEAP-1 causes disruption in its interaction with Nrf-2 resulting in nuclear translocation of Nrf-2 [11].

NF-κB is another redox sensitive transcription factor which is known as a key regulator of the expression of immune cell receptors, growth factors, adhesion molecules and cytokines (IL-1, IL-2, IFN-γ, IL-6) in lymphocytes, epithelial cells and monocytes [12], and its dysregulation is associated with immune disorders [13], T cell mediated inflammation, impaired cellular immunity and higher susceptibility to bacterial infections [14,15]. Several investigators have highlighted the role of KEAP-1/Nrf-2 pathway in regulating NF-κB activation as well as NF-κB mediated inflammatory response [16–19]. KEAP-1 functions as an adapter protein for CUL-3 based E3 ligase to degrade IκB kinase β (IKKβ), which is responsible for suppression of NF-κB activation and modulation of immune responses [19]. Hence, the cross talk between NF-κB and KEAP-1/Nrf-2 immunoregulatory transcription factors is a potential target for developing anti-inflammatory drugs [20].

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Table 1
List of primer sequences.

Gene	Sequence (5'-3')
HO-1	Forward: AGGTACACATCCAAGCCGAGA Reverse: CCATCACCAGCTTAAAGCCTT
GCLC	Forward: CTACCACGCAGTCAAGGACC Reverse: CCTCCATTCAGTAACAACTGGAC
Nrf-2	Forward: CTTTAGTCAGCGACAGAAGGAC Reverse: AGGCATCTTGTTTGGGAATGTG
β-Actin	Forward: GCGGGAAATCGTGCGTGACATT Reverse: GATGGAGTTGAAGGTAGTTTCGTG

Earlier studies from our laboratory have shown that pro-oxidants protect against radiation injury via activation Nrf-2/HO-1 pathway [21]. Considering the distinctive ability of Nrf-2 to act as a sensor of cellular redox status and its crucial role as an immunoregulatory transcription factor, studies were carried out to investigate the effect of pro-oxidants like hydrogen peroxide (H_2O_2), *tert*-butylhydroquinone (*t*-BHQ) and 1,4-naphthoquinone (NQ) on lymphocyte activation. NQ is also a model molecule for biologically active clinically approved quinones possessing anticancer, anti-parasitic and anti-infective properties. Higher redox potential and electrophilic nature of quinones imparts high affinity for cellular nucleophiles like thiols of cysteine group present in proteins and glutathione contributing to its biological activity has led us to investigate the anti-inflammatory activity of 1,4-naphthoquinone and mechanism of action.

2. Materials and methods

2.1. Chemicals

NQ, RPMI 1640, DMEM, HEPES, EDTA, EGTA, phenylmethanesulfonyl fluoride (PMSF), leupeptin, aprotinin, benzamidine, dithiothreitol (DTT), H_2O_2, *t*-BHQ, Trizol reagent and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (MO, USA). Fetal calf serum (FCS) was obtained from GIBCO BRL (MD, USA). Concanavalin A (Con A), tin-protoporphyrin (SnPP) and all-trans-retinoic acid (ATRA) were purchased from Calbiochem (Darmstadt, Germany). Carboxyfluorescein succinimidyl ester (CFSE) and LIVE/DEAD fixable dead cell stain kit were procured from Molecular Probes (NY, USA). cDNA synthesis kit and SYBR green PCR mix were procured from Roche Chemical Co. (USA). ELISA sets for detection of cytokines (IL-2, IL-3, IL-4, IL-6, IL-1β, TNF-α and IFN-γ) and monoclonal antibodies against CD3 and CD28 were procured from BD Pharmingen (CA, USA). Antibodies against IκBα, β-actin, IKKβ and KEAP-1 were procured from Cell Signalling Technologies (CA, USA). Antibody against GSH was procured from Abcam (CA, USA). Oligonucleotide probes for NF-κB, Nrf-2 and anti-Cox-2 antibody were purchased from Santacruz Biotechnology (CA, USA).

2.2. Animal maintenance

Six to eight weeks old Balb/c male mice weighing approximately 20–25 g, 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.

2.3. Murine lymphocytes preparation

Spleen was aseptically removed from the mice and placed in a sterile petri dish containing RPMI 1640 medium. Single cell suspensions were prepared as described earlier [6]. Gut associated lymphoid tissue (GALT) was aseptically removed from the mice and placed in a sterile petri dish containing 1× PBS. Single cell suspensions were prepared by gently squeezing the GALT on a cell strainer (100 μm) followed by centrifugation at 2000 rpm for 5 min. Cells were washed twice with PBS and resuspended in RPMI 1640 medium containing 10% heat inactivated fetal bovine serum.

2.4. Cell line and culture

RAW 264.7 cells were obtained from National Centre for Cell Sciences (NCCS), Pune, India and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C in an atmosphere of 5% CO_2.

2.5. NQ treatment

NQ stocks were prepared in DMSO. For all in vitro experiments, cells were treated with different concentrations of NQ for 2 h or with 5 μM NQ for different time intervals. DMSO was used as vehicle control. For all in vivo experiments, 2 mg/kg body weight of NQ was administered intra-peritoneally to mice.

2.6. Proliferation assay

Lymphocytes were stained with CFSE (20 μM, 8 min, 37 °C) as described earlier [22]. Two million lymphocytes were treated with NQ (0.5 μM to 5 μM, 2 h) or H_2O_2 (50 μM, 10 min) or *t*-BHQ (20 μM, 2 h) and were stimulated with Con A (5 μg/ml) or plate coated anti-CD3 and soluble anti-CD28 antibody (1 μg/ml) for 72 h at 37 °C in 2 ml RPMI with 10% FCS 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.

2.7. Live and dead assay

Lymphocytes were treated with different concentrations of NQ for 24 h and percentage cell death was calculated using LIVE/DEAD fixable dead cell stain kit as per manufacturer's instructions.

2.8. Measurement of cytokine secretion

Cytokine concentration (IL-2, IL-3, IL-4, IL-6, IL-1β, TNF-α and IFN-γ) was measured in the supernatant of unstimulated cells and cells stimulated with Con A or anti-CD3/CD28 antibodies or LPS for 6 h or 24 h after NQ treatment (2 h) using cytokine ELISA sets (BD Pharmingen, USA) [23].

2.9. Measurement of nitric oxide

The concentration of nitric oxide in the supernatant of RAW 264.7 cells pre-treated with NQ and cultured for 24 h at 37 °C in the presence of LPS was measured by using Griess reagent as described earlier [24].

2.10. Intracellular antibody staining

Two million RAW 264.7 cells in the presence or absence of NQ were stimulated with LPS (1 μg/ml) for 24 h at 37 °C. Cultured cells were fixed with 4% paraformaldehyde for 10 min at room temperature and excess paraformaldehyde was removed by

washing once with wash buffer (PBS containing 1% bovine serum albumin). Cells were permeabilized with PBS containing 0.02% Tween 20 three times for 5 min each at room temperature and then stained with PE-labeled Cox-2 mAb. Changes in Cox-2 levels were measured using a flow cytometer (Partec CyFlow) and analyzed using FlowJo software.

2.11. Determination of intracellular glutathione and glutathione disulfide levels

Glutathione levels were measured spectrophotometrically by conventional enzyme cycling method [25].

2.12. Determination of nuclear levels of Nrf-2 using confocal microscopy

Lymphocytes were treated with NQ (5 μM) for various time intervals. The cells were labelled with anti-Nrf-2 antibody as described previously [26]. Further, these cells were stained with FITC-labelled secondary antibody followed by Hoechst staining. Slides were examined using an LSM510 confocal microscope (Carl Zeiss, Jena, Germany) with a krypton–argon laser coupled to an Orthoplan Zeiss photomicroscope.

2.13. Electrophoretic mobility shift assay

Lymphocytes were treated with NQ (5 μM, 2 h) and were stimulated with Con A (5 μg/ml) for 3 h at 37 °C or treated with 5 μM NQ for various time intervals. Nuclear extracts were prepared as described earlier [27]. EMSA for NF-κB (Con A stimulated samples in presence or absence of NQ) or Nrf-2 (NQ treated samples) was performed by incubating 8 μg of nuclear proteins with 16 fmol of ^{32}P-end-labeled, 45-mer double-stranded NF-κB oligonucleotides from the human immunodeficiency virus long terminal repeat (5′-TTGTTACAA*GGGACTTT*CCGCTG*GGGACTTT*C-CAGGGAGGCGTGG-3′; italic indicates NF-κB binding sites) or the antioxidant response element (ARE; 5′-CGGTCACCGTTACT-CAGCACTTTG-3′), respectively for 30 min at 37 °C. The DNA–protein complex was separated from free oligonucleotide on 7.6% native polyacrylamide gel and the dried gel was exposed on Molecular Dynamics phosphorImager Screen. Visualization of radioactive bands was done in a phosphorImager (Amersham Biosciences, USA).

2.14. Quantitative real time PCR

RNA isolation, cDNA synthesis and RT-PCR were performed as described previously [28]. Briefly, total RNA was isolated from the

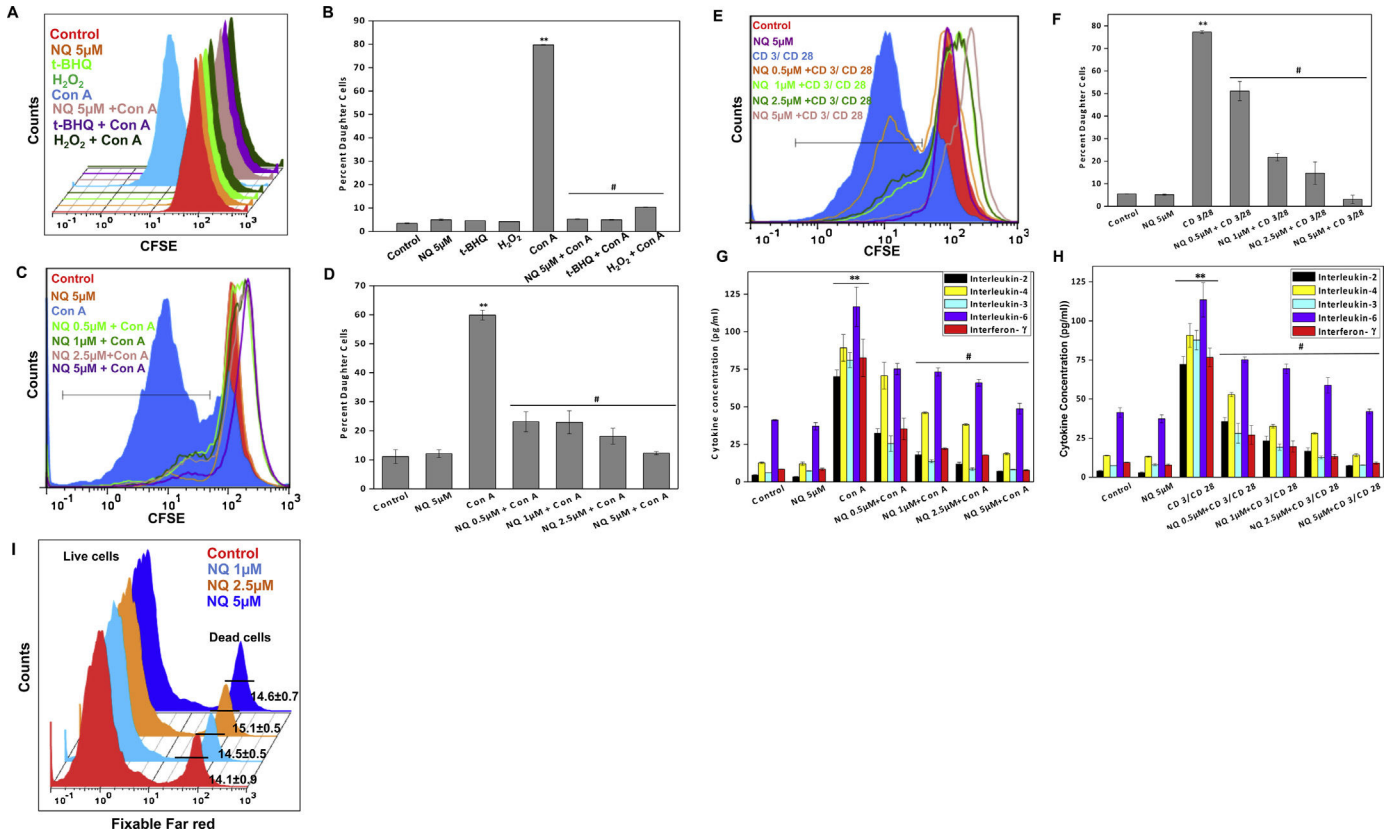

Fig. 1. Effect of pro-oxidants on mitogen induced proliferation and cytokine secretion by lymphocytes in vitro: (A) CFSE labeled lymphocytes were treated with NQ (5 μM, 2 h) or *t*-BHQ (20 μM, 2 h) or H_2O_2 (50 μM, 10 min) and then stimulated with Con A at 37 °C for 72 h. Cells were treated with H_2O_2 for 10 min and then washed before being stimulated. Thirty thousand cells in each group were acquired in a flow cytometer. Vehicle treated cells served as control. Percent daughter cells were calculated from decrease in mean fluorescence intensity as shown in overlaid flow cytometric histograms. (B) Graph represents mean ± S.E.M. from three replicates in each treatment group. (C) CFSE labeled lymphocytes were treated with NQ (0.5–5 μM) for 2 h and then stimulated with Con A at 37 °C for 72 h. Thirty thousand cells in each group were acquired in a flow cytometer. Vehicle treated cells served as control. Percent daughter cells were calculated from decrease in mean fluorescence intensity as shown in overlaid flow cytometric histograms. (D) Graph represents mean ± S.E.M. from three replicates in each treatment group. (E) CFSE labeled lymphocytes were treated with NQ (0.5–5 μM) for 2 h and then stimulated with plate bound anti-CD3 mAb (1 μg/ml) and soluble anti-CD28 mAb (1 μg/ml) at 37 °C for 72 h. Thirty thousand cells in each group were acquired in a flow cytometer. (F) Graph represents mean ± S.E.M. from three replicates in each treatment group. (G and H) Lymphocytes were treated with NQ (0.5–5 μM) for 2 h and then stimulated with either Con A (5 μg/ml) or anti-CD3/CD28 mAb at 37 °C for 24 h. Supernatants were harvested and cytokine concentration was estimated by ELISA. Graphs represent mean ± S.E.M. of IL-2, IL-4, IL-6 and IFN-γ cytokines. (I) Lymphocytes were treated with NQ (1–5 μM) for 24 h and stained with Live/Dead assay kit as described in Materials and Methods section. Two such independent experiments were carried out. Thirty thousand cells in each group were acquired in a flow cytometer. Vehicle treated cells served as control. Percentage cell death was calculated from representative histograms. $^{**}p < 0.01$, as compared to vehicle treated cells and $^{\#}p < 0.01$, as compared to Con A or anti-CD3/CD28 stimulated cells.

samples using Trizol reagent (Sigma). 1 μg of total RNA was used for preparation of cDNA by reverse transcription (cDNA synthesis kit, Roche) following the manufacturer's instructions. Quantitative PCR was carried out using a Rotor Gene 3000 (Corbett Research) machine. The threshold cycle values were used for calculating the expression levels of genes by REST-384 version-2 software [29]. PCR efficiency was calculated for individual primer pairs. Specific primer sequences were obtained from primer bank (Table 1). Expression of the genes was normalized against a house keeping gene, β-actin and plotted as relative change in expression with respect to control.

2.15. Molecular docking

Molecular docking was performed with Autodock Vina [30] molecular docking software. NQ coordinates were obtained from Zinc database [31] (Zinc id: ZINC00901405).The structure of Kelch domain of mouse KEAP-1 was obtained from Protein Data Bank (PDB id: 2DYH). The X-ray structure of KEAP-1 contained residues 301–609. The protein was prepared for docking by removing the bound ligands and water molecules. The missing atoms and the polar hydrogen were added to the structure in Autodock Tools [32]. "Blind docking" of NQ was carried out against Kelch domain of KEAP-1 using a box that encompassed the entire protein. The size of the grid box was 40 A × 40 A × 40 A with centre *x*, *y*, *z* as 16, 18, 8. The value for the exhaustiveness parameter was 20 and all other parameters had default values. Best 20 poses were analyzed. The figures were prepared in PyMol molecular graphics system.

2.16. Immunoprecipitation

Lymphocytes were treated with NQ for different time points and whole cell lysates were prepared as described earlier [33]. Immunoprecipitation was performed following manufacturer's protocol (Protein G Immunoprecipitation Kit, Sigma, USA).

2.17. Western blot analysis

For monitoring total glutathionylation, lymphocytes were treated with NQ for 4 h and whole cell lysates were prepared. Effect of NQ on IκBα was monitored in cytoplasmic fraction. Lymphocytes were pre-treated with 5 μM NQ for 2 h and then stimulated with Con A (5 μg/ml) for 3 h at 37 °C. Cytosolic extracts were prepared as described earlier [34]. Vehicle treated cells served as a control. Equal amount of protein were resolved by SDS–PAGE (10%), transferred to nitrocellulose membrane, blocked and incubated overnight with the primary antibody specific to GSH, IκBα, IKKβ and KEAP-1. After subsequent washing, membrane was further incubated with horseradish peroxidase-labelled secondary antibody for 2 h and specific bands were visualized on X-ray films using Enhanced Chemiluminiscence Kit (Roche, Germany). β-actin was used as loading control.

2.18. Statistical analysis

Data are presented as mean ± S.E.M. The statistical analysis was done using Student's *t*-test with Microcal Origin 6.0 software. ** refers to $p < 0.01$, as compared to control, # refers to $p < 0.01$, as compared

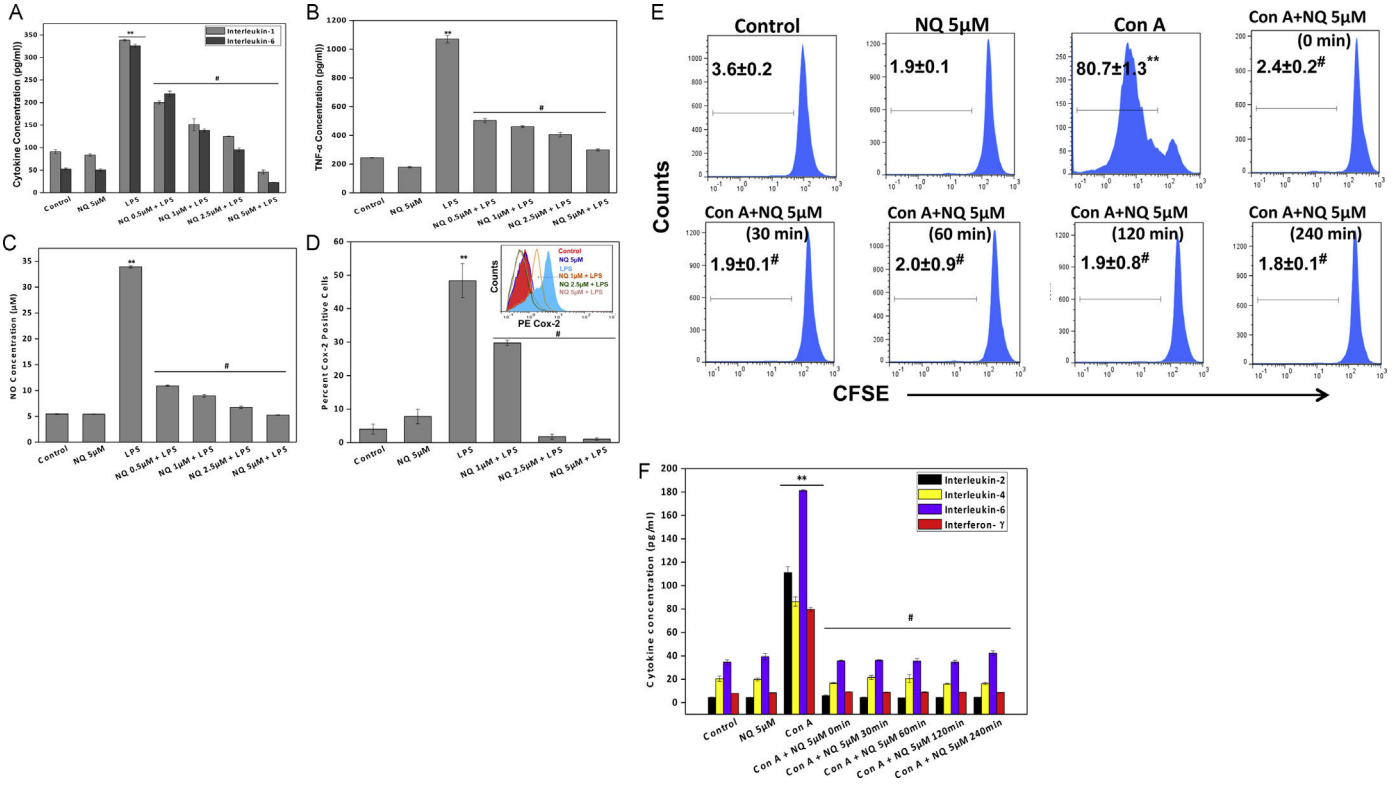

Fig. 2. NQ inhibited LPS induced secretion of inflammatory mediators in macrophage cells: RAW 264.7 cells were treated with NQ (0.5–5 μM) prior to stimulation with LPS (1 μg/ml) and cultured for 6 h or 24 h. (A) Cytokine (IL-1β and IL-6) concentration was measured in supernatant after 24 h and (B) TNF-α concentration was measured after 6 h of LPS treatment by ELISA. (C) Nitrite concentration was measured in culture supernatant after 6 h of stimulation with LPS using Griess reagent. (D) RAW 264.7 cells were treated with NQ (1–5 μM) prior to stimulation with LPS (1 μg/ml) and cultured for 24 h. Cells were harvested, fixed, permeabilized and stained with PE-labeled anti-Cox-2 antibody. Thirty thousand cells in each group were acquired in a flow cytometer. Graph represents the percent Cox-2 positive cells calculated from representative histograms. (E) CFSE labeled lymphocytes were stimulated with Con A (5 μg/ml) and then treated with NQ (5 μM) at indicated time intervals at 37 °C for 72 h. Thirty thousand cells in each group were acquired in a flow cytometer. Vehicle treated cells served as control. Percentage daughter cells were calculated from representative histograms. (F) Cytokine concentration was measured in supernatant after 24 h of Con A treatment by ELISA. Each bar represents mean ± S.E.M. from three replicates and two such independent experiments were carried out. ** $p < 0.01$, as compared to vehicle treated cells and # $p < 0.01$, as compared to Con A or LPS stimulated cells.

to stimulated cells and $ refers to $p < 0.01$, as compared to stimulated cells after NQ treatment.

3. Results

3.1. Pro-oxidants abrogated mitogen induced lymphocytes proliferation and cytokines secretion in vitro

CFSE stained lymphocytes were pre-treated with H_2O_2 (50 μM, 10 min exposure followed by washing with RPMI media) or *t*-BHQ (20 μM, 2 h) or NQ (5 μM, 2 h) prior to stimulation with Con A or anti-CD3/CD28 mAb. Effect of these pro-oxidants on mitogen induced increase in percent daughter cells was assessed by CFSE dye dilution using a flow cytometer. Mitogen stimulated group showed a significant increase in percentage of daughter cells as well as cytokine secretion. Pre-treatment of cells with H_2O_2, *t*-BHQ and NQ significantly inhibited Con A induced proliferation (Fig. 1A and B). Further, pre-treatment with different concentrations of NQ significantly inhibited mitogen induced proliferation and cytokine secretion (Fig. 1C–H). Live and dead assay showed that NQ did not induce any cytotoxicity in naive lymphocytes (Fig. 1I).

3.2. NQ inhibited the LPS induced secretion of inflammatory mediators in macrophage cells

RAW 264.7 cells were stimulated with LPS in the presence or absence of NQ and cultured for 6 h or 24 h. Pre-treatment of cells with NQ significantly inhibited LPS induced secretion of IL-1β, IL-6 and TNF-α (Fig. 2A and B). Further, NQ also significantly inhibited LPS induced nitric oxide release and Cox-2 levels in RAW 264.7 cells (Fig. 2C and D).

3.3. NQ inhibited the proliferation and cytokine secretion in activated lymphocytes

Experiments were carried out using activated lymphocytes to determine the therapeutic potential of NQ as an immunomodulatory agent. Lymphocytes were stimulated with Con A and then

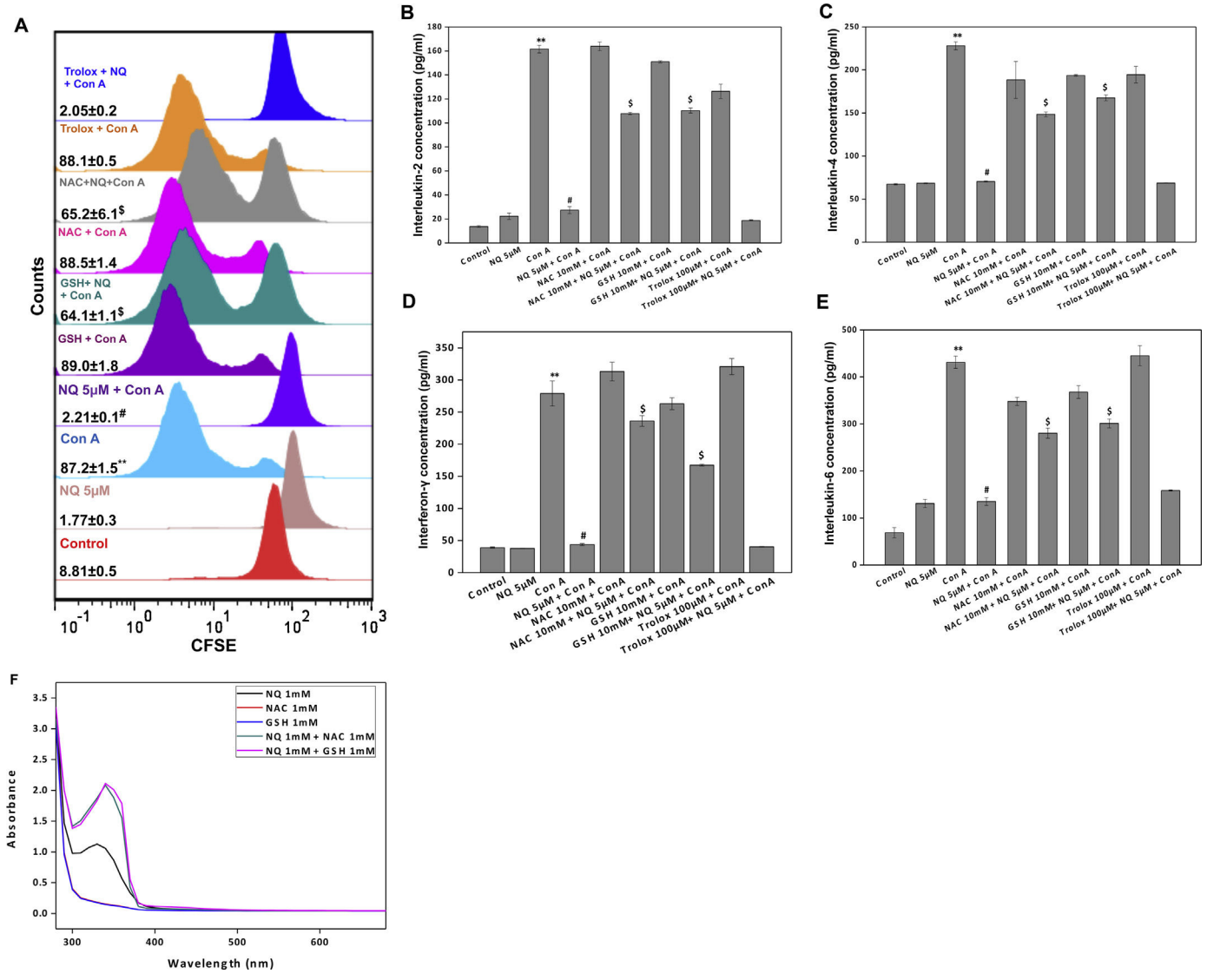

Fig. 3. Thiol antioxidants abrogated the immunosuppressive effect of NQ: CFSE labeled lymphocytes were treated with NAC (10 mM), GSH (10 mM) or Trolox (100 μM) for 2 h followed by treatment with NQ (5 μM) for 2 h and then stimulated with the Con A at 37 °C for 72 h. Thirty thousand cells in each group were acquired in a flow cytometer. (A) Representative flow cytometric histograms showing daughter cells with their respective mean ± S.E.M. in each treatment group. (B–E) Supernatant was harvested 24 h after stimulation and cytokine (IL-2, IL-4, IL-6 and IFN-γ) concentrations were estimated by ELISA. Each bar represents mean ± S.E.M. from three replicates and two such independent experiments were carried out. ** $p < 0.01$, as compared to vehicle treated cells, # $p < 0.01$, as compared to Con A stimulated group and $ $p < 0.01$, as compared to NQ treated and Con A stimulated group. (F) Direct interaction of NQ with NAC and GSH. To monitor the interaction, changes in absorption spectra of NQ was observed in presence or absence of NAC and GSH.

treated with 5 μM of NQ at different time points. NQ completely inhibited Con A induced proliferation and cytokines secretion (IL-2, IL-4, IL-6 and IFN-γ) even when added 2 h after mitogenic stimulation (Fig. 2E and F).

3.4. Thiol antioxidants abrogated the anti-inflammatory action of NQ in lymphocytes

To confirm the role of cellular redox in the observed anti-inflammatory activity of NQ, different thiol antioxidants (NAC, GSH) and non-thiol antioxidant (Trolox) were used. Lymphocytes were pre-treated with 10 mM NAC or 10 mM GSH or 100 μM Trolox for 2 h. These lymphocytes were then treated with 5 μM NQ for 2 h and stimulated with Con A for 24 h or 72 h. Both thiol and non thiol antioxidants per-se did not show any effect on proliferation and cytokines secretion of Con A stimulated cells. Thiol antioxidants, NAC and GSH, were able to abrogate the anti-inflammatory effects of NQ (Fig. 3A–E), whereas, non thiol antioxidant had no effect. As thiol antioxidants reverted the effect of NQ, experiments were carried out to investigate whether NQ interacts with NAC and GSH. Interestingly, NQ binds with NAC and GSH as evident from changes in absorption spectra of NQ in presence of NAC and GSH (Fig. 3F).

3.5. NQ activated redox sensitive transcription factor Nrf-2 by inducing oxidative stress in lymphocytes

Splenic lymphocytes incubated with NQ showed a decrease in GSH/GSSG ratio, in dose and time dependent manner (Fig. 4A and B). Modulation of cellular redox status is known to affect the redox sensitive transcription factors and other stress related regulatory proteins including Nrf-2 [35]. NQ treatment significantly increased the nuclear levels of Nrf-2 as seen by confocal microscopy and EMSA (Fig. 4C and D). NQ treatment also led to a significant increase in mRNA copy number of Nrf-2 and its dependent genes HO-1 and GCLC (Fig. 4E).

3.6. NQ induced protein S-thiolation and KEAP-1 glutathionylation in lymphocytes

Changes in redox status have been shown to induce post translational modifications of KEAP-1 thereby activating Nrf-2 and phase 2 detoxification enzymes [36,37]. We found that treatment of lymphocytes with NQ led to a significant increase in the total protein S-thiolation (Fig. 5A). Further, immunoprecipitation using anti-GSH antibody followed by Western blot analysis with anti-KEAP1 antibody revealed that NQ treatment

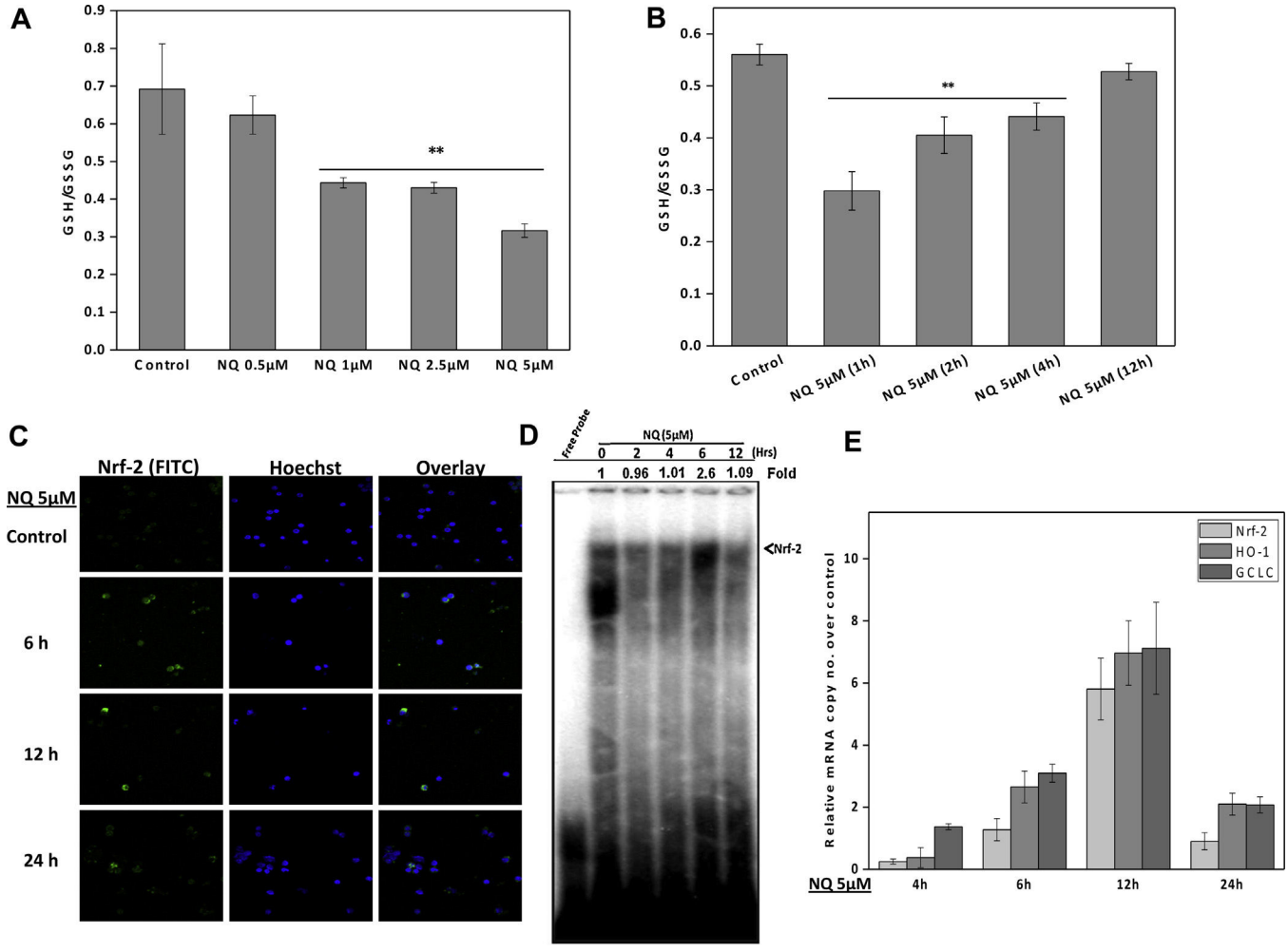

Fig. 4. NQ modulated cellular redox and induced Nrf-2 activation: (A and B) NQ reduced cellular GSH/GSSG ratio. Lymphocytes were treated with (0.5–5 μM) NQ for 2 h or NQ (5 μM) for indicated time intervals at 37 °C. GSH/GSSG ratio was estimated using enzyme recycling method. Data points represent mean ± S.E.M. from three replicates and two such independent experiments were carried out. ** $p < 0.01$, as compared to vehicle treated cells. (C) Lymphocytes were treated with NQ (5 μM) for 6 h, 12 h and 24 h, stained with FITC labelled anti-Nrf-2 antibody and Hoechst 33342. FITC (left)/Hoechst (mid)/overlay (right) is shown. (D) Lymphocytes were treated with NQ (5 μM) for 2 h, 4 h, 6 h and 12 h and EMSA was performed using nuclear extracts and specific signal intensities were subsequently quantified by Syngene Gene Tools software (Cambridge, UK) (E) Lymphocytes were treated with NQ for 4 h–24 h, mRNA was isolated and used for real time RT-PCR. Bar diagram shows relative mRNA copy number of Nrf-2, HO-1 and GCLC over control. Each bar represents mean ± S.E.M. from three replicates and two such independent experiments were carried out.

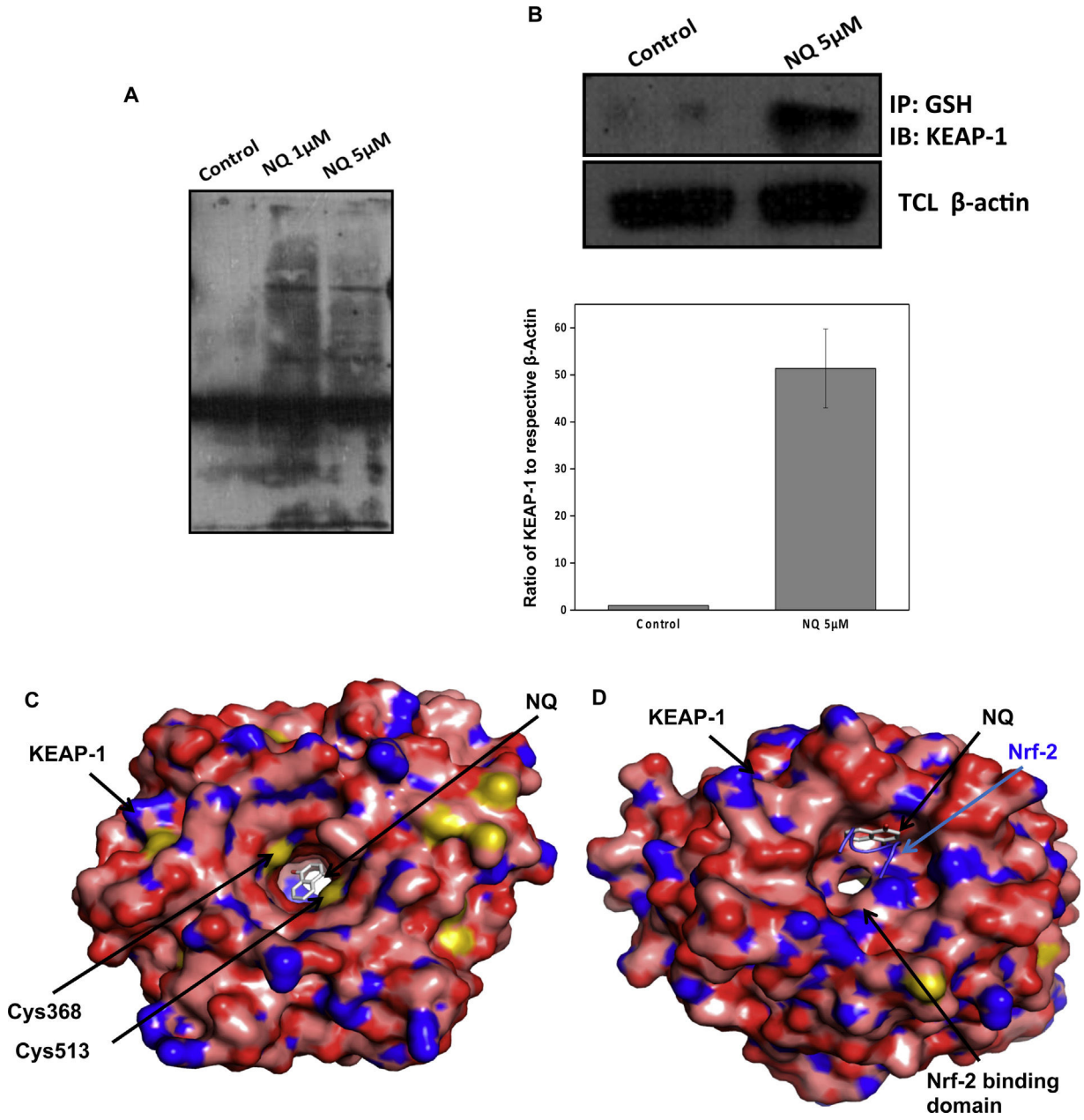

Fig. 5. NQ induced glutathionylation of KEAP-1: (A) NQ induced protein glutathionylation. Lymphocytes were treated with NQ (1 μM or 5 μM) for 4 h and whole cell lysates were fractionated on 10% non reducing SDS–PAGE, and electrotransferred to nitrocellulose membrane. Western blot analysis was performed using anti-GSH antibody. (B) Lymphocytes were treated with NQ (5 μM) for 4 h. Glutathionylated proteins were immune-precipitated from whole cell lysates using anti-GSH antibody followed by immunoblotting with anti-KEAP1 antibody. Three such independent experiments were carried out and specific signal intensities were subsequently quantified by Syngene Gene Tools software (Cambridge, UK) (C and D) "Blind docking" of NQ was done with KEAP-1 using Autodock Vina molecular docking software.

to lymphocytes induces glutathionylation of KEAP-1protein (Fig. 5B).

3.7. NQ binds to Kelch domain of KEAP-1 at two different locations

The "blind docking" of NQ against the Kelch domain of KEAP-1 revealed that NQ, apart from inducing KEAP-1 glutathionylation, can also activate Nrf-2 by interacting with the Kelch domain of KEAP-1 at two distinct locations with almost equal affinity. In the first place, it binds the central hole with a binding affinity ~6.7 kcal/mol. In this orientation, NQ is in close proximity of two cysteine residues, Cys368 and Cys513 (Fig. 5C). Since NQ is an electrophile, it is possible that it may form an adduct with nucleophilic sulfuhydryl group of these cysteine residues. But how these adducts would regulate/restrict the binding of Nrf-2 is not apparent. An alternate location where NQ binds KEAP-1 with almost similar binding affinity (6.0 kcal/mol) is at the Nrf-2 binding pocket of KEAP-1 (Fig. 5D). In this orientation, NQ superposes on Nrf-2 peptide backbone (shown in blue ribbon). Therefore, NQ could directly compete and restrict the binding of Nrf-2 to KEAP-1 and thereby prevent its ubiquitination and subsequent degradation.

3.8. NQ treatment induced IKKβ degradation and abrogated mitogen induced activation of NF-κB pathway

Since up-regulation of Nrf-2 is known to down-regulate NF-κB activation pathway via IKKβ degradation, experiments were performed to investigate the effect of NQ on mitogen induced activation of NF-κB pathway. Pre-treatment of cells with NQ for 2 h abrogated Con A induced NF-κB nuclear translocation and IκBα

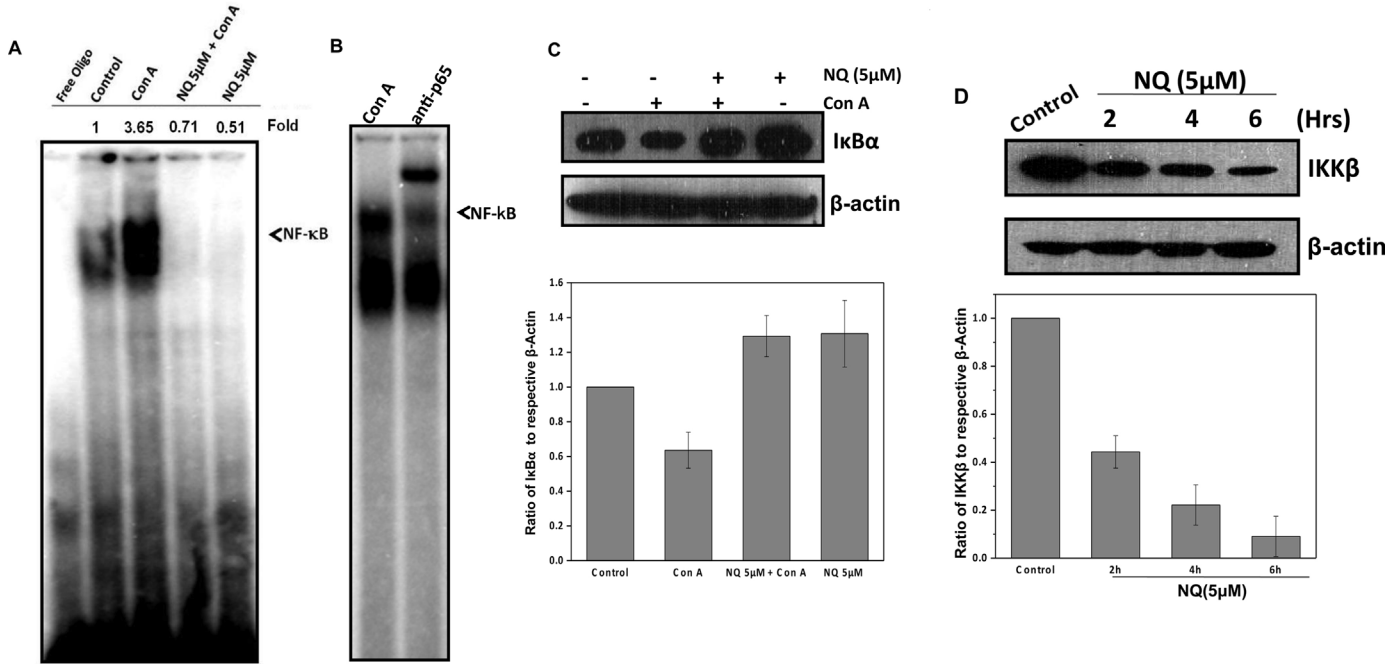

Fig. 6. NQ inhibited Con A induced NFκB activation: (A) Lymphocytes were treated with NQ (5 μM, 2 h) and stimulated with Con A (5 μg/ml) for 3 h. EMSA was performed using nuclear extracts to assay for NF-κB nuclear translocation. (B) Nuclear extracts prepared from Con A treated lymphocytes were incubated for 15 min with anti-p65 antibody and used for super shift assay. (C) Lymphocytes were incubated with NQ (5 μM, 2 h) and stimulated with Con A for 3 h. Western blot analysis was performed with cytosolic extracts using antibodies specific for IκBα and β-actin. (D) Lymphocytes were incubated NQ (5 μM) for 2 h, 4 h and 6 h followed by Western blot analysis with cytosolic extract using antibodies against IKKβ and β-actin. Specific signal intensities were subsequently quantified by Syngene Gene Tools software (Cambridge, UK). Three such independent experiments were carried out.

degradation (Fig. 6A and C). Anti-p65 antibody shifted the band to a higher molecular weight (Fig. 6B) confirming the specificity of retarded band as visualized by EMSA. Treatment of lymphocytes with NQ resulted in a time dependent decrease in the levels of IKKβ (Fig. 6D).

3.9. Anti-inflammatory and anti-proliferative effects of NQ were reverted by inhibition of Nrf-2/HO-1

To ascertain the role of Nrf-2 and its dependent genes in the observed anti-inflammatory effects of NQ, experiments were

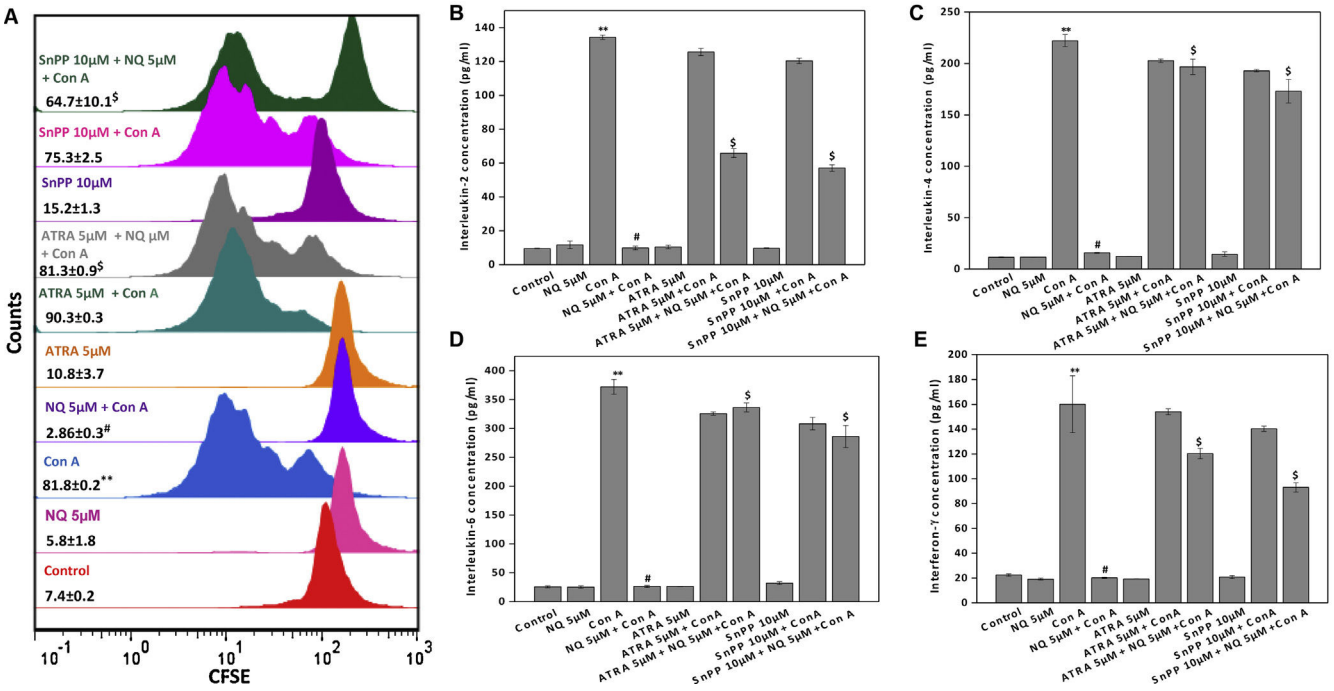

Fig. 7. Inhibitors of Nrf-2 and HO-1 abrogated the immunosuppressive effect of NQ: (A) CFSE labelled lymphocytes were treated with ATRA (5 μM) or SnPP (10 μM) for 2 h prior to incubation with NQ (5 μM, 2 h) and stimulated with Con A for 72 h at 37 °C. Thirty thousand cells in each group were acquired in a flow cytometer. Representative flow cytometric histograms with respective mean ± S.E.M. percent daughter cells of each group are given. (B–E) Cytokine levels were measured in supernatant 24 h after Con A treatment by ELISA. Each bar represents mean ± S.E.M. from three replicates. Two such independent experiments were carried out. ** $p < 0.01$, as compared to vehicle treated cells, # $p < 0.01$, as compared to Con A stimulated cells and $ $p < 0.05$, as compared to NQ treated and Con A stimulated cells.

performed using pharmacological inhibitors of Nrf-2/HO-1. Lymphocytes were pre-treated with 5 μM ATRA (Nrf-2 inhibitor) or 10 μM SnPP (HO-1 inhibitor) for 2 h. These cells were then incubated with 5 μM of NQ for 2 h prior to stimulation with Con A and were further monitored for cell proliferation and cytokine secretion. Pre-treatment with ATRA or SnPP significantly abrogated NQ mediated suppression of Con A induced proliferation (Fig. 7A) and cytokines secretion (Fig. 7B–E).

3.10. Lymphocytes from NQ injected mice showed decreased responsiveness to Con A and anti-CD3/CD28 mAb stimulation

In order to ascertain the in vivo anti-inflammatory activity of NQ, 2 mg/kg body weight of NQ was injected intra-peritoneally to mice. Splenic lymphocytes were isolated from vehicle and NQ administered mice 24 h after administration and were stimulated with Con A or plate bound anti-CD3/soluble anti-CD28 mAb for monitoring proliferation and cytokine secretion. Lymphocytes isolated from NQ injected mice showed reduced proliferation and cytokine secretion when stimulated with Con A (Fig. 8A, B and G) or anti-CD3/CD28 mAb (Fig. 8C, D and H) as compared to control group. Further, stimulation of lymphocytes isolated from NQ injected mice with Con A showed significantly less degradation of IκBα in cytoplasmic fraction and reduced nuclear translocation of NF-κB (Fig. 8E and F) as compared to control mice. Lymphocytes were also isolated from gut associated lymphoid tissue (GALT) and were stimulated with anti-CD3/CD28 mAb for 24 h. GALT lymphocytes isolated from NQ injected mice showed reduced cytokine secretion as compared to those from control mice (Fig. 8I).

4. Discussion

Balance between intracellular redox couples like GSH/GSSG, cysteine/cystine and reduced/oxidized thioredoxin maintains the

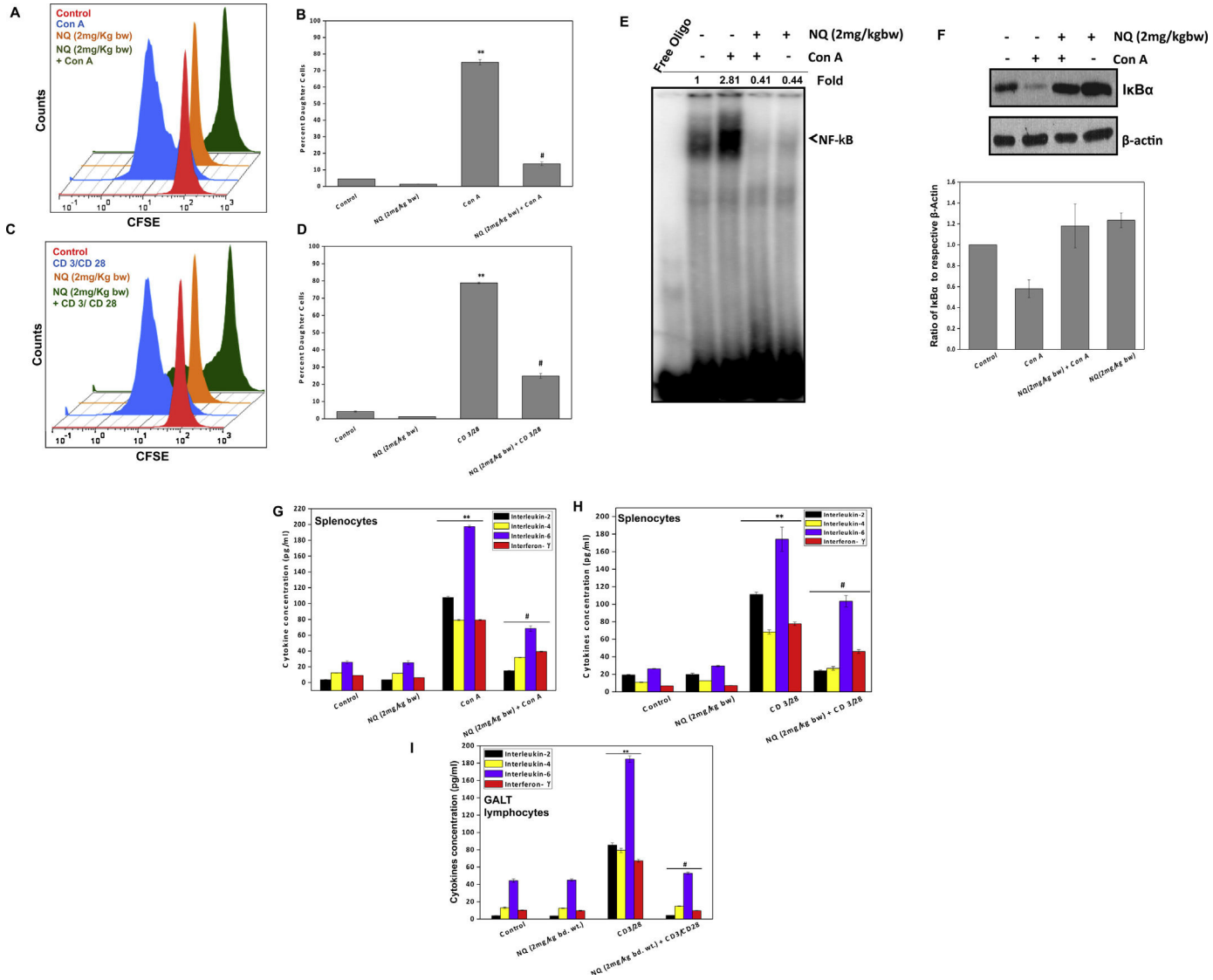

Fig. 8. NQ inhibited Con A and anti-CD3/CD28mAb induced proliferation and cytokine secretion by lymphocytes ex vivo: (A–D) Mice were injected i.p. with NQ (2 mg/kg body weight) or vehicle and splenic lymphocytes were isolated 24 h after injection. Cells were stained with CFSE and stimulated with either Con A (A and B) or anti-CD3/anti-CD28 mAb (C and D) to assess proliferation at 72 h or secretion of IL-2, IL-4, IL-6 and IFN-γ cytokines at 24 h (G and H) ex vivo. (E) Isolated splenic lymphocytes were stimulated with Con A for 3 h, nuclear extracts were prepared and subjected to EMSA. (F) Cytosolic extracts were subjected to Western blot analysis with antibodies specific for IκBα and β-actin and specific signal intensities were subsequently quantified by Syngene Gene Tools software (Cambridge, UK). Three such independent experiments were carried out. (I) Mice were injected i.p. with NQ (2 mg/kg bw) or vehicle and 24 h post injection, lymphocytes from GALT were isolated and stimulated with anti-CD3/anti-CD-28 mAb for 24 h. Supernatants were used to measure cytokine secretion. Each bar represents mean ± S.E.M. from three replicates. Two such independent experiments were carried out. ** $p < 0.01$ as compared to vehicle treated cells, $^{\#}$ $p < 0.01$ as compared to Con A stimulated cells.

cellular redox status requisite for generation of efficient immune response [38–40]. Depending on the extent of modulation in cellular redox levels, Nrf-2 and NF-κB may be activated [40–44]. Small increase in the basal ROS levels or depletion in cellular GSH/GSSG induces Nrf-2. An intermediate amount of ROS triggers an inflammatory response through the activation of NF-κB and AP-1, and a high level of ROS disrupts mitochondrial membrane potential resulting in cell death [45].

Thus we hypothesized that pro-oxidants may exhibit potent anti-inflammatory activity by perturbing cellular redox and activating Nrf-2 pathway. Pro-oxidants like NQ, H_2O_2 and *t*-BHQ indeed inhibited mitogen induced proliferation of splenic lymphocytes and NQ also inhibited cytokine secretion by activated lymphocytes (Fig. 1). The anti-inflammatory activity of NQ was evident from its ability to suppress LPS induced cytokine secretion, nitric oxide release and Cox-2 expression (Fig. 2A–D). Abrogation of NQ mediated immunosuppression only by thiol anti-oxidants and its interaction with thiol containing molecules further confirmed the crucial role of thiol modification in the anti-inflammatory activity of NQ (Fig. 3A–F).

Several studies have shown an indispensable role of Nrf-2 and its dependent gene HO-1 in the regulation of inflammatory responses via regulation of cytokines and pro-inflammatory protein and its deficiency was shown to increase susceptibility to inflammatory disorders [3,4,46–51]. NQ mediated decrease in GSH/GSSG ratio was associated with increased activation of Nrf-2 and an increased transcription of its dependent genes, hemoxygenase-1 and GCLC (Fig. 4A–E).

NQ induced S-thiolation of proteins suggested the involvement of oxidative stress and thiol depletion in inducing protein glutathionylation (Fig. 5A). Specific S-glutathionylation of KEAP-1 (Fig. 5B) may be responsible for Nrf-2 induction. Cys368 residue present in the DC domain of KEAP-1 directly associates with Nrf-2 and is known to be modified during Nrf-2 activation [52–55]. Docking studies indicated that NQ can come in close proximity of Cys368 and Cys513 of KEAP-1 located in the central hole (the barrel surrounded by propeller) of the Kelch domain (Fig. 5C) or it may prevent the binding of Nrf-2 to KEAP-1 by directly blocking the binding site of Nrf-2 in the KEAP-1 protein (Fig. 5D).

Activation of Nrf-2 has been shown to inhibit mitogen induced activation of NF-κB and its dependent pro-inflammatory genes [56–59]. NQ inhibited mitogen induced degradation of IκBα and activation of NF-κB in murine lymphocytes (Fig. 6A–C). It is well known that IKKβ plays an important role in immune responses by regulating the activation of NF-κB pathway [60–62]. KEAP-1 functions as IKKβ E3 ubiquitin ligase by directly interacting with E(T/S)GE motif of IKKβ. Depletion in KEAP-1 leads to accumulation and stabilization of IKKβ and up-regulation of NF-κB [19,63]. We observed that NQ induced IKKβ degradation in a time dependent manner (Fig. 6D). Based on these results we propose that KEAP-1 mediated IKKβ degradation in response to NQ might be responsible for the suppression of NF-κB pathway. To corroborate our hypothesis that activation of Nrf-2 pathway mediates anti-inflammatory action of NQ, pharmacological inhibitors of Nrf-2 and HO-1 were used. Both ATRA and SnPP significantly abrogated NQ mediated suppression of mitogen induced proliferation (Fig. 7A–E) confirming the involvement of Nrf-2/HO-1 pathway. Finally, we also explored the in vivo anti-inflammatory potential of NQ and observed that lymphocytes (splenic and GALT) isolated from NQ treated mice were hyporesponsive for mitogen induced activation as compared to control mice (Fig. 8A–I).

In conclusion, the present study demonstrates that pro-oxidants (H_2O_2, NQ and *t*-BHQ) inhibit immune responses and NQ induced oxidative stress leads to KEAP-1 protein modification and disruption of KEAP-1/Nrf-2 interaction resulting in activation of Nrf-2 pathway. Our results also demonstrate that induction of mild oxidative stress in lymphocytes by NQ leads to IKKβ degradation and suppression of NF-κB pathway. Further, we also highlight the potential of redox modifiers as promising anti-inflammatory and immune-suppressive agents.

Author contributions

SKS and DS conceived and designed the experiments; LG, MT and RSP performed the experiments; MK performed the in silico analysis; SKS, DS, and RC analyzed the data; SKS, DS, RC and wrote the manuscript in consultation with the other authors.

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