STUDIES ON THE PERTURBATION OF IMMUNE SYSTEM DURING INFLAMMATION AND ITS MODULATION BY PLUMBAGIN

By

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BHABHA ATOMIC REASERCH CENTRE, MUMBAI

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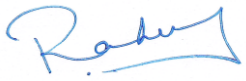

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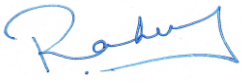

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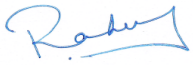

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PREAMBLE

Inflammation (Latin, inflammare, to set on fire) is the body's protective reaction to injurious stimuli in the form of infections, ionizing radiation, harmful chemicals, foreign particles, trauma, auto-antibodies and physical or thermal stress. This protective response of the tissue is mediated by the organism's immune system to repair the damage caused to its cells and vascularized tissues. While acute inflammation is an important element of the organism's defense repertoire, chronic inflammation can lead to several undesired side effects including arthritis, multiple sclerosis, cancer, diabetes, cardiovascular, pulmonary, and neurological diseases. Suppression of this unwanted and chronic activation of immune cells is desirable to avoid occurrence of the aforementioned inflammation associated disorders. Immunosuppression can be achieved by inhibiting lymphocyte response pathways, depleting lymphocytes, or diverting lymphocyte traffic. The immunosuppressive regimens currently employed involves the use of multiple drugs such as inhibitors of transcription (Cyclosporin A, tacrolimus), inhibitors of nucleotide synthesis (azathioprine, mycophenolate mofetil, mizoribine, leflunomide), inhibitors of growth factor signal transduction (sirolimus, leflunomide), and inhibitors of differentiation [1]. Each of these drugs target a discrete site in the lymphocyte/leukocyte cascade and each has distinct side effects on normal tissues which limits their long term usage. One of the major complications in patients receiving repeated blood transplantation is Graft-versus-host disease (GVHD) which is characterized by undesired immune activation and proinflammatory cytokine production leading to tissue destruction. The objective remains to identify novel anti-inflammatory & immunosuppressive agents that can be used in combination with the current drugs to optimize treatment for acute inflammation and prolong graft survival while limiting the side effects.

It is well known that transcription factor NF-κB is central to a series of cellular processes like inflammation, cell proliferation and apoptosis and is of particular importance in modulating the expression of immunoregulatory genes [2, 3]. Lately, plumbagin was shown to inhibit constitutive as well as inducible NF-κB activation and NF-κB regulated genes in tumor cells. It also inhibited DNA binding ability of NF-κB [4]. Several studies have shown that inhibition of NF-κB activation is a relevant strategy for alleviation of GVHD induced tissue damage [5, 6]. It was shown that cardiac graft rejection was slower when the transplantation was performed in p50 and p52 deficient mice [7]. Further, the mice expressing transdominant IκB-α did not reject transplanted hearts from

allogenic donors [8]. Based on these observations, we speculated that plumbagin, which was recently shown to inhibit constitutive as well as inducible NF-κB activation and NF-κB regulated genes in tumor cells, may show immunomodulatory effects and might have significant clinical application in prevention of inflammatory disorders. To test this hypothesis, the immunomodulatory effects of plumbagin were studied in murine lymphocytes in vitro and its in vivo anti-inflammatory efficacy was also investigated using mouse models of inflammatory disorders.

AIMS OF THE PRESENT STUDIES:

- To establish the immunosuppressive activity of plumbagin in lymphocytes in vitro in terms of inhibition of mitogen induced activation, cytokine secretion and proliferation of T cells.
- Comparison of sensitivities of different lymphocyte subsets (CD4+ T cells, CD8+ T cells and B cells) to plumbagin in response to mitogenic stimuli.
- To study the effect of plumbagin on different activation markers and co-stimulatory molecules involved in T cell activation.
- To study the mechanism of action of plumbagin mediated immunosuppression and compare it with clinically used immunosuppressive drugs.
- To study the in vivo efficacy of plumbagin in modulating inflammatory conditions like graft-versus-host-disease, allograft rejection and endotoxin shock.

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

(1) Introduction and Review of literature,
(2) In vitro anti-inflammatory effects of plumbagin,
(3) Mechanism of action of plumbagin,
(4) In vivo anti-inflammatory efficacy of plumbagin,
(5) Discussion and Conclusions
(6) Bibliography.

CHAPTER 1: INTRODUCTION AND REVIEW OF LITERATURE:

This chapter gives an introduction to the research problem and reviews the relavent literature in the field of inflammation, anti-inflammatory agents and their mechanism of

action. It is well known that the inflammatory response is finely orchestrated sequence of events initiated by any kind of trauma to a healthy vascularized tissue. This infection or injury to the tissue is followed by activation and migration of cells of the immune system to the site of trauma followed by the killing of microbes and clearance of infected cells. Finally, the surrounding tissue is removed to prevent microbial metastasis which is followed by the healing of tissues damaged by trauma or by the immune response. If at any step this inflammatory process is deranged, the outcome may include undesired infiltration of a tissue with aggregates of leukocytes (granulomas) that are sometimes embedded in proliferating synovial fibroblasts. Thus, development of immunosuppressive drugs is the key to successful inhibition of this chronic inflammation which can induce a wide variety of diseases (like cardiovascular diseases, cancer, diabetes, arthritis, Alzheimer's disease, pulmonary diseases, and autoimmune diseases). There is little doubt that immunosuppressive agents like cyclosporin A, tacrolimus, azathioprine, mycophenolate mofetil, sirolimus, leflunomide and glucocorticoids will continue to be used for the treatment of inflammatory disorders in the foreseeable future. However, decreasing the dose of administration of these drugs to the patients with inflammatory and autoimmune disorders, by improved combination or alternative therapies, is a major objective of current clinical research.

Engagement of antigen receptors on B and T lymphocytes results in activation of several immunoregulatory transcription factors like NF-κB, AP-1 and NF-AT. Further, several additional factors that play important roles in TCR-mediated NF-κB activation, including kinases of the Src (Lck and Fyn) and Syk (ZAP70) families, as well as adapters such as LAT and SLP-76, and intracellular signaling components including phospholipase C (PLC) γ1, Vav1, BCL10, 3-phosphoinositide-dependent kinase 1 (PDK1), CARMA1 [caspase recruitment domain (CARD) membrane-associated guanylate kinase (MAGUK) protein 1], and MALT1 (mucosa-associated lymphoid tissue 1) [9-11]. The importance of NF-κB in innate and adaptive immunity has been demonstrated in various disease and infection models [12]. Thus NF-kB has long been considered as an important target for development of novel anti-inflammatory drugs.

Phytochemicals derived from traditional medicine have shown promising results as immunosupressors and immunomodulators[13]. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), a quinone found in the plants of Droseraceae, Plumbaginaceae, Ancestrocladaceae and Dioncophyllaceae families, has been shown to possess potent anti-tumor activity [14]. It has been shown to inhibit the growth of Raji, Calu-1, HeLa and Wish

cell lines in vitro [15]. Plumbagin has been shown to exert several biological effects including anticancer, antiproliferative, chemopreventive, chemotherapeutic, and radiosensitizing properties in experimental animals as well as in tumor cells in vitro [16, 17]. A recent randomized double blind study using plant based formulation containing plumbagin as one of the active components showed positive results in the management of chronic obstructive pulmonary disease in humans [18]. Lately, plumbagin was shown to inhibit constitutive as well as inducible NF-κB activation and NF-κB regulated genes in tumor cells. Based on these observations, we speculated that plumbagin may show immunomodulatory effects and might have significant clinical application in the treatment of inflammatory disorders.

CHAPTER 2: IN VITRO ANTI-INFLAMMATORY EFFECTS OF PLUMBAGIN: This chapter summarizes the in vitro immunomodulatory properties of plumbagin as measured in terms of its ability to inhibit mitogen induced activation, cytokine secretion and proliferation of T cells. This chapter also describes effects of plumbagin on different lymphocyte subsets (CD4+, CD8+ and B cells) as well as on different activation markers and co-stimulatory molecules involved in activation of T cells and B cells.

Plumbagin inhibited T cell proliferation in response to polyclonal mitogen Concanavalin A (Con A) and anti-CD3/CD28 monoclonal antibodies in a dose dependent manner in vitro. Plumbagin inhibited cell cycle progression in Con A stimulated lymphocytes by arresting them in G1 phase of cell cycle. At these immunosuppressive doses (up to 5 µM), plumbagin did not reduce the viability of lymphocytes as assessed by propidium iodide staining, annexin-V staining and DNA fragmentation assay. Further, the inhibition of lymphocyte proliferation by plumbagin was accompanied by a decrease in the levels of Con A and anti-CD3/CD28 mAb induced IL-2, IL-4, IL-6 and IFN-γ cytokines. Plumbagin also inhibited anti-CD3/CD28 mAb induced proliferation and cytokine secretion of CD4+ T cells, CD8+ T cells as well as LPS induced proliferation B cells. Plumbagin treatment also inhibited nitric oxide, TNF-α, IL-6 and Prostaglandin E2 production by RAW264.7 cells as well as TNF-α, IL-1β and IL-6 production by splenic macrophages.

Further, we studied the effect of plumbagin on mitogen induced upregulation of co-stimulatory and activation markers on T cells and B cells. Treatment of lymphocytes with plumbagin prior to Con A stimulation significantly inhibited mitogen induced upregulation of CD69 (early T cell activation marker), CD25 (IL-2 receptor alpha), CD54

(ICAM-1), CD71 (Transferrin receptor protein 1) and CD28 (co-stimulatory molecule) expression on T cells. The inhibitory effects of plumbagin on lipopolysaccharide induced upregulation of co-stimulatory markers CD80 and CD86 on B cells emphasize its ability to inhibit B cell activation and antigen presentation. We also observed that lymphocytes that were washed after treatment with plumbagin did not secrete IL-2 even after they were rested for upto 72h indicating that plumbagin treatment may render these cells anergic.

CHAPTER 3: MECHANISM OF ANTI-INFLAMMATORY EFFECTS OF PLUMBAGIN: This chapter summarizes the studies that were carried out to elucidate the mechanism of immunomodulatory effects of plumbagin in murine splenic lymphocytes. This chapter details the identification of possible molecular targets of plumbagin in resting and activated lymphocytes. The biochemical and signaling mechanisms responsible for the novel anti-inflammatory action of plumbagin are described in this chapter.

Plumbagin was earlier shown to generate reactive oxygen species (ROS) in tumor cells leading to DNA damage and cytotoxicity [19-21]. Oxidative stress has been shown to modulate signaling pathways through modulation of thiol groups present and induce protein glutathionylation [22-25]. We hypothesized that the anti-inflammatory effects of plumbagin may be due to its ability to perturb the redox balance in lymphocytes leading to modification of critical signaling molecules required for activation of lymphocytes. We found that plumbagin disrupted cellular redox homeostasis and increased basal ROS levels. Plumbagin-induced increase in intracellular ROS was not abrogated by specific inhibitors of NADPH oxidase, xanthine oxidase, and mitochondrial complex I, but thiol containing antioxidant N-acetylcysteine significantly abrogated plumbagin induced ROS levels in these cells. Further, we observed a decrease in the GSH levels in plumbagin treated cells. Therefore plumbagin may exerts its effects by depleting GSH in lymphocytes and this depletion of intracellular thiols by plumbagin could be responsible for increased ROS levels in lymphocytes. It was also observed that the activity of catalase was significantly higher in plumbagin-treated cells. This may be due to cellular response to increased levels of H_2O_2 following treatment with plumbagin. In order to determine whether increased ROS levels or decreased thiol content or both were responsible for immunosuppressive action of plumbagin, effect of thiol and non-thiol antioxidants was investigated on suppression of mitogen-induced T-cell activation by plumbagin. It was observed that suppressive effects of plumbagin on mitogen induced cytokines and T-cell proliferation were ameliorated by thiol-containing antioxidants GSH, DTT, and NAC, but

not by non-thiol antioxidants (Trolox and MnTBAP)suggesting that modulation of cellular thiol levels is very critical for immunosuppression by plumbagin. Our results showed that disruption of cellular redox by plumbagin affected specific signaling events following TCR ligation with mitogen (including NF-κB, ERK, Bcl-2, Bcl-xL, and cyclin A) which are involved in cellular survival and proliferation. However, plumbagin did not inhibit activation of P38MAPkinase and JNK and signals emanating from costimulatory molecule in activated T cells. The suppressive effects of plumbagin on mitogen-induced signaling events were also sensitive to GSH. These results indicate that the mechanism of action of plumbagin is via modulation of cellular redox status.

The modulation of thiol groups on proteins could be in terms of direct reaction with plumbagin or glutathionylation of proteins or formation of disulfide bridge between proteins. Our results demonstrated that plumbagin indeed formed an adduct with GSH and also converted GSH to GSSG in cell-free systems. Further, it was observed that plumbagin modulates thiol groups present on the proteins as evinced from decrease in iodoacetamide reactive thiol groups of the proteins. The results using Bio-GEE demonstrated formation of PSSG (protein–glutathione adducts) in plumbagin treated cells. Plumbagin also induced glutathionylation of proteins as assessed by Western blot using anti-GSH antibody and induced glutathionylation of P65 subunit of NFκB.

CHAPTER 4: IN VIVO ANTI-INFLAMMATORY EFFICACY OF PLUMBAGIN: This chapter details the studies carried out to establish the in vivo anti-inflammatory efficacy of plumbagin using mouse models of graft-versus-host disease (GVHD), allograft transplantation and lipopolysaccharide induced septic shock. The mechanism of in vivo action of plumbagin was also elucidated in detail using LPS induced septic shock model and is described in this chapter.

It was observed that lymphocytes from plumbagin treated mice showed decreased responsiveness to Con A stimulation. The cytokine production and proliferation response of lymphocytes isolated from plumbagin (2 mg/kg body weight, 24h) treated mice was measured and compared to that from vehicle treated control mice. The lymphocytes from plumbagin treated mice showed significantly lower production of IL-4, IL-6 and IFN-γ cytokines as compared to that in lymphocytes taken from vehicle treated control mice when stimulated with Con A. Interestingly, the Con A induced proliferation response of T cells was also significantly diminished by plumbagin administration as compared to that in cells taken from control mice.

To test immunosuppressive activity of plumbagin in GVHD model, splenocytes from C57BL/6J mice were incubated with plumbagin (1µM, 4h) and transferred to immunocompromised Swiss mice. The recipient mice that receieved vehicle treated cells developed GVHD that led to 70% death within 45 days, demonstrating typical symptoms of GVHD, including alopecia, scleroderma, hunched posture, diarrhea, and progressive weight loss. However, in the recipients injected with plumbagin treated cells, 90% of the mice survived in better health. Furthermore, the mice injected with plumbagin treated cells experienced inconspicuous weight loss as compared to control group.

The in vivo immunosuppressive efficacy of plumbagin was also studied in a mouse model of allograft transplant. In this model allo-skin grafts were obtained from tail skin of C57BL/6 mice. A graft bed of approximately $0.6cm^2$ was made by surgical incision on the dorso-lateral side of the recipient Swiss mice. Tail skin graft of the size approximately $0.6cm^2$ were grafted on to the excised upon wound to cover it and the survival/rejection of graft was monitored by daily observation. The day of complete necrosis and physical rejection of the grafted tissue was scored as the day of rejection. It was observed that administration of plumbagin (mean allograft survival 13.4 days) significantly delayed rejection of allograft in mice as compared to untreated mice (mean allograft survival 8 days).

Further, the ability of plumbagin to ameliorate acute inflammatory response was studied using a mouse model of LPS induced septic shock. It was observed that plumbagin rescued mice from lethal LPS-induced septic shock. Plumbagin administration to mice decreased LPS induced increase in the serum levels of nitric oxide, tumor necrosis factor-α and IL-12, IL-6, IL-10 and IFN-γ. In agreement with these results, LPS induced increase in the serum levels of Serum Glutamic Oxaloacetate Transaminase, Serum Glutamic Pyruvate Transaminase and blood urea nitrogen were also inhibited by plumbagin administration. Plumbagin administration to mice also inhibited LPS induced activation of NF-κB and ERK in liver. These results clearly demonstrate the in vivo anti-inflammatory efficacy of plumbagin in ameliorating inflammatory disorders.

CHAPTER 5: DISCUSSION AND CONCLUSIONS: This chapter discusses the implications of the studies described in chapters 2, 3 and 4. In the present study, we have shown the novel anti-inflammatory and immunosuppressive effects of plumbagin in vitro and in vivo. These results highlight a potential application of plumbagin as an immunosuppressive agent which may be used in the treatment of inflammatory disorders.

This study also showed a ROS-independent mechanism of anti-inflammatory action of plumbagin. Modulation of cellular thiols played a more significant role than increased ROS levels in biological actions of plumbagin. For the first time, evidence for a role for glutathionylation of cellular proteins as a mechanism of anti-proliferative action of plumbagin is provided. Further, mechanistic basis for potential therapeutic application of plumbagin as an immunosuppressive or anti-inflammatory drug is highlighted.

The major conclusions drawn from this study are:

1. Plumbagin inhibits mitogen induced lymphocyte cell activation, proliferation and cytokine secretion.
2. Plumbagin also inhibits upregulation of activation markers and co-stimulatory molecules involved in T cell and B cell activation.
3. Plumbagin modulated cellular redox status and acts via a novel redox dependent mechanism which is independent of ROS but dependent on GSH.
4. Anti-inflammatory effects of plumbagin are mediated by inhibition of NF-kappaB and MAPKinase activation in lymphocytes.
5. Plumbagin interacts with free thiol groups present on proteins and also induces protein S-glutathionylation in lymphocytes.
6. Plumbagin (2mg/Kgbw) administration to mice could prevent mortality and morbidity associated with graft-versus-host disease and septic shock. It was also able to significantly delay allograft rejection in mice model of allograft transplant.

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List of Abbreviations

PAMP	Pathogen-associated molecular patterns
PRRs	Pattern-recognition receptors
TLR	Toll like receptor
LPS	Lipopolysaccharide
NADPH	Nicotinamide adenine dinucleotide phosphate
ROS	Reactive oxygen species
CD	Cluster of differentiation
MHC	Major histocompatibility complex
APCs	Antigen presenting cells
Th	Helper T cells
Tc	Cytotoxic T cells
TCR	T cell receptor
CTLA	Cytotoxic T-Lymphocyte Antigen 4
ICAM-1	Intercellular adhesion molecule-1
NF-κB	Nuclear factor kappaB
LFA-1	Lymphocyte function-associated antigen 1
ITAM	Immunoreceptor-based tyrosine activation motif
Lck	lymphocyte-specific protein tyrosine kinase
ZAP-70	Zeta-chain-associated protein kinase 70
PTK	Protein tyrosine kinase
LAT	Linker for Activation of T Cells
SLP-76 `	SH2 domain–containing leukocyte phosphoprotein
PLCγ	Phosphoinositide phospholipase C
PI3K	Phosphoinositide 3-kinase
DAG	Diacylglycerol
PIP2	Phosphatidylinositol (4,5) bisphosphate
IP3	Inositol 1,4,5-triphosphate

PKC	Protein kinase C
MAPK	Mitogen activated protein kinase
ERK	Extracellular signal-regulated kinase
AP-1	Activator protein-1
JAK	Janus activated kinase
STAT	Signal transducer and activator of transcription
NFAT	Nuclear factor of activated T cells
IL	Interleukin
RHD	Rel homology domain
IKK	IκB kinase
IFN	Interferon
TNF	Tumor necrosis factor
GSH	Glutathione
GSSG	Glutathione disulphide
Cox	Cycloxygenase
NOS	Nitric oxide synthase
GVHD	Graft-versus-host disease
HLA	human leukocyte antigen
SIRS	Systemic Inflammatory Response Syndrome
NSAIDs	Non-steroidal anti-inflammatory drugs
IBD	Inflammatory bowel disease
Akt	Atypical kinase
Con A	Concanavalin A
CFSE	Carboxy fluorescein diacetate succinimidyl ester
FBS	Fetal Bovine serum
PBS	Phosphate buffered saline
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
PE	R-phycoerythrin

DCFDA	5-(and-6)-carboxy-2,7-dichlorofluorescein diacetate
Bio-GEE	Glutathione ethyl ester biotin amide
MCB	Monochlorobimane
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel Electrophoresis

CHAPTER - I

INTRODUCTION

AND

REVIEW OF LITERATURE

1.1 INTRODUCTION

Inflammation (Latin, inflammare, to set on fire) is the body's protective reaction to injury from infections, ionizing radiation, harmful chemicals, foreign particles, trauma, auto-antibodies and physical or thermal stress. It involves pathogen killing as well as tissue repair processes and helps to restore homeostasis at infected or damaged sites. A well regulated inflammatory response which is self-limiting and resolves rapidly without causing excessive damage to the host is called as acute inflammatory reaction. This involves the activation of negative feedback mechanisms such as the secretion of anti-inflammatory cytokines, inhibition of pro-inflammatory signaling cascades, shedding of receptors for inflammatory mediators and activation of regulatory cells. However, inflammatory responses that fail to regulate themselves can become chronic and contribute to the initiation and progression of disease [4]. Typical characteristics of chronic inflammatory responses include loss of barrier function, responsiveness to a normally benign stimulus, infiltration of inflammatory cells into compartments where they are not normally found in such high numbers, and overproduction of oxidants, cytokines, chemokines, eicosanoids and matrix metalloproteinases [5 6]. The levels of these mediators amplify the inflammatory response are harmful to self tissue and thus contribute to the clinical symptoms. Inflammation may be classified into four types, the mechanisms of which in part overlap: (i) inflammation caused by innate and acquired immunity against infectious agents, in which cells are activated and mediators are released to prevent or combat infection and remove foreign material; (ii) inflammation caused by different inhaled agents ('irritants') like diesel exhaust particles, ozone and endotoxin; (iii) allergic inflammation, in which specific IgE antibodies bound to mast cells upon cross-linking by allergen cause the immediate release of a number of inflammatory mediators and

activation of inflammatory cells; and (iv) neurogenic inflammation, mediated by the neural system.

Suppression of this unwanted and chronic activation of immune cells is desirable to avoid occurrence of the aforementioned inflammation associated disorders. Conditions with a well recognised inflammatory component are usually treated with general or specific anti-inflammatory pharmaceuticals. Immunosuppression can be achieved by inhibiting lymphocyte response pathways, depleting lymphocytes, or diverting lymphocyte traffic. The immunosuppressive regimens currently employed involve the use of multiple drugs such as inhibitors of calcium signaling and translation (Cyclosporin A, tacrolimus), inhibitors of nucleotide synthesis (azathioprine, mycophenolate mofetil, mizoribine, leflunomide), inhibitors of growth factor signal transduction (sirolimus, leflunomide), and inhibitors of differentiation (deoxyspergualin) [7 8]. Each of these drugs target a discrete site in the T-cell activation cascade and each of them induce a distinct set of side effects on normal tissues which limits their long term usage.

There is little doubt that immunosuppressive agents like Cyclosporin A, tacrolimus, azathioprine, mycophenolate mofetil, sirolimus, leflunomide and glucocorticoids will continue to be used for the treatment of inflammatory disorders in the near future. However, decreasing the dose of administration of these drugs in patients with inflammatory and autoimmune disorders, by improved combination or alternative therapies, is a major objective of current clinical research. Phytochemicals derived from traditional medicine have shown promising results as immunosupressors and immunomodulators. Plants derived molecules have been the basis of many traditional medicine systems throughout the world for thousands of years and continue to provide mankind with new remedies. Many natural dietary bioactive compounds have established

pharmacological effects and/or can significantly alter activity of therapeutic agents by modulating biochemical pathways [9, 10].

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), a quinone found in the plants of *Droseraceae, Plumbaginaceae, Ancestrocladaceae and Dioncophyllaceae* families, has been shown to possess potent anti-tumor activity [11]. A recent randomized double blind study using plant based formulation containing plumbagin as one of the active components showed positive results in the management of chronic obstructive pulmonary disease in humans [12]. However, there are no reports on the immunomodulatory effects of plumbagin in lymphocytes. A thorough examination of the immunomodulatory properties of plumbagin may facilitate its use for application in human clinical trials. It is well known that transcription factor NF-κB is central to a series of cellular processes like inflammation, cell proliferation and apoptosis and is of particular importance in modulating the expression of immunoregulatory genes [13]. Lately, plumbagin was shown to inhibit constitutive as well as inducible NF-κB activation and NF-κB regulated genes in tumor cells. It also inhibited DNA binding ability of NF-κB [14].

Based on these observations, we speculated that plumbagin may show immunomodulatory effects and might have significant clinical application in prevention of inflammatory disorders. To test this hypothesis, the immunomodulatory effects of plumbagin were studied in murine lymphocytes in vitro and in vivo.

1.2 AIMS OF THE PRESENT STUDIES:

1. To establish the immunosuppressive activity of plumbagin in lymphocytes in vitro in terms of inhibition of mitogen induced activation, cytokine secretion and proliferation of T cells.
2. Comparison of sensitivities of different lymphocyte subsets (CD4+ T cells, CD8+ T cells and B cells) to plumbagin in response to mitogenic stimuli.
3. To study the effect of plumbagin on different activation markers and co-stimulatory molecules involved in T cell activation.
4. To study the mechanism of action of plumbagin mediated immunosuppression and compare it with clinically used immunosuppressive drugs.
5. To study the in vivo efficacy of plumbagin in modulating inflammatory conditions like graft-versus-host-disease, allograft rejection and endotoxin shock.

1.3 REVIEW OF LITERATURE

To maintain its integrity, it is vital for an organism to discriminate between biological entities that have to be eradicated (anything that may harm the organism) and structures that must not be attacked, e.g., the cells of our own body or useful microflora. The human body is continually exposed to organisms that may be inhaled, swallowed, or inhabits our skin and mucous membranes. These organisms are primarily microbes such as bacteria, parasites and fungi that can cause infections. The human body provides a conducive environment for many of these microbes and at the same time offers limitless resources that microbes can use for generation of energy and reproduction. The immune system is responsible for keeping out these microbes and, if they manage to penetrate the organism, to seek them out and neutralize them.

1.3.1 The immune system

The term immunity is derived from the Latin word *Immunis* meaning "to make safe". All multicellular organisms need to guard themselves against infectious organisms, collectively called as pathogens. The immune system is an interactive network of lymphoid cells, tissues, organs, humoral factors and cytokines that work in co-ordination to defend the organism against attacks by “foreign” invaders. The immune response is divided into two “lines of defense” based on the speed and specificity of the reaction (Table 1.1). The first line of defense is a non-specific (no memory) response to an antigen (substance to which the body regards as foreign or potentially harmful) known as the innate immune system. It includes physical, chemical, and microbiological barriers as well as cells of the immune system (neutrophils, monocytes, macrophages, complement proteins and acute phase proteins) and provides immediate defense against invading microbes [15]. This type of response is even in simple multicellular organisms and

underscores its important role in survival of the organism [16]. The innate immune response has the ability to thwart the growth of nascent infection as well as it can check the spread of an infection for a few days. This response is rapid, and is unable to "memorize" the same pathogen in case the body be exposed to it in the future [17]. The second line of defense celled as the adaptive immune system, displays a high degree of memory and specificity which is a hallmark of immune system of higher animals. The adaptive immune response is very precise and includes antigen-specific reactions mediated by T lymphoctyes and B lymphocytes, however, it takes several days to develop. Further, it possesses a greater degree of specificity as compared to the innate responses and has immunological memory because of which it evokes a more potent response on subsequent exposure to the same pathogen [18, 19].

1.3.2 The innate immune response

The term "innate" refers to that part of the immune system with which does not change or adapt to specific pathogens. It provides an early line of defense to keep early infection in check, giving the adaptive immune system adequate time to put together a more specific response. The innate immune response is based on its ability to recognize a few highly conserved structures present in large groups of pathogenic microbes. These conserved structures are referred to as pathogen-associated molecular patterns (PAMP) and the receptors of the innate immune system that recognize them are called pattern-recognition receptors (PRRs). The recognition of PAMPs by a limited number of evolutionarily conserved PRRs helps in recognition and elimination of majority of pathogens belonging to diverse species of bacteria, virus and fungi. Bacterial lipopolysaccharide, peptidoglycan, lipoteichoic acids, mannans, bacterial DNA, double-stranded RNA, and glucans are among the pathogen-associated molecular patterns that are

recognized by PRRs [20, 21]. These PAMPs are produced only by microbial pathogens and not by their hosts, for example, lipopolysaccharide is synthesized only by bacteria. CD14, a member of Toll like receptor (TLR) family is a PRR that recognizes lipopolysaccharide and alerts the host against invading bacteria. Further, the ligands recognized by PRRs present on the cells of innate immune system are evolutionarily conserved structures essential for the survival or pathogenicity of these microorganisms and are usually invariant structures shared by a wide sphere of pathogens [22]. For example, all gram-negative bacteria have lipopolysaccharides (LPS) and therefore, the lipopolysaccharide pattern-recognition receptor of the host can detect the presence of virtually any gram-negative bacterial infection. The LPS receptor is hardwired to downstream signaling cascade which would ensure bacterial killing by the effector cells without triggering their proliferation which is responsible for the swift kinetics of innate immune responses. The signals induced in the cells of the innate immune response upon recognition of pathogen-associated molecular pattern further controls the activation of adaptive immune responses; the adaptive immune system responds to a pathogen only after it has been recognized by the innate immune system.

Table1.1: Features of Innate and adaptive immune system

	INNATE SYSTEM	ADAPTIVE SYSTEM
COMPONENTS		
PHYSICAL & CHEMICAL BARRIERS	Skin, mucous, anti-microbial chemicals	Cutaneous and mucosal immune system
CELLS	Phagocytes (macrophages Neutrophils,, Mast cells) Natural Killer cells	Lymphocytes (B cells and T cells)
CIRCULATING EFFECTOR MOLECULES	Complement, Lysozyme, Acute phase proteins, Interferons	Cytokines,Antibodies
CHARACTERISTICS		
SPECIFICITY	Recognition of crude molecular patterns	Recognition of specific microbial and non-microbial antigens
DIVERSITY	Limited, germline encoded	Very large, generated by somatic recombination of gene segments
MEMORY	No	Yes

1.3.3 Characteristics/Components of innate immune response:

1. **Neutrophil recruitment**: The initial phase of the innate reaction is recruitment and activation of neutrophils to the site of infection, which ultimately leads to eradication of harmful pathogens [23]. Neutrophils possess a characteristic multilobular nucleus and play a central role in the development of acute inflammation. During an infection neutrophils migrate to the site of inflammation using a multistep process involving proinflammatory mediators, adhesion molecules, chemoattractants, and chemokines [24]. These neutrophils then phagocytose the infectious microbes and form a membrane-

bound vesicle around it called the phagosome. Inside the neutrophil, this is followed by fusion of the phagosome with neutrophil cytoplasmic granules leading to the formation of the phagolysosome. In the phagolysosome, killing of the organism then occurs by an oxygen-dependent response called as the respiratory burst. Respiratory burst involves sequential reduction of oxygen by an NADPH oxidase leading to generation of reactive oxygen species (ROS), such as hydrogen peroxide, hydroxyl radicals and singlet oxygen. The neutrophil cytoplasmic granules also contain acidic and alkaline phosphatases, defensins, myeloperoxidase, elastase, proteinase, cathepsin G, gelatinase, tryptase and peroxidase which play a crucial role in the successful elimination of the phagocytosed microbes. Neutrophils also these enzymes to kill extracellular bacteria using neutrophil extracellular traps (NETS). Besides these, they use lactoferrin and lipocalin 2 to chelate iron and restrict its supply to bacteria [25].

2. **Complement system:** The complement pathway consists of a series of over thirty proteins in plasma that are part of the immune response (denoted by the letter C and followed by a number) which are secreted by both hepatocytes and monocytes. The complement cascade can be activated, with amplification stages, by both the adaptive immune system (classical pathway) and innate immune system (alternative pathway). Regulation of the complement cascade is essential to protect host cells from damage and this is achieved by a series of regulatory proteins, which are expressed on the host cells [26].

   The functions of the complement system may be summarized as follows:

   a. Opsonisation: enhances the phagocytosis of antigens

   b. Lysis or destruction of foreign cells through damage of plasma membrane

   c. Chemotaxis: attracting macrophages and neutrophils

   d. Activation of mast cells

e. Increasing vascular permeability

f. Smooth muscle contraction

3. **Natural Killer cells:** Natural killer (NK) cells are large, granular, bone marrow-derived lymphocytes that do not express any specific antigen receptors. They are identified by the surface expression of CD16 and CD56 and comprise 5–15% of the peripheral blood mononuclear cells in normal individuals [27, 28]. NK cells are particularly important in responding to viral infections and play a crucial role in the initial defense of the organism against pathogens [29]. They mount a swift response to infected or transformed cells by either directly killing the abnormal cells or by releasing immunomodulatory chemokines and cytokines that initiate subsequent adaptive immune response at the site of infection [30]. The activity of NK cells is normally controlled by inhibitory receptors that recognize MHC class I expression on target cells. This allows NK cells to survey tissues for normal MHC class I expression. When MHC class I molecules are down-regulated or absent, NK cells are released from the inhibitory influence of these receptors which leads to killing of these target cells [31]. Target cell killing is achieved by secretion of perforins onto the surface of the cell to which the natural killer cell has adhered. Perforins make holes in the cell membrane and granzymes are injected through the pores which lead to induction of apoptosis in the target cell.
4. **Mast cells and basophils:** Both mast cells and basophils are very similar in that both contain electron dense granules in the cytoplasm. Basophils derive their name from the affinity of their cytoplasmic granules for certain basic dyes. Mast cells are traditionally considered tissue-resident cells, whereas basophils are normally found only in peripheral blood. Mast cells and basophils bear high-affinity receptors for IgE FcγRI (CD23) which rapidly absorb any local IgE. Crosslinking of these receptors by

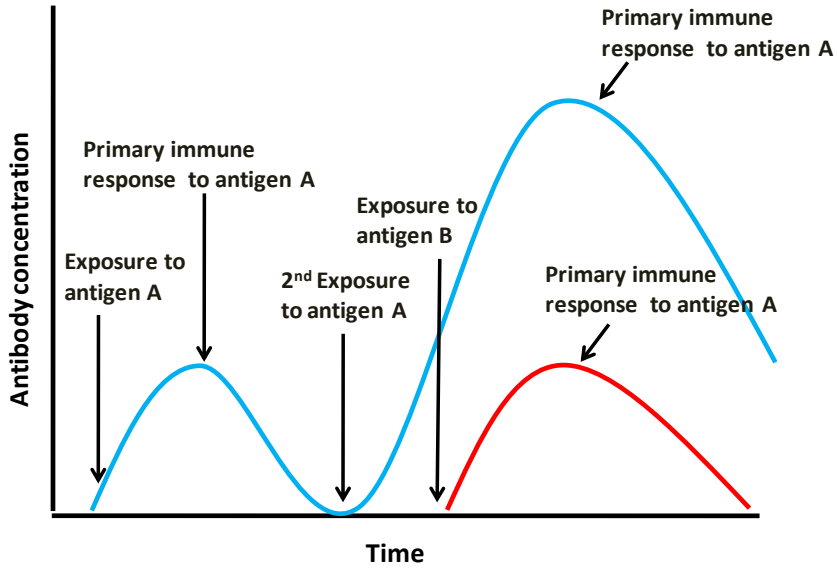
the binding of antigen to IgE leads to degranulation and release of preformed mediators, such as the vasoactive amines, histamine, serotonin and cytokines. Histamine release leads to vasodilation, increased vascular permeability which play an important role in attracting both neutrophils and eosinophils [32]. Another important feature is release of membrane derived mediators such as leucotrienes B4, C4, D4 and E4, prostaglandins and platelet activating factor which lead to induction of an inflammatory response [33, 34].

5. **Eosinophils:** Eosinophils (so called because their granules stain with eosin) are granulocytes that possess phagocytic properties. The main physiological role of eosinophils to protect host from parasitic (particularly nematode) infections. Eosinophils circulate at relatively low levels in the bloodstream, comprising only 1-3% of circulating white blood cells. They have large granules containing major basic protein, eosinophilic cationic protein, eosinophil peroxidase, and eosinophil-derived neurotoxin, which are highly cytotoxic when released onto the surface of organisms [35].

1.3.4 The adaptive immune system

The adaptive immune system is so-called because it "adapts" itself to recognize specific kinds of pathogens and also retains a "memory" of them for a more rapid response towards a subsequent exposure to the same pathogen. In vertebrates, the innate responses work together in conjunction with the adaptive immune responses to eliminate harmful microbes. Whereas the innate immune responses are non-specific defense reactions against a wide range of pathogens, the adaptive responses are highly specific to a particular pathogen and provide long-lasting protection. This memory is during a "primary response" against a pathogen that is encountered for the first time by the immune system. This

primary response is slow and becomes apparent several days after the initial infection and it takes a few weeks for it to clear the infection. Once the infection is cleared, the immune system retains a memory of the pathogen that caused the infection and mounts a much swifter and efficient response called the "secondary response" in case the body is infected again with the same pathogen (Scheme 1.1). The immune memory that the organism achieves during the primary response can confer protection up to the life-time of the organism.



Scheme 1.1: Kinetics of primary and secondary immune responses

Although the innate immunity can adequately control infections, many pathogenic microbes have evolved mechanisms to evade innate immunity. The adaptive immune response provides defense against such infections and hence, defects in the adaptive immune system result in increased susceptibility towards infections. The adaptive immune system primarily consists of certain types of white blood cells, called lymphocytes which circulate in the body via blood and lymphatic systems. Whereas the receptors of innate

immunity recognize only a limited diversity of structures shared by certain classes of microbes, lymphocytes express an incredibly wide range of receptors that specifically recognize different substances produced by microbes as well as noninfectious molecules called as antigens. Adaptive immune responses are triggered only if microbes or their antigens pass through epithelial barriers and are delivered to lymphoid organs where they can be recognized by lymphocytes.

1.3.5 Types of adaptive immunity

There are two broad classes of adaptive immune responses – **humoral/antibody responses** and **cell-mediated immune responses** which are carried out by different classes of lymphocytes called B lymphocytes and T lymphocytes, respectively. For example, antibodies secreted by B cells function to eliminate microbes in extracellular fluids whereas activated T cells eliminate microbes living inside the cells. Adaptive immune responses often use cells and molecules of the innate immune system to eliminate microbes and functions by significantly enhancing the antimicrobial mechanisms of innate immunity.

A. **Humoral immunity**: In humoral responses, B lymphocytes are activated to secrete antibodies which belong to a class of proteins called immunoglobulins (gammaglobulin proteins). B cells secrete antibodies into the circulation and mucosal fluids where they neutralize and eliminate microbes and microbial toxins that are present outside the host cells, in the blood and in the lumens of mucosal organs, such as the gastrointestinal and respiratory tracts. These antibodies bind to and inactivate viruses and microbial toxins (such as tetanus toxin or diphtheria toxin) by blocking their ability to bind to receptors on host cells. Antibody binding on these invading pathogens makes it easier for phagocytic cells of the innate immune system to ingest them. When antibodies bind to

microbes, these coated microbes avidly bind to and activate phagocytes (a component of innate immunity) which ingest and destroy the microbes.

B. **Cell-mediated immune responses:** Immune response against intracellular microbes which are inaccessible to antibodies is called cell-mediated immunity because it is mediated by cells called T lymphocytes (Table 1.2). T cells, when activated, react directly against a foreign antigen that is presented to them on the surface of a host cell called as antigen-presenting cells (APCs). These activated T helper (Th) lymphocytes trigger phagocytes to destroy the ingested microbes that have been ingested by the phagocytes into intracellular vesicles. Other T lymphocytes, called as cytotoxic T lymphocytes (Tc cells) kill any type of host cells that are harboring infectious microbes in the cytoplasm. Thus, the antibodies produced by B lymphocytes recognize extracellular microbial antigens, whereas T lymphocytes recognize antigens produced by intracellular microbes. Another important difference between B and T lymphocytes is that most T cells recognize only protein antigens, whereas antibodies are able to recognize many different types of molecules, including proteins, carbohydrates, and lipids.

1.3.6 Danger hypothesis:

Burnet's self-nonself model suggested that each lymphocyte expresses multiple copies of a single surface receptor specific for a foreign entity and signaling through this surface antibody initiates the immune response whereas the self-reactive lymphocytes are deleted early in life [36]. This theory gained further acceptance when Medawar et al. showed that adult mice would accept foreign skin grafts if they had been injected as babies with cells from the donors [37]. The danger hypothesis, first proposed by Matzinger [38], states that the immune system does not care about self and non-self, but rather that its primary driving force is to protect against danger [39].

Table 1.2:Properties of adaptive immune responses.

Feature	Functional significance
Specificity	Ensures that distinct antigens elicit specific responses
Diversity	Enables immune system to respond to a large variety of antigens
Memory	Leads to enhanced responses to repeated exposures to the same antigens
Clonal expansion	Increases number of antigen-specific lymphocytes to keep pace with microbes
Specialization	Generates responses that are optimal for defense against different types of microbes
Contraction and homeostasis	Allows immune system to respond to newly encountered antigens
Nonreactivity to self	Prevents injury to the host during responses to foreign antigens

It also states that the immune system does not works alone, but receives positive and negative signals from an extended network of other bodily tissues. The hypothesis states that presentation of an antigen in the absence of any danger signal results in tolerance, while the presence of a danger signal will result in a generation of immune response. The APCs are activated by danger/alarm signals from injured cells, such as those exposed to pathogens, toxins or mechanical damage. The "Danger model" has been supported by the discovery of endogenous, non-foreign alarm signals [40], including mammalian DNA [41], RNA, heat shock proteins (HSPs), interferon-α (an inducible protein often made by virus-infected cells), interleukin-1β, CD40-L (a surface molecule on activated platelets and activated T cells), and breakdown products of hyaluron (made when vessels are damaged).

1.3.7 Generation of adaptive immune response

1. **T cell development and formation of antigen-specific receptors**: T and B cells are derived from the hematopoietic stem cell present in the liver in the foetus or in the bone-marrow in adults. B cells remain within the bone-marrow during their development

whereas precursor cells already committed to T cell lineage called as the early thymic precursors migrate to the thymus and give rise to thymocytes, where all the subsequent stages of T-cell maturation occur. Pre-T cells express recombinase activating genes and terminal deoxynucleotidyl transferase, enabling recombination of TCR genes. The production of antigen-specific receptors is a result of random rearrangement and splicing together of multiple DNA segments that code for the antigen-binding areas of the receptors (complementarity-determining regions). Gene rearrangement occurs early in the development of the cells, before exposure to antigen, which leads to the production of a repertoire of over 10^8 T-cell receptors and 10^{10} antibody specificities, adequate to cover the range of pathogens likely to be encountered in life [42]. There are four segments of gene involved in receptor formation called the variable (V), diversity (D), joining (J), and constant (C) regions. The genes for TCRα and TCRβ chains contain large number of domains coding for these variable regions and a limited number of constant regions are found on separate chromosomes. The variable domain segments from DNA are cut out by nucleases and spliced together using ligases (a product of the recombination activation genes, RAG-1 and RAG-2). This forms the final gene sequence from which protein will be transcribed to form the receptor molecule. Further, clonal diversity can be generated in several ways. First, there are multiple regions within the DNA (V=25–100 genes, D~25 genes, and J~50 genes), but only one of each is used for protein coding. There is combinational freedom in that any one of the genes can join with any one other to form the final VDJ region. Second, the splicing is inaccurate and frameshift in base pairs leads to the production of a different amino acid (junctional diversity). Third, the enzyme deoxyribonucleotidyl transferase can insert nucleotides to further alter the sequence. The β-chain of the TCR is rearranged first and is expressed together with pre-TCR α-chain. Signals from the immature TCR complex inhibit rearrangement of the second β-chain

allele and induce proliferation and expression of the CD4 and CD8 molecules. Subsequently the TCR α-chain is recombined, and low levels of TCRs appear on the cell surface. From here, the T cell undergoes many differentiation and selection steps modulated by the thymic microenvironment, in particular, by thymic epithelial cells, macrophages, and dendritic cells. Thymic stromal cells regulate T-cell proliferation by secreting lymphopoietic growth factors, such as IL-7. Interaction of the TCR with MHC molecules expressed on epithelial cells and on dendritic cells/macrophages determines the fate of the thymocyte. Low-avidity recognition of peptide/MHC complexes on thymic epithelial cells by the TCR results in positive selection. This recognition event rescues cells from apoptotic cell death and ensures that only T cells with functional receptors survive. Thymocytes that express a receptor not fitting to any MHC antigen complex die by neglect. High-affinity interaction between the TCR and peptide/MHC complex induces apoptotic cell death of the recognizing T cell. This process of negative selection eliminates T cells with specificity for self-antigens and is responsible for central tolerance to many autoantigens. It has been estimated that approximately 1% of thymocytes survive the stringent selection process. While undergoing selection, T cells continue to differentiate with orderly expression of cell surface molecules. Thymocytes expressing CD4 and CD8 molecules develop into single-positive CD4+ helper T cells that have been selected on MHC class II complexes and CD8+ cytotoxic T cells that are restricted to MHC class I complexes. A small proportion of self-reactive CD4+ T cells differentiate into natural regulatory (nTreg) T cells.

1.3.8 T cell activation

Naive T cells have receptors that bind to adhesion molecules on the post-capillary high endothelial venules of the inflamed tissue of lymph nodes. Inside the lymph node they bind transiently to the multiple antigen-presenting cells. T-cell activation is initiated when TCR complexes recognize antigenic peptides in the context of the appropriate MHC molecule on the surface of an antigen-presenting cell [43]. The antigen is brought to the lymphoid tissue directly in the lymphatics, or within dendritic (or other antigen-presenting cells) cells that have endocytosed the antigen locally. However, if there is inflammation within a tissue, the dendritic cells are activated to leave the site and migrate to the draining lymph node. Antigen recognition by T cells results in proliferation and differentiation and triggers different effector functions. Stimulation of the TCR is not sufficient and needs to be complemented by the interaction of accessory molecules on the T cell and their ligands on the antigen-presenting cell. The co-receptors, CD4 and CD8, interact with MHC class II and class I molecules and support activation signals through the TCR. Adhesion molecules (integrins) stabilize the interactions between T cells and antigen-presenting cells. The stimulation of a T-cell–antigen receptor is necessary, but not sufficient, to induce complete T-cell activation, and it does not lead to cell proliferation or cytokine secretion. Complete T-cell activation requires a second signal called as the co-stimulatory signals which help to initiate, maintain and regulate the activation cascade (Table 1.3). In the absence of a co-stimulatory signal, T cells undergo apoptosis or are rendered nonresponsive and anergic. Several ligand–receptor pairs function as co-stimulators. One of the best characterized is the B7:CD28/CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4) co-stimulatory pathway. CD28 is expressed by both resting and activated T cells and is the predominant receptor for B7 on resting T cells. B7 binding to CD28 co-stimulates T-cell activation, leading to the expression of cytokines, cytokine receptors and genes for cell

survival [44, 45]. CD28 mediated signals are mandatory for the expression of many activation markers on the responding T cells and, in particular, the secretion of IL-2 [46, 47]. In contrast to CD28, CTLA-4 is expressed only on activated T cells. B7-1 and B7-2 have a higher affinity for binding with CTLA-4 than with CD28, which raises the possibility that CTLA-4 is the predominant B7 receptor on activated T cells. Unlike CD28, CTLA-4 can transduce an inhibitory signal, and its function may be to terminate T-cell activation [48].

Table 1.3: T cell activation and co-stimulatory markers.

COSTIMULATORY MOLECULE	PROLIFERATION EARLY/LATE	INCREASED IL-2	PROTECTION FROM APOPTOSIS	CYTOKINE POLARIZATION
CD28	+ / +	+	+	Th1 and Th2
ICOS (Inducible COStimulator)	- / +	-	N.D.	Th2
CTLA-4	- / -	-	-	-
PB-1	- / -	-	-	-
LFA-1	+ / -	-	-	Th1
ICAM-1	+ / +	+	+	Th1
41-BB	- / +	+	+	Th1
OX-40	- / +	+	N.D.	Th2

N.D.: No direct evidence. 1: Early refers to costimulatory events that contribute immediately to onset of the proliferative response. Late refers to events that are thought to contribute to sustaining proliferation because of markedly later expression of the signaling molecule by the T cell or because of continued use. 2: Early proliferation is delayed compared to LFA-1 or CD28. Adapted with modifications from Kohlmeier JE at al 2006 [2].

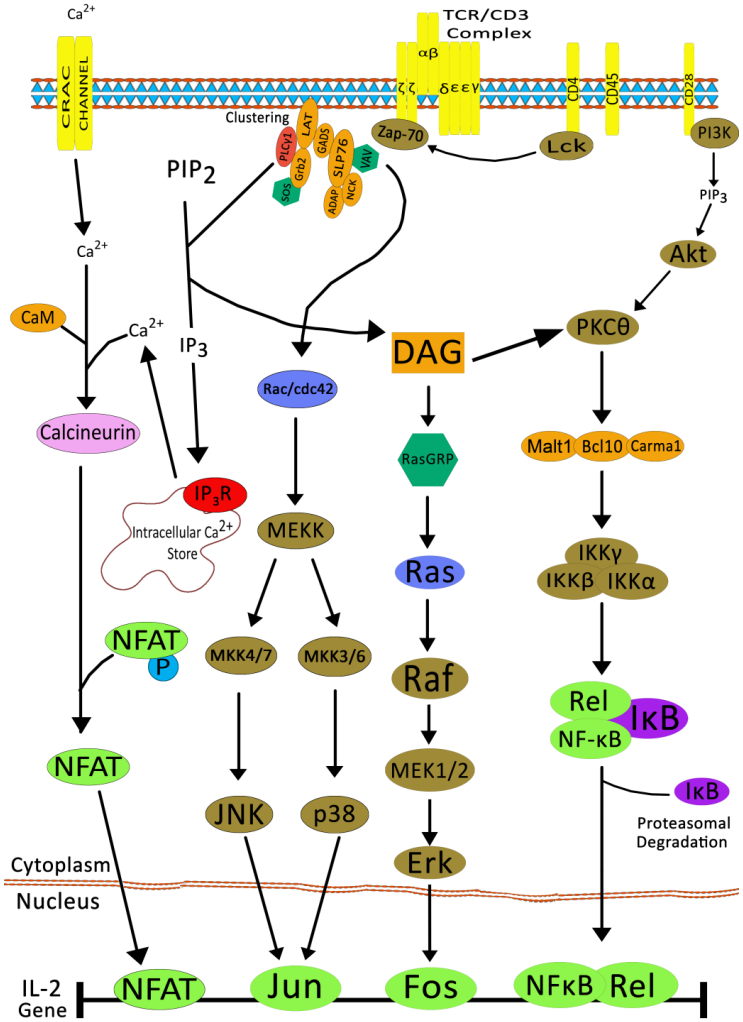
Intercellular adhesion molecule (ICAM)-1 (CD54) is a member of the immunoglobulin gene superfamily and is expressed on endothelial cells, epithelial cells, and fibroblasts, as well as T-cells, B-cells, dendritic cells, macrophages, and eosinophils. ICAM-1 is expressed at low but detectable levels on resting and naïve T cells, and is upregulated by inflammatory cytokines or after stimulation through the TCR. In vitro, T-cell activation by mitogen gradually increases ICAM-1 expression from 15 to 80% of T-lymphocytes over the course of 2–3 days of culture. NF-κB has also been reported to play a pivotal role in ICAM-1 gene regulation where RelA (p65)/RelA, RelA/c-Rel, and RelA/NF-κB1 (p50) dimers can potently induce ICAM-1 expression in several cell types

[49]. The presence of ICAM-1 on T-cells may play an important role when the expression of other co-stimulatory molecules on T-cell is suboptimal. Emerging evidence suggests that ligation of ICAM-1 on T-cells can have an important effect on cytokine production. Co-engagement of CD3 with ICAM-1 enhances the activation of murine CD4+ and CD8+ T-cells in an accessory cell-free culture system and induces production of IL-3 and IFN-γ. Interaction between LFA-1 expressed on B-cells and ICAM-1 on activated T-cells is important in early events of T-cell-dependent B-cell activation, proliferation and differentiation [50, 51].

1.3.9 T cell activation markers: CD25 is the alpha chain of the trimeric IL-2 receptor, which is expressed by T lymphocytes after triggering of the T-cell receptor. CD25 expression is low on resting immune cells (with the exception of FoxP3+ Treg cells) but is upregulated on all T cells by both T cell receptor and IL-2 mediated signaling [52]. CD69 is expressed earlier after T-cell activation than other activation antigens. CD69 expression is triggered by several mechanisms, including T-cell receptor and IL-2 receptor (CD25) stimulation. CD69 transcripts are detectable 30–60 min after T-cell receptor stimulation; they decline rapidly after 4–6 h [53].

1.3.10 T cell receptor signaling: The initial event that leads to the activation and differentiation of mature T-cells is the triggering of their antigen-specific T cell receptor (TCR) by its specific ligand, which consists of a processed antigenic peptide presented in association with MHC molecules on the surface of antigen-presenting cells or appropriate target cells. This interaction triggers several signal transduction pathways that involve secondary messengers, protein kinases, protein phosphatases and other enzymes and key intermediates (Scheme 1.2) [54, 55]. The key element during T cell responses to antigen is a complex receptor, consisting of two clonally distributed, highly polymorphic

heterodimeric αβ or γδ subunits of the TCR, the three invariant CD3 polypeptides (γ, δ and ε) and two additional homodimeric (ζ-ζ) or heterodimeric (ζ-η) subunits. The function of the αβ (or γδ) TCR is to recognize and specifically bind antigenic peptides presented by MHC molecules, while the CD3 complex, ζ and η transduce the signal generated by ligand binding to the TCR. The cytosolic components of these molecules contain a unique motif, the ITAM (immunoreceptor-based tyrosine activation motif), which has a consensus sequence of YxxI/L x YxxI/L [56-58]. Each of the CD3 chains (γ, δ and ε) contains one such motif, whereas ζ chain contains three. The increased phosphorylation of ITAM tyrosines in TCR subunits by the Src family kinases Lck and Fyn is the key initiating event in T cell activation by antigen [59, 60].



Scheme 1.2: T cell activation and receptor signaling.

The most important consequence of ITAM tyrosine phosphorylation is the binding of ZAP-70 (Zeta-chain-associated protein kinase 70), a member of a second family of PTKs involved in TCR signaling [61]. ZAP-70, once bound to the TCR in this fashion is activated by phosphorylation of the kinase domain activation loop mediated by the Src PTKs [62]. Other phosphorylations of ZAP-70 allow additional proteins to bind, like linker for the activation of T cells (LAT) and the cytosolic adapter protein Src homology 2 (SH2) domain–containing leukocyte phosphoprotein of 76 kDa (SLP-76), giving ZAP-70 itself the role of a scaffold [63, 64]. LAT contains nine tyrosines that are phosphorylated upon TCR engagement which bind the C-terminal SH2 domain of PLCγ1, the p85 subunit of phosphoinositide 3-kinase (PI3K), and the adapters growth factor receptor-bound protein 2 (GRB2) and GRB2-related adapter downstream of Shc (Gads) [65]. SLP-76 is then recruited to phosphorylated LAT via their mutual binding partner Gads [66]. SLP-76 itself contains three modular domains: an N-terminal acidic domain with three phosphorylatable tyrosines that interact with the SH2 domains of Vav1, Nck, and IL-2-induced tyrosine kinase (Itk) [67]. This proximal signaling complex results in the activation of PLCγ1-dependent pathways including Ca2+- and DAG-induced responses, cytoskeletal rearrangements, and integrin activation pathways [68].

Ligation of co-stimulatory receptors such as CD28 augments these pathways. Following TCR ligation, PLCγ1 is found in the proximal signaling complex bound to SLP-76, Vav1, and LAT, where it is phosphorylated and activated by Itk. Activated PLCγ1 then hydrolyzes the membrane lipid PI (4,5)P2 (phosphatidylinositol 4,5-bisphosphate) producing the second messengers IP3 (inositol trisphosphate) and DAG (diacylglycerol). 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, which initiates a mitogen-

associated protein kinase (MAPK) phosphorylation and activation cascade. Raf-1 is a MAPK kinase kinase (MAPKKK) that phosphorylates and activates MAPKkinases (MAPKKs),which in turn phosphorylate and activate the MAPK's extracellular signal-regulated kinase 1 (Erk1) and Erk2. Erk kinase activity results in the activation of the transcription factor Elk1, which contributes to the activation of the activator protein-1 (Jun/Fos) transcription complex via regulation of Fos expression. Additionally, Erk activity can result in the transcriptional activation of signal transducer and activator of transcription 3 (STAT3) and in the serine phosphorylation of Lck [69]. The second major signaling pathway regulated by DAG is mediated by PKCθ, a PKC family member that contains a lipid-binding domain specific for DAG, which is important for recruiting PKCθ to the plasma membrane following T cell activation. One critical pathway that PKCθ regulates is NF-κB activation and is described in detail in later section.

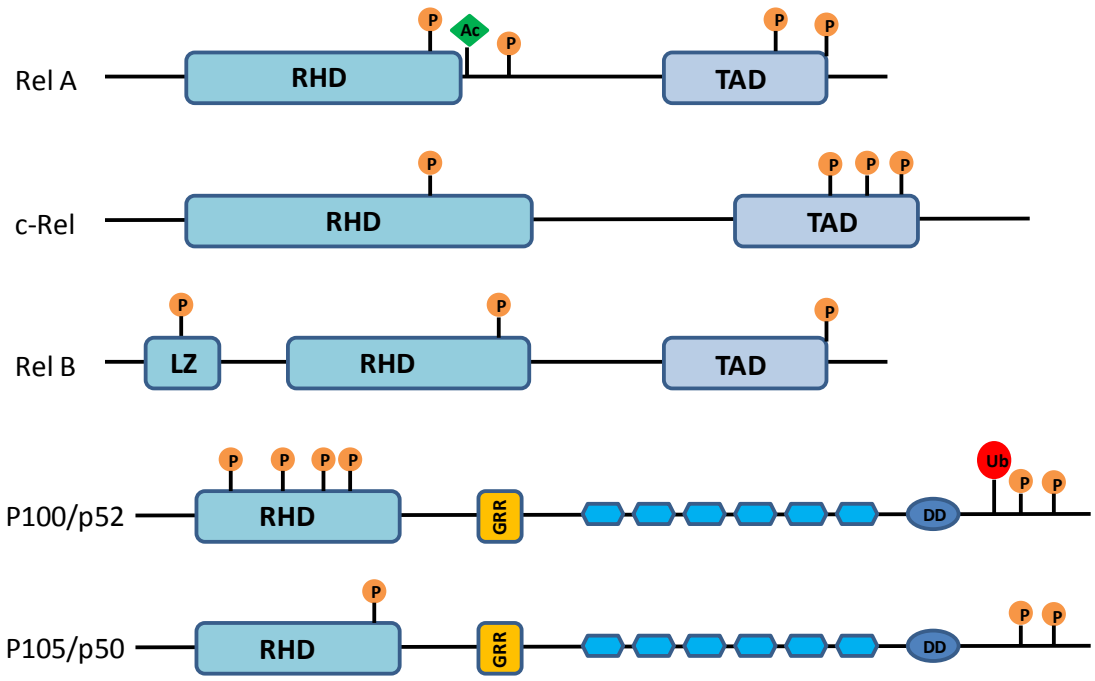
The IP3 generated by TCR-stimulated PLCγ1 activity also stimulate Ca2+-permeable ion channel receptors called inositol trisphosphate receptor (IP3R) on the endoplasmic reticulum (ER) membrane, leading to the release of ER Ca2+ stores into the cytoplasm. TCR-induced increases in intracellular Ca2+ levels result in the activation of Ca2+ and calmodulin-dependent transcription factors, as well as signaling proteins, including the phosphatase calcineurin and the Ca2+-calmodulin-dependent kinase (CaMK), that in turn activate a variety of transcription programs [70]. Activated calcineurin dephosphorylates members of the nuclear factor of activated T cells (NFAT) family, leading to their translocation to the nucleus [71, 72]. In the nucleus, NFAT isoforms can form cooperative complexes with a variety of other transcription factors, thereby integrating signaling pathways, resulting in differential gene expression patterns and functional outcomes, depending on the context of the TCR signal. NFAT/AP-1 interaction integrates Ca2+ and Ras signals and results in the expression of genes important for T cell activation

including IL-2. In contrast, NFAT activity in the absence of AP-1 activation induces a pattern of gene expression that ultimately results in T cell anergy and a characteristic lack of IL-2 production [73]. The regulatory T cell lineage–specific transcription factor forkhead box protein 3 (FOXP3) also cooperates with NFAT and antagonizes NFAT/AP-1 gene transcription, resulting in Treg functional gene expression and a lack of IL-2 production [74]. Finally, NFAT family members can also cooperate with STAT proteins to induce either Th1 or Th2 differentiation through expression of T-bet or GATA3, respectively [75].

Like NF-κB activation, the activation of AP-1 requires PKCθ activation, and PKCθ$^{-/-}$ mice fail to activate AP-1 in response to TCR stimulation [76, 77]. The AP-1 transcription factor is composed of dimers of c-Jun and c-Fos family proteins and can be activated both by phosphorylation of c-Jun by Jnk and by upregulation of c-Fos and c-Jun expression [78]. AP-1 is also activated by PKCθ-independent pathways including the Ras/Raf/Mek/Erk pathway, which signals for increased expression of c-Fos. An important aspect of AP-1 function is its ability to form complexes with the NF-AT and NF-κB transcription factors. It is particularly notable that the proximal NFAT binding sites of the IL-2 promoter cooperatively bind both NFAT and AP-1. Likewise the CD28RE site of the IL-2 promoter is a cooperative binding site for NF-κB and AP-1 [71, 72].

1.3.11 Activation of NF-κb signaling pathway during T-cell activation: 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 [79, 80]. Many different stimuli activate NF-κB transcription factors to induce their nuclear accumulation. Several diseases, including cancer, atherosclerosis, and diabetes, are associated with dysregulation of NF-κB and hence NF-κB emerged as a major regulator of innate and adaptive immunity and inflammatory responses. The NF-κB family of transcription factors consists of NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), RelA (also called

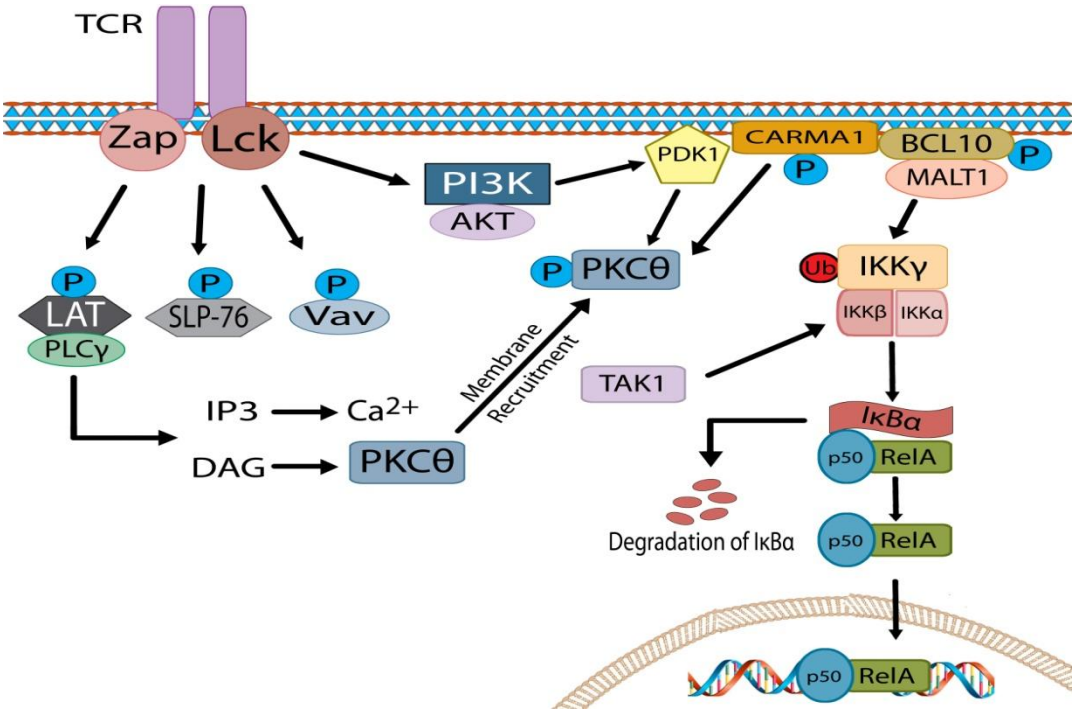
p65), c-Rel, and RelB, all of which are characterized by presence of an N-terminal Rel homology domain (RHD) responsible for homo- and heterodimerization as well as for sequence-specific DNA binding (Scheme 1.3). RelA, c-Rel, and RelB also contain a C-terminal transcription activation domain (TAD), whereas the p52 and p50 subunits do not and therefore rely on interactions with other factors to positively regulate transcription [81]. Whereas RelB preferentially heterodimerizes with p100 [82] as well as its processed form p52 [83], RelA and c-Rel predominantly heterodimerize with p50 [84].



Scheme 1.3: Schematic diagram for different NF-κB family members: Different domains including Rel homology domain (RHD), transcription activation domain (TAD), leucine zipper motif (LZ), glycine-rich region (GRR), and ankyrin repeats (ANK) are shown. Posttranslational modifications such as phosphorylation, acetylation, and ubiquitination at indicated amino acid positions are shown by P, Ac, and Ub, respectively. p105 is ubiquitinated at multiple sites. Adapted with modifications from Vallabhapurapu S et al,. 2009 [3].

In resting cells, NF-κB is found in the cytosol associated with inhibitor of NF-κB (IκB) family members that keep NF-κB from moving into the nucleus by interfering with the Nuclear Localization Signals present in NF-κB. The C-terminal halves of p105 and p100 also harbor multiple ankyrin repeats that allow them to serve an IκB-like function [82].

Proteasome-mediated processing removes the C-terminal half of p105 to generate the p50 subunit [85]. Engagement of antigen receptors on B and T lymphocytes also results in IKK and NF-κB activation. The protein kinase C isozymes PKCθ in T cells [86] and PKCβ in B cells [87] play central roles in recruiting additional factors and in IKK activation. Genetic studies have identified several additional factors that play important roles in TCR-mediated NF- κB activation, including kinases of the Src (Lck and Fyn) and Syk (ZAP70) families, as well as adapters such as LAT and SLP-76, and intracellular signaling components including phospholipase C (PLC) γ1, Vav1, BCL10, 3-phosphoinositide-dependent kinase 1 (PDK1), CARMA1 [caspase recruitment domain (CARD) membrane-associated guanylate kinase (MAGUK) protein 1], and MALT1 (mucosa-associated lymphoid tissue 1) [88]. Recent biochemical studies led to the postulation that TCR activation results in sequential recruitment of these factors to the immunological synapse (IS) as well as PKCθ-dependent formation of a complex between CARMA1, BCL10, and MALT1 [89]. This complex, called CBM, promotes the K63- linked polyubiquitination of IKKγ/NEMO and subsequent IKK activation [90]. IκB kinase (IKK) complex comprises of catalytic subunits IKKα and IKKβ and the regulatory/scaffold subunit IKKγ (also called NEMO for NF-κB essential modulator). NF-κB activation depends on the IKK catalyzed phosphorylation of IκB proteins. IKKγ/NEMO is obligatory for classical NF-κB signaling, as in its absence the IKK complex can no longer be activated [91]. The activated IKK complex phosphorylates IκBα on Ser32 and Ser36, leading to its polyubiquitination at Lys19 by the Skp1, Cdc53/Cullin1, and F-box proteinβ transducin repeat-containing protein (βTRCP). The ubiquitinated IκBα is degraded via the 26S proteasome, thereby exposing the strong NLS on RelA and inducing nuclear translocation of RelA:p50 dimers and leading to transcription of its dependent genes (Scheme 1.4) [84].



Scheme 1.4: Activation of NF-κB pathway following MHC-TCR interaction in T cells. Adapted with modifications from Vallabhapurapu S et al,. 2009 [3].

1.3.12 Proliferation, Survival, and Cellular Responses Mediated by NF-κB: NF-κB is involved in the control of transcription of many genes whose functions extend beyond the immediate 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 1.4). The majority of proteins encoded by NF-κB target genes participate in the host immune response. These target genes alone would merit NF-κB the designation as a central mediator of the immune responses.

Table 1.4: Target genes of NF-κB

CLASS	TARGET GENE
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
Cell adhesion molecules	• Endothelial leukocyte adhesion molecule 1 (ELAM-1), Vascular cell adhesion molecule 1 (VCAM-1) • Intercellular cell adhesion molecule 1(ICAM-1), Mucosal addressin cell adhesion molecule (MadCAM-1)
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 Colony Stimulating Factor), VEGF C (Vascular Endothelial Growth Factor) and G-CSF (granulocyte colony-stimulating factor)
Chemokines	• Macrophage inflammatory protein 1α (MIP-1α), Macrophage inflammatory protein 2 (MIP-2) • Macrophage chemotactic protein (MCP-1/JE), Regulated upon Activation Normal T lymphocyte Expressed and Secreted (RANTES)
Acute-phase proteins	• C-reactive protein, Lipopolysaccharide-binding protein, Angiotensinogen, Tissue factor-1 • Urokinase-type Plasminogen activator
Transcription factors and subunits	• IκBα, NF-κB precursor p 105, NF-κB precursor p 100
Regulators of apoptosis	• Bfl1/A1, Bcl-xL, NRL3 (Pro-survival Bcl-2 homologues) • CD95 (Fas) (Pro-apoptotic receptor), Fas-Ligand (Inducer of apoptosis), IAPs (Inhibitors of Apoptosis)
Others	• Nitric oxide synthase, Cyclooxygenase-2, Phospholipase A2, CD69, Angiotensin II

1.3.13 Reactive oxygen species in T cell activation and signaling:

Several investigators have reported that reactive oxygen species (ROS) play an important in the regulation of T cell signal transduction, gene expression, and functions. Mitogens induce strong T cell proliferation, and the addition of antioxidants to the cultures inhibited proliferation and production of IL-2, which is required for optimal T cell expansion [92-94]. Extending these observations to alloantigen-stimulated cultures suggested that antigen mediated T cell activation required ROS production [95]. In contrast to these studies, others reported that exposure to exogenous oxidative stress suppressed mitogen or antigen-induced T cell activation [96, 97]. These types of data have created a disagreement over the

importance and role(s) of oxidants in T cell signaling and activation, although it is clear that redox balance is critical to the activation of lymphocytes.

A) Reactive oxygen species regulate inflammatory proteins via s-glutathionylation:

The interaction of ROS with cellular components has become increasingly important in the study of both normal and pathological processes. At low concentrations they function in many normal processes, for example, hydrogen peroxide (H_2O_2), nitric oxide (•NO), and possibly superoxide ($O2^-$•) function in signal transduction pathways under normal physiologic conditions [98]. Additionally, ROS regulate immune activity and host defense, cell proliferation, differentiation, and death, and the aging process [99] [100]. Under oxidative stress conditions, ROS may damage nearly all biomolecules, including nucleic acids, proteins, lipids, and sugars, which is known to be associated with a vast array of diseases, including neoplastic, autoimmune, neurodegenerative, and cardiovascular disease [101] [102]. Further, several investigators have shown that excessive ROS can lead to oxidative stress, inflicting damage to cells and tissues, thereby amplifying inflammation [103]. ROS also fine-tunes the inflammatory responses, depending on the circumstances of production and amounts produced [104], and can induce reversible or irreversible oxidative modifications to Cys thiols of susceptible proteins. Reversible modifications may protect proteins against permanent oxidative damage and/or modulate their functions, but excessive ROS can lead to permanent loss of function and/or cell apoptosis and necrosis that may promote pathogenesis [105]. Glutathione (GSH) is an abundant anti-oxidant in cells and plays a critical role in protection from oxidative damage [106]. Protein S-glutathionylation, the disulfide coupling of a GSH moiety to Cys residues, is the prevalent S-thiolation reaction in biological systems [107, 108], regulating numerous physiological processes [109]. This modification may be driven by oxidative/ nitrosative stress in the presence of endogenous GSH but can also persist under basal conditions and in reducing environments [110] [111]. It

can occur via thiol-disulfide exchange with oxidized GSH (GSSG) or reaction of oxidative thiol intermediates such as S-nitrosothiols (SNO) with GSH [112]. Removal of GSH is promoted by reduced thiols and changes in intracellular redox or is catalyzed by enzymes such as thioredoxin and glutaredoxin [113]. Altered levels of S-glutathionylation in some proteins are associated with pathologies such as hyperlipidemia [114], diabetes [115] Mol. Med. 7, 619–623), Friedreich's ataxia [116], diabetes, atherosclerosis, and cancer [117], and identification of targets and their functional consequences may be clinically important. The number of known proteins that can undergo S-glutathionylation is relatively large, and a partial list is given in Table 1.5. Some targets include transcription factors (Jun, NF-κB), enzymes (creatine kinase, human immunodeficiency virus-1 protease), and cytoskeletal proteins (actin, tubulin), all of which influence critical pathways in growth, differentiation, and metabolism [112]. S-glutathionylation of the p50 and p65 subunits of NF-κB, a transcription factor with pivotal roles in inflammation and proliferation, inhibits its binding to promoter regions of genes [118, 119] .

S-glutathionylation of protein thiols can be reversed via direct thiol/disulfide exchange reactions with GSH once the intracellular redox balance i.e. the GSH/GSSG ratio has been restored [120] by means of an enzymatically mediated reaction. Enzymes capable of reducing S-glutathionylated proteins include the glutaredoxins (GRX; also known as thioltransferases) [121], the thioredoxins (TRX) [113] and sulfiredoxin [122].

Table 1.5: Proteins known to undergo s-glutathionylation

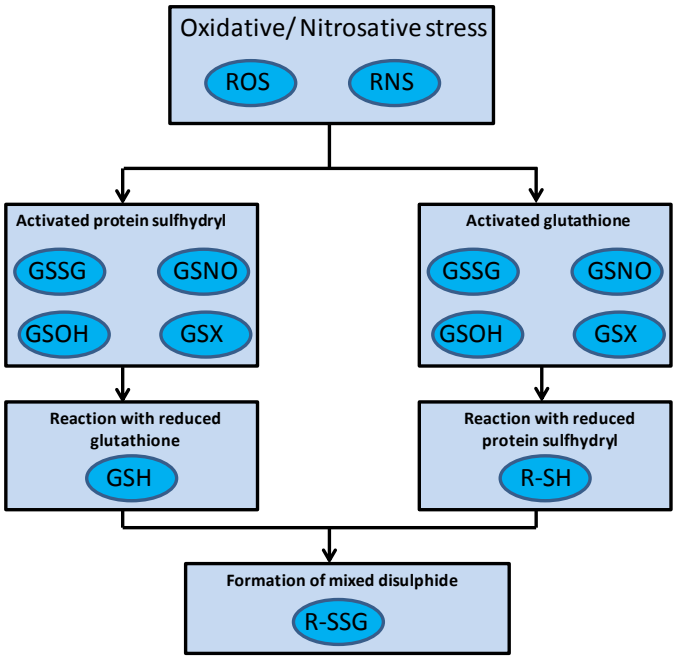
A PARTIAL LIST OF PROTEINS KNOWN TO UNDERGO PROTEIN S-GLUTATHIOLATION	
Carbonic anhydrase III	**Calbindin**
Protein kinase C	**Bovine serum albumin**
Glyceraldehyde-3-phosphate dehydrogenase	**Creatine kinase III**
Alcohol dehydrogenase	**Matrix metalloproteinases**
Enolase	**Caspase-3**
Protein tyrosine phosphatase 1B	**Calcium ATPase**
H-ras	**Malate dehydrogenase**
Glutathione transferases	**Aldose reductase**
HIV-1 protease	**Ubiquinating enzymes**
c-Jun	**Hemoglobin**
p50	**Cathepsin K**
Glycogen phosphorylase	**Nuclear factor-1**
Glutaredoxin	**Glycerol phosphate dehydrogenase**
Thioredoxin	**γ-Glutamyl transpeptidase**
Annexin II	**Aryl sulfotransferase IV**
Superoxide dismutase	**Transthyretin**

Adapted with modifications from Shackelford et al ., 2005 [123].

B) Mechanisms of ROS/RNS-induced protein S-glutathionylation:

ROS and RNS (reactive nitrogen species) may induce S-glutathionylation of protein thiols by two distinct pathways. The initial activation step may involve the oxidative or nitrosative modification of a protein thiol. Such activated protein species include a protein thiyl radical (R-S´), sulfenic acid (R-SOH) or S-nitrosothiol (R-SNO). Depending on the structural context of the targeted thiol, these modifications may either be stabilized or react with GSH to the mixed disulfide (R-SSG). Alternatively, the oxidation or nitrosation of GSH may trigger the incorporation of the glutathione moiety into target proteins. This pathway involves the formation of glutathione disulfide (GSSG), S-nitrosoglutathione (GSNO), glutathione sulfenate (GSOH) or other reactive intermediates, which may arise from the breakdown of activated GSH derivatives (GSX). Finally, these more or less reactive glutathione species may react with reduced protein thiols (R-SH) or a stabilized

anionic form of the thiol, i.e. a protein thiolate (R-S2), yielding the corresponding mixed disulfide (R-SSG) (Scheme 1.5).



Scheme 1.5: Mechanisms of ROS/RNS-induced protein S-glutathiolation

1.3.14 Inflammation

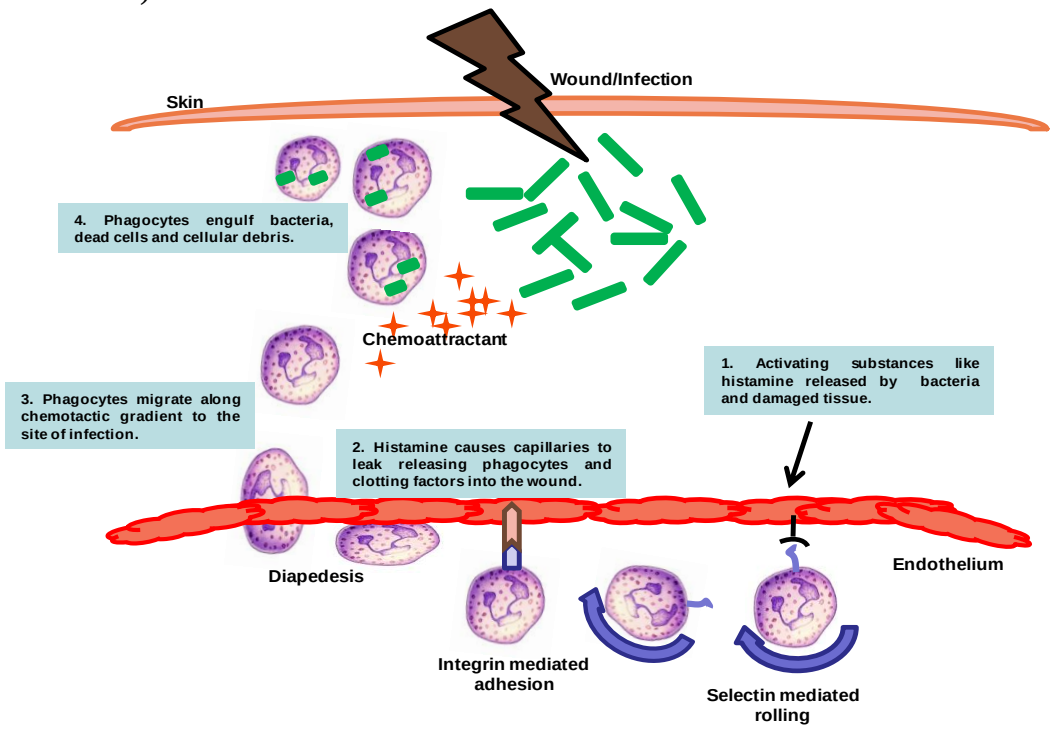
Inflammation is the universal response of the host to tissue damage by a wide range of harmful stimuli including mechanical trauma, tissue necrosis and infection (Scheme 1.7). Based on visual observation, the ancients characterized inflammation by five cardinal signs, namely redness (rubor), swelling (tumour), heat (calor; only applicable to the body' extremities), pain (dolor) and loss of function (functio laesa). The first four of these signs were named by Celsus in ancient Rome (30–38 B.C.) and the last by Galen (A.D 130–200) (Table 1.6) [124]. The purpose of inflammation is to destroy (or contain) the damaging agent, initiate repair processes and return the damaged tissue to useful function. It consists of a long chain of reactions and cellular activities that serve to repair a tissue in many circumstances of life, from a small skin cut or repair of tissue after birth to healing of the most severe burn injuries.

Table 1.6: Physiologic Rationale for Cardinal Signs of Inflammation

Cardinal Signs of Inflammation	Physiologic Rationale
Rubor (redness)	Increased vascularity
Tumor (swelling)	Exudation of fluid
Calor (heat)	A combination of increased blood flow and the release of inflammatory mediators
Dolor (pain)	The stretching of pain receptors and nerves by the inflammatory exudates, and by the release of chemical mediators
Functio laesa (loss of function)	A combination of the above effects

The inflammatory cascade at the tissue and cellular level involves a cascade of events which begins with dilation of the arterioles and venules, as well as increased blood vessel permeability and blood flow, followed by stasis and thrombosis, infiltration of leukocytes into the tissue, escape of plasma into the tissue, a breakdown of tissue by proteolytic activity and oxygen free radical formation leading to necrosis and apoptosis. The tissue

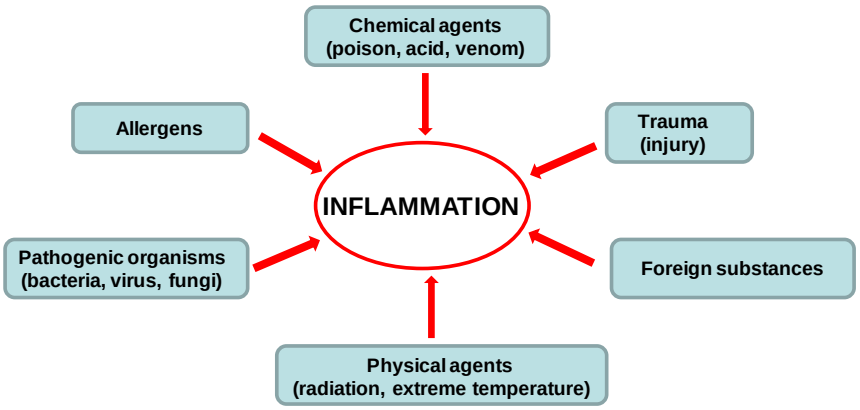
debri is removed by phagocytic cells which is followed by generation of new humoral mediators for cell growth and regeneration of new functional and connective tissue (Scheme 1.6).



Scheme 1.6: Steps of inflammatory response

A) Causes of Inflammation:

Different types of trauma or microtrauma, infections, and toxins can induce inflammation (Scheme 1.7). It results from the production of large quantities of proinflammatory chemicals such as cytokines and eicosanoids.



Scheme 1.7: Agents capable of stimulating an inflammatory response

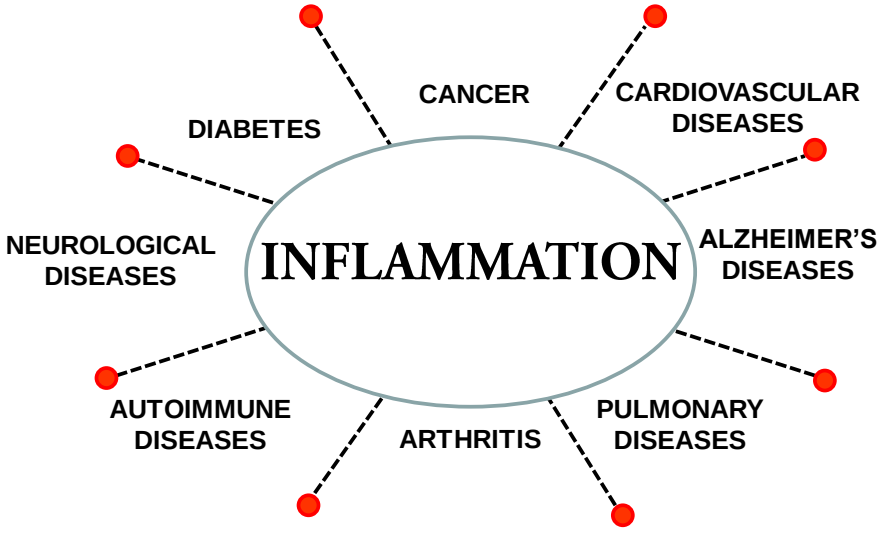
B) Acute inflammation: Acute inflammation may develop over minutes or hours depending on the type and severity of the tissue damage and generally lasts hours to days. It manifests with exudation of fluid and plasma proteins, and emigration of leukocytes, most notably neutrophils. Clinically, patients who have significant acute inflammation feel unwell and have a fever. This is mediated by cytokines released into the blood (interleukins-1, imterleukin-6, tumour necrosis factor (TNF) and prostaglandins), acting on the hypothalamus.

Components of acute inflammation:

- Vascular dilatation: relaxation of vascular smooth muscle leading to engorgement of tissue with blood (hyperaemia).
- Endothelial activation: increased endothelial permeability allows plasma proteins to pass into tissues and 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.

C) Chronic inflammation is of prolonged duration and manifests histologically by the presence of lymphocytes and macrophages and results in fibrosis and tissue necrosis. There are two general causes of such persistence: the inability to eliminate or continual reacquisition of the offending agent. When inflammation continues for prolonged periods of time, it can lead to deleterious changes can occur to localized tissues as well as the entire body (Scheme 1.8). This happens during persistent infections and autoimmune diseases. Infection with a microorganism of low virulence that cannot be eliminated easily may result in chronic rather than acute inflammation like in case of tuberculosis. Sometimes an individual's immune system may attack her/his own cells. This condition is

known as autoimmunity. In these cases, the affected patient's cells serve as a source of constant stimulation of the chronic inflammatory process. Systemic lupus erythematosus and rheumatoid arthritis are autoimmune diseases characterized by chronic inflammation. In chronic inflammation, macrophages and lymphocytes are the predominant cells along with a few neutrophils.



Scheme 1.8: Inflammation associated disorders

1.3.15 Inflammation and disease

Acute inflammatory reactions are usually self-limiting and resolve rapidly, due to the involvement of negative feedback mechanisms. Thus, regulated inflammatory responses are essential to remain healthy and maintain homeostasis. However, inflammatory responses that fail to regulate themselves can become chronic and contribute to the perpetuation and progression of disease [4]. Characteristics typical of chronic inflammatory responses underlying the pathophysiology of several disorders include loss of barrier function, responsiveness to a normally benign stimulus, infiltration of inflammatory cells into compartments where they are not normally found in such high numbers, and overproduction of oxidants, cytokines, chemokines, eicosanoids and matrix metalloproteinases [5, 6]. The levels of these mediators amplify the inflammatory response,

are destructive and contribute to the clinical symptoms. These various diseases and disorders have been linked to increased expression of pro-inflammatory mediators which activates inflammatory cells by increasing the expression of pro-inflammatory cytokines, up-regulating genes that produce NF-κB, NADPH oxidase, phospholipase A2, COX-1 and -2, 5-LOX, myeloperoxidase, iNOS, increasing oxygen consumption and producing many oxygen-free radicals that can finally lead to certain degenerative diseases.

The molecules/processes that play prominent roles in inflammatory processes include:

1. Leukocyte migration [125 126],
2. NO and Arachidonic acid metabolism [127-129]
3. Reactive oxygen species [130 131 132]
4. NF kappa B [133 134] and
5. Pro-inflammatory cytokines [89, 135-137].

Examples of chronic inflammatory diseases:

- Tuberculosis.
- Chronic cholecystitis.
- Bronchiectasis.
- Rheumatoid arthritis.
- Hashimoto's thyroiditis.
- Inflammatory bowel disease (ulcerative colitis and Crohn's disease).
- Silicosis and other pneumoconioses.

Other examples of chronic inflammatory diseases:

- Allergy — inflammatory cytokines induce autoimmune reactions
- Alzheimer's — Chronic inflammation destroys brain cells

• Anemia — inflammatory cytokines attack erythropoietin production

• Aortic valve stenosis — chronic inflammation damages heart valves

• Arthritis — inflammatory cytokines destroy joint cartilage and synovial fluid

• Cancer — chronic inflammation causes many cancers

• Congestive heart failure — chronic inflammation contributes to heart muscle wasting

• Fibromyalgia — Inflammatory cytokines are elevated

• Fibrosis — inflammatory cytokines attack traumatized tissue

• Heart attack — chronic inflammation contributes to coronary atherosclerosis

• Kidney failure — inflammatory cytokines restrict circulation and damage nephrons

• Lupus — inflammatory cytokines induce an autoimmune attack

• Pancreatitis — inflammatory cytokines induce pancreatic cell injury

• Psoriasis — inflammatory cytokines induce dermatitis

• Stroke — chronic inflammation promoted thromboembolic events

• Surgical complications — inflammatory cytokines prevent healing

1.3.16 Autoimmune Disorders:

Autoimmune diseases are pathological conditions identified by abnormal autoimmune responses and characterized by auto-antibodies (proteins, nucleic acids, or other molecules from one's own body, also known as autoantigens) and T-cell responses (as in multiple sclerosis or the animal model experimental autoimmune encephalomyelitis (SLE)) to self-molecules by immune system reactivity [138]. Autoimmune diseases, with the exception of rheumatoid arthritis and autoimmune thyroiditis, are individually rare, but together they affect approximately 5 percent of the population in Western countries [139]

[140]. There are currently more than eighty various kinds of autoimmune diseases, and many of them have similar symptoms.

Autoimmune disease also can be classified as systemic or organ specific. Systemic autoimmune diseases, such as SLE, involve multiple organs or tissues, whereas organ-specific autoimmune diseases involve a single organ or tissue, such as the thyroid gland in autoimmune thyroiditis or the islets of Langerhans in type I diabetes (TID).

1.3.17 Graft-versus-host Disease:

Haemopoietic-cell transplantation (HCT) is an intensive therapy used to treat high-risk haematological malignant disorders and other life-threatening haematological and genetic diseases. The main complication of HCT is graft-versus-host disease (GVHD), an immunological disorder that affects many organ systems, including the gastrointestinal tract, liver, skin, and lungs [141]. GVHD can also develop in various other clinical settings when tissues containing T cells (blood products and solid organs) are transferred from one person to another who is not able to eliminate those cells [142, 143]. Patients whose immune systems are suppressed and who receive white blood cells from another individual are at especially high risk for the disease. These patients require continued treatment with immunosuppressive drugs that increases their susceptibility to serious infections and other complications.

The three basic requirements for GVHD were formulated about 50 years ago are [144].

(1) Immunocompetent transplanted cells,

(2) antigens in the host, which can be recognized by the transplanted cells but are lacking in the donor, and

(3) Sufficient time for complete engraftment to mount an immune response.

GVHD arises when donor T cells respond to genetically defined proteins on host cells. The most important proteins are human leukocyte antigens (HLAs), which are highly polymorphic and are encoded by the major histocompatibility complex (MHC) [145]. Class I HLA (A, B, and C) proteins are expressed on almost all nucleated cells of the body at various densities. Class II proteins (DR, DQ, and DP) are mainly expressed on haemtopoietic cells (B cells, dendritic cells, and monocytes), but their expression can be induced on many other cell types after inflammation or injury.. The frequency of acute GVHD is directly related to the degree of mismatch between HLA proteins and thus ideally, donors and recipients are matched at HLA A, B, C, and DRB1 (referred to as 8/8 matches) [146]. On the basis of experimental data, progression of GVHD can be summarized in three sequential steps or phases: (1) activation of APCs; (2) donor T-cell activation, proliferation, differentiation, and migration; and (3) target tissue destruction [147].

Types of GVHD

a. Acute GVHD: This happens up to day 100 after the bone marrow transplant. This is graded from mild to severe [148]. The most common symptoms of acute GVHD is a maculopapular, erythematous rash in the extremities, which may spread to other parts of the body [149] [150] (Table 1.7).

b. Chronic GVHD: This occurs after day 100 and can be limited or extensive. Chronic GVHD is the main long-term complication and limitation to successful hematopoietic stem cell transplantation (HSCT) [151]. It affects over 50% of all patients undergoing HSCT, and the majority of those with acute GVHD

Table 1.7: Actue GVHD symptoms

Symptoms of acute GVHD	
Skin	Maculopapular skin rash
Upper gastrointestinal tract	Nausea, anorexia, or both, and positive histological findings
Lower gastrointestinal tract	Watery diarrhoea (≥500 mL) Severe abdominal pain Bloody diarrhoea or ileus (after exclusion of infectious causes)
Liver	Cholestatic hyperbilirubinaemia

Management of GVHD: GVHD can thus be considered an exaggerated and dysregulated response of a normal immune system of the donor to tissue damage that is intrinsic to transplantation.

a. Steroids, with their potent anti-lymphocyte and anti-inflammatory activity, are the gold standard for treatment of GVHD [152].

b. An increasingly frequent treatment for GVHD is extracorporeal photopheresis. During this procedure, the patient's white blood cells are gathered by apheresis, incubated with the DNA-intercalating agent 8-methoxypsoralen, exposed to ultraviolet light, and returned to the patient. Extracorporeal photopheresis is known to induce cellular apoptosis, which has strong anti-inflammatory effects in several systems, including prevention of rejection of solid organ grafts [153].

c. Another strategy to treat GVHD is blockade of the inflammatory cytokine TNF-α [154].

1.3.18 Sepsis

Sepsis is a life-threatening medical condition characterized by an overwhelming infection and the body's infammatory response to that infection. It is a leading cause of death in critically ill patients despite the use of modern antibiotics and resuscitation therapies [155]. The expected and appropriate inflammatory response to an infectious process

becomes amplified leading to organ dysfunction or risk for secondary infection [156]. The complex toll-like receptor signaling and associated downstream regulators of immune cell functions play a crucial role in the innate system as a first line of defense against pathogens [157]. However, signaling is sometimes conflicting and a sustained inflammatory response can result in tissue damage. Septic shock is a form of severe sepsis with associated low blood pressure that is life threatening. It leads to dysfunction of essentially all organs because of poor oxygenation and blood perfusion.

Pathogenesis of sepsis: A series of pathogenic events are responsible for the transition from sepsis to severe sepsis/septic shock. The initial reaction to infection is a neurohumoral, generalized pro- and anti-inflammatory response. This begins with a cellular activation of monocytes, macrophages, and neutrophils that interact with endothelial cells through numerous pathogen recognition receptors. A further host response includes the mobilization of plasma substances as a result of this cellular activation and endothelial disruption. These plasma substances include cytokines such as tumor necrosis factor, interleukins, caspase, proteases, leukotrienes, kinins, reactive oxygen species, nitric oxide, arachidonic acid, platelet activating factor, and eicosanoids. Activation of the complement and coagulation cascades further amplifies this elaborate chain of events [158-160]. The vascular endothelium is the predominant site of these interactions, and, as a result, there is microvascular injury, thrombosis, and a loss of endothelial integrity (capillary leak), resulting in tissue ischemia. This diffuse endothelial disruption is responsible for the various organ dysfunctions and global tissue hypoxia that accompany severe sepsis/septic shock [161]. Sepsis exists as a spectrum of severity from the Systemic Inflammatory Response Syndrome (SIRS) through to Multiple Organ Dysfunction Syndrome (MODS) [162] (Table 1.8).

Table 1.8: SEPSIS, SIRS AND SEPTIC SHOCK

Clinical definition of sepsis	
Systemic Inflammatory Response Syndrome (SIRS)	1. Body temperature <36 C or >38 C 2. Heart rate >90 beats/min 3. Respiratory rate >20 breaths/min or PCO_2 <4.3 kPa (32 mmHg) 4. White cell count <4 or >12 x 109/l or the presence of greater than 10% immature neutrophils.
Sepsis	Systemic response to infection, manifested by two or more of the conditions mentioned under SIRS (SIRS + evidence of infection)
Severe sepsis	Sepsis associated with organ dysfunction, hypoperfusion, or hypotension including lactic acidosis, oliguria, or acute alteration in mental state
Septic shock:	Sepsis-induced hypotension which persists despite adequate fluid resuscitation, along with the presence of perfusion abnormalities that may include lactic acidosis, oliguria, or an acute alteration in mental state.
Multiple Organ Dysfunction Syndrome (MODS):	The presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention

1.3.19 Therapeutic strategies for inflammatory and autoimmune diseases:

One of the major thrust area of current clinical research is to generate reproducible and reliable serologic and clinical methods of assessing the risk of a specific disease and of identifying active disease and remission. Several therapeutic approaches are being currently explored that include altering the thresholds of immune activation, modulating antigen-specific responses, reconstituting the immune system with autologous or allogeneic stem cells, and sparing of target organs (Table 1.9) . Interference with costimulation, signaling, chemokines, cytokines, and other molecules critical to immune activation is designed to restore homeostasis in the immune system and dampen the autoimmune response. It is based on the concept that small changes in the availability of

proteins that control interactions between cells or participate in intracellular signaling can divert the immune system away from autoreactivity [163].

1.3.20 Immunosuppression:

Immunosuppression can be achieved by depleting lymphocytes, diverting lymphocyte traffic, or blocking lymphocyte response pathways. Immunosuppressive drugs have three effects: the therapeutic effect (suppressing rejection), undesired consequences of immunodeficiency (infection or cancer), and nonimmune toxicity to other tissues. Pharmacological immunosuppression is required for treatment of inflammatory and immune disorders as well as in all types of organ transplantation to prevent rejection of the allograft. Immunosuppressive drugs include small-molecule drugs, depleting and

Table 1.9: Therapeutic approaches employed for inflammatory disorders

THERAPEUTIC APPROACHES	
Alteration of thresholds of immune activation	Blockade of costimulatory factors Antagonism of inflammatory cytokines or protective cytokines Inhibition of signaling cascades by small molecules Antagonism of complement, Antagonism of chemokines Use of anti-inflammatory agents, Inhibition of matrix metalloproteases Inhibition of nitric oxide synthase
Modulation of antigen-specific cells	Induction of regulatory cells (intravenous, subcutaneous, or oral delivery of antigen), Alteration in peptide ligands Formation of complexes of peptide and major-histocompatibility-complex molecules, Development of T-cell receptor vaccines Induction of B-cell tolerance, Immune deviation from type 1 to type 2 helper T cells
Reconstitution of the immune system	Bone marrow ablation with autologous stem cells Bone marrow ablation with donor stem cells Bone marrow ablation without stem cells

non-depleting protein drugs (polyclonal and monoclonal antibodies), fusion proteins, intravenous immune globulin, and glucocorticoids [1, 7] (Table 1.10). Due to the strength of

the inflammatory and alloimmune response, the drugs that are currently available are not best suited as single agents to prevent rejection without unacceptable toxicity (Table 1.11) [8]. Current practice is therefore to use combinations of agents with complementary immunosuppressive actions but differing adverse effects to achieve synergistic immunosuppression with the lowest possible toxicity [164]. Despite the remarkable advances of the last half-century, current immunosuppressive drugs are not ideal approach to support long term acceptance of solid organ transplant and survival. Each agent is associated with its own set of toxicities in addition to the shared adverse consequences of long-term immunosuppression: infection and malignancy. It seems unlikely that additional permutations of currently available therapies will be able to alter this dynamic.

Table 1.10: Classification of Immunosuppressive Drugs

CLASS	Mechanism of Action
Glucocorticoids	Regulation of gene expression
Small-molecule drugs	Immunophilin-binding drugs Calcineurin inhibitors Cyclophilin-binding drugs: cyclosporine, ISA(TX)247 FKBP12-binding drugs: tacrolimus Target-of-rapamycin inhibitors: sirolimus,everolimus
Inhibitors of nucleotide synthesis	Purine synthesis (IMPDH) inhibitors:Mycophenolate mofetil Mizoribine Pyrimidine synthesis (DHODH) inhibitors:Leflunomide,FK778
Antimetabolites:	azathioprine Sphingosine-1-phosphate–receptor antagonists: FTY720
Protein drugs	Depleting antibodies (against T cells, B cells, or both) Polyclonal antibody: horse or rabbit antithymocyte globulin Mouse monoclonal anti-CD3 antibody (muromonab-CD3) Humanized monoclonal anti-CD52 antibody (alemtuzumab) B-cell–depleting monoclonal anti-CD20 antibody (rituximab)
Nondepleting antibodies and fusion proteins	Humanized or chimeric monoclonal anti-CD25 antibody (daclizumab, basiliximab) Fusion protein with natural binding properties: CTLA-4–Ig LEA29Y†)
FKBP12 denotes FK506-binding protein 12, IMPDH inosine monophosphate dehydrogenase, DHODH dihydroorotate dehydrogenase, and CTLA-4 cytotoxic T-lymphocyte–associated antigen 4. Adapted with modifications from Halloran PF,. 2004 [1].	

Table 1.11: Mechanism of action and associated toxicities of clinically used Immunosuppressive Drugs

Drug	Description	Mechanism	Non-immune Toxicity
Small-Molecule Immunosuppressive Drugs			
Cyclosporine	11-amino-acid cyclic peptide from *Tolypo cladium inflatum*	Binds to cyclophilin; complex inhibits calcineurin phosphatase and T-cell activation	Nephrotoxicity, hemolytic–uremic syndrome, hypertension, neurotoxicity, post-transplantation diabetes mellitus, hyperlipidemia
Tacrolimus (FK506)	Macrolide antibiotic from *Streptomyces tsukubaensis*	inds to FKBP12; complex inhibits calcineurin phosphatase and T-cell activation	Effects similar to those of cyclosporine but with a lower incidence of hypertension, hyperlipidemia,
Sirolimus (rapamycin)	Triene macrolide antibiotic from *S. hygroscopicus*	Binds to FKBP12; complex inhibits target of rapamycin and interleukin-2 driven T-cell proliferation	Hyperlipidemia, increased toxicity of calcineurin inhibitors, thrombocytopenia, delayed wound healing, delayed graft function, mouth ulcers, pneumonitis,
Mycophenolate mofetil	Mycophenolic acid from penicillium molds	Inhibits synthesis of guanosine monophosphate nucleotides; blocks purine synthesis, preventing proliferation of T and B cells	Gastrointestinal symptoms (mainly diarrhea), neutropenia, mild anemia
Azathioprine	Pro-drug that releases 6-mercaptopurine	Converts 6-mercaptopurine to tissue inhibitor of metalloproteinase, which is converted to thioguanine nucleotides that interfere with DNA synthesis	Leukopenia, bone marrow depression, macrocytosis
Protein Immunosuppressive Drugs			
Polyclonal anti-thymocyte globulin	Polyclonal IgG from horses or rabbits immunized with human thymocytes	Blocks T-cell membrane proteins (CD2, CD3, CD45, and so forth), causing altered function, lysis, and prolonged T-cell depletion	The cytokine-release syndrome (fever, chills, hypotension), thrombocytopenia, leukopenia, serum sickness
Muromonab-CD3	Murine monoclonal antibody against CD3 component of T-cell–receptor signal transduction complex	Binds to CD3 associated with T-cell receptor, leading to initial activation and cytokine release, followed by blockade of function, lysis, and T-cell depletion	Severe cytokine-release syndrome, pulmonary edema, acute renal failure, gastrointestinal disturbances, changes in central nervous system
Rituximab	Chimeric monoclonal antibody against membrane-spanning four-domain protein CD20	Binds to CD20 on B cells and mediates B-cell lysis	Infusion reactions, hypersensitivity reactions (uncommon)

Adapted with modifications from Halloran PF,. 2004 [1]

Future Directions:

a. Increasing ability of new approaches to define subgroups of transplant recipients at different immunological risk using novel biomarkers and microarrays.
b. Development of novel immunosuppressant pharmacotherapies: A wider choice of immunosuppressive agents will probably help transplant physicians to minimize the burden of drug toxicity in their patients.

1.3.21 Natural products in drug discovery:

Beyond just providing essential nutrition and minerals, the food we consume affects our bodies in many other ways. Many natural dietary bioactive compounds have established pharmacological effects and/or can significantly alter activity of therapeutic agents by modulating biochemical pathways [9]. Natural plant products have been used throughout human history for various purposes. Several of these products are produced as secondary metabolites by higher plants as a natural defense mechanism against disease and infection. Many of these natural products have pharmacological or biological activity that can be exploited in pharmaceutical drug discovery and drug design. Medicines derived from plants have played a pivotal role in the health care of many cultures, both ancient and modern. The Indian system of holistic medicine known as "Ayurveda" uses mainly plant-based drugs or formulations to treat various ailments, including cancer and inflammation. Excessive inflammation is considered to be a critical factor in many human diseases and conditions, including obesity, cardiovascular diseases, neurodegenerative diseases, diabetes, aging, and cancer. In chronic inflammation, the persistent tissue damage and cell proliferation, as well as the enrichment of ROS and reactive carbonyl species (RCS), contribute to a cancer-prone microenvironment. Several classes of drugs, such as corticosteroids, and NSAIDs (Non-steroidal anti-inflammatory drugs), are used to

treat the inflammatory disorders. The main problem is that these drugs possess several adverse effects or are too expensive to be used. Corticosteroids have long been used for the management of rheumatoid arthritis and IBD diseases, but they suffer from some serious adverse effects, such as hypertension, hyperglycemia, muscular weakness, increased susceptibility to infection, osteoporosis, glaucoma, psychiatric disturbances, growth arrest, etc. Anti-inflammatory activity of natural bioactive compounds is attracting growing interest among researchers and physicians [165].

Natural products have been the single most productive source of leads for the development of drugs. Plants have been the basis of many traditional medicine systems throughout the world for thousands of years and they represent an exhaustive source of "raw materials" in order to find and synthesize new molecules with pharmacological activity [10]. Of the at least 877 small-molecule drugs introduced worldwide between 1981 and 2002, the origins of most (61%) can be traced to natural products [166]. Almost half of the drugs approved since 1994 are based on natural products: thirteen natural product-related drugs were approved from 2005 to 2007 and five of these represented the first members of new classes of drugs [167], [168].

Inflammatory pathways as potential targets for natural dietary bioactive compounds:

Inflammation is a complex process that engages molecular and cellular mechanisms, resulting in widespread physiological alterations. The initial inflammation involves the recruitment of a wide range of immune cells to inflamed sites and the release of various pro-inflammatory cytokines and other agents [169]. This adaptive response evolved as a general reaction to a variety of stimuli and conditions, including infection and injuries, and the controlled acute inflammatory response is an essential part of the host's defense system. In sharp contrast with acute inflammation, systemic chronic inflammation, which

is not apparently triggered by infection or injury, is strongly associated with a wide variety of diseases, including cancer, type 2 diabetes, and cardiovascular diseases. Chronic inflammation is linked to the malfunction of tissues and is believed to be functionally unrelated to tissue repair or to organism defense. It is well-established that inflammatory processes contribute to pathophysiology of cancer development and progression.

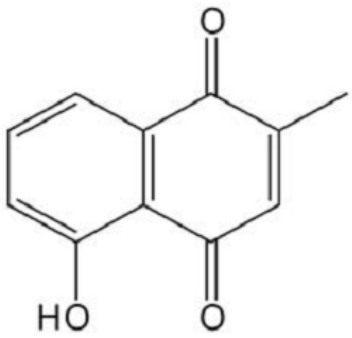
The network of inflammatory pathways includes, besides cytokines and chemokines, a variety of transcription factors and enzymes that should be recognized for their critical regulatory functions during this complicated process. Key molecular players linking cancer and inflammation include STAT, NF-κB, NFAT, AP-1, CCAAT enhancer binding protein (C/EBP), cAMP response element binding protein/p300 (CBP/p300), and activator transcription factor (ATF) [170]. It has been recently recognized that NF-κB, the central coordinator of innate and adaptive immune responses, also plays a critical role in cancer development and progression [171]. Activated NF-κB often facilitates transcription of numerous genes, including iNOS, COX-2, interleukin-6 (IL-6), IL-1β, tumor necrosis factor-R (TNF-R), 5-lipoxygenase (5-LOX), hypoxia inducible factor-1R (HIF-1R), and vascular endothelial growth factor (VEGF), resulting in inflammation and tumorigenesis. Activation of NF-κB is induced by a cascade of events leading to the activation of IKK, which in turn phosphorylates IκB. The subsequent ubiquitination and proteasomal degradation of IκB leaves NF-κB free to translocate to the nucleus. These kinases can be activated through phosphorylation by upstream kinases, including NFκB-inducing kinase, mitogen-activated protein kinase and protein kinase C [172] . In addition, many studies have confirmed the cytokine function in the induction of transcription activity of NFκB through Janus kinase (JAK), Erk1/2, p38 MAPK, Ras and PI3K)/Akt pathways [173]. NF-κB provides a mechanistic link between inflammation and cancer and is a major factor controlling the ability of both preneoplastic and malignant cells to resist apoptosis-based

tumor surveillance mechanisms. Recent studies revealed that constitutive activation of STATs, particularly STAT3, is found in a number of primary human epithelial tumors and cancer cell lines. Persistently active STAT3 induces tumor angiogenesis by up-regulation of VEGF and its immune evasion. An understanding of molecular mechanisms linking inflammation and cancers is beneficial for the development of efficacious prevention and treatment of inflammation-associated tumorigenesis. Growing evidence clearly demonstrates that inflammatory pathways are critical targets in cancer treatment and prevention [174]. Many natural bioactive compounds have been reported to interfere with the initiation, promotion/progression, and invasion/metastasis of cancer through control of intracellular signaling cascades of inflammation process progresses [169]. There is growing research on the effects of plant-derived compounds on the attenuation of pro-inflammatory gene expression which may find application in the treatment of immune disorders.

1.3.22 PLUMBAGIN:

Plumbagin is a naphthoquinone (5-hydroxy-2-methyl-1,4-naphthoquinone) (Scheme 1.9) that has been known for several hundred years before the structural investigations were carried out on its constitution (Table 1.12). Naphthoquinones constitute one of the largest and diverse groups of plant secondary metabolites with a broad range of properties that include antimicrobial, anti-feedent, and allelopathic activity which contribute toward plant defense. They also possess important pharmacological activities, such as antioxidant, anti-inflammatory, anticancer, and antibacterial, respectively [175-179]. The oldest reference to this plant is found in the ancient Indian Ayurvedic texts of Charaka (second century B.C.) where the plant was known as ‘‘Chitraka,’’ whose roots were credited with therapeutic properties against dyspepsia, piles, diarrhea, and skin diseases [180-182]. Plumbagin is the active principle found in

Plumbagenaeace, *Droseraceae* and *Ebenceae* families. *Plumbaginaceae* is a family of ten genera and about 300 families mostly found in semiarid regions of the Mediterranean and central Asia. Three species are found in India, which include *Plumbago rosea*, *Plumbago zeylanica* (Scheme 1.10) and *Plumbago capensis*.



Scheme 1.9: Structure of plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone)

Table 1.12: Physico-chemical properties of plumbagin

Chemical name	5-hydroxy-2-methyl-1,4-naphthoquinone
Molecular Formula	C11H8O3
Formula weight	188.18
Melting point	78-79°C
Density	1.345
Appearance	Orange crystalline powder or crystals
Solubility	Slightly soluble in hot water; soluble in alcohol, acetone, chloroform,benzene, and acetic acid

Scheme 1.10: ***Plumbago zeylanica*** **(A) plant and (B) root**

A) Isolation of plumbagin: Isolation of plumbagin from the respective plant material is usually carried out by solvent extraction of the coarsely powdered roots by solvents of increasing polarity using cold percolation method [183]. Three locally available plumbago species (*Plumbago auriculata, P. rosea, and P zeylanica*) have been quantitatively screened for the bioactive marker plumbagin by high performance thin layer chromatography. In general, the root parts were found to be the rich sources of plumbagin.

B) Bioavailibility and pharmacokinetic studies of plumbagin: Due to potent biological activities associated with plumbagin, studies have been carried out to assess its bioavailability. An automated blood sampling method, coupled to liquid chromatography tandem mass spectroscopy (LC-MS/MS), has been standardized to evaluate the orally bioavailable plumbagin in rats. Pharmacokinetics of plumbagin in rats indicated the oral bioavailability of plumbagin to be 38.7±5% [184].

C) Biological activities of plumbagin:

a. Antioxidant Activities: To identify the possible mechanism of action of several beneficial effects of plumbagin, Tilak et al., studied the in vitro anti-oxidant properties of aqueous/alcoholic extracts of root of *Plumbago zeylanica*. Further, studies conducted by Demma et al., 2009 showed that non-DNA damaging concentrations of plumbagin diminished the DNA damage induced by catechol indicating that plumbagin may act as an antioxidant agent at low concentrations [185].

b. Radio-sensitizing effect of plumbagin: Nair et al. have reported a potential role of plumbagin as a radio sensitizer. It was reported that plumbagin, in combination with radiation, was able to augment cell growth inhibition very effectively as compared to a higher dose of radiation alone [186]. Similar sensitizing effects of plumbagin on the radiation induced cytogenetic and cell cycle changes have been reported in mouse Ehrlich ascites carcinoma in vivo [187].

c. Anticancer Activities: The anti-proliferative effects of plumbagin on tumor cells has been extensively studied by several investigators using cell lines as well as mouse tumor models (Table 1.13).

d. Anti-bacterial and anti-fungal properties: Plumbagin has been reported to possess significant antimicrobial activity against *Aspergillus niger, Bacillus subtilis, Candida albicans, Colletotrichum gloeosporioides,* and *Staphylococcus aureus* [188]. The antimycobacterial and antigonorrhoeal activities of plumbagin isolated from *Diospyros canaliculata* and *Diospyros crassiflora* have also been evaluated [189]. The antifungal activity of plumbagin has been demonstrated by its ability to significantly inhibit the

growth of 12 different strains of yeast pathogens and filamentous fungi *Aspergillus flavus, A. niger, Alternaria sp., C. albicans, Candida glabrata, Candida krusei, Cryptococcus neoformans, Candida tropicalis, Cladosporium sp., Geotrichum candidum, Fusarium sp., and Penicillium* sp. This study suggested a potential use of plumbagin as a promising antifungal agent compared to ketoconazole which was used in the study as the control standard antifungal agent [190].

e. Effect of plumbagin on immunoregulatory proteins:

Sandur SK et al., for the first time reported that that plumbagin inhibited NF-κB activation induced by TNF, and other carcinogens and inflammatory stimuli (e.g. phorbol-12-myristate-13-acetate, H_2O_2, cigarette smoke condensate, interleukin-1β, lipopolysaccharide, and okadaic acid). It was also shown that plumbagin suppressed the constitutive NF-κB activation in certain tumor cells. Further, the suppression of NF-κB activation correlated with sequential inhibition of the TNF induced activation of IκBα kinase, IκBα phosphorylation, IκBα degradation, p65 phosphorylation, p65 nuclear translocation, and the NF-κB-dependent reporter gene expression activated by TNF, TNFR1, TRAF2, NIK, IKK-β, and the p65 subunit of NF-κB. Plumbagin was shown to suppress direct binding of nuclear p65 and recombinant p65 to the DNA, and this binding was reversed by dithiothreitol both *in vitro* and *in vivo*. Gomathinayagam R et al., also reported that plumbagin significantly inhibited the growth of H460 and down-regulated the expression of EGFR/Neu and its downstream signaling (Akt, NF-κB, Bcl-2 and survivin) in H460 cells [191]. Shieh JM et al., recently reported that plumbagin strongly inhibited TPA-induced phosphorylation and degradation of inhibitor of IκBα (IkappaBalpha), and the nuclear levels of NF-κB, c-Fos, and c-Jun an A549 cells [192].

Table 1.13: Anti-tumor activity of plumbagin

Type of cancer	Cell lines used	Reference
Leukemia	human acute promyelocytic (NB4) cells	193
	HL-60 cells	194
	NB4 tumor xenograft in NOD/SCID mice	195
	chronic myelogenous leukemia	196
Lung Cancer	non-small cell lung cancer cells (A549) cells	197
	A549 and H460 cells	191
	human non-small lung epithelium carcinoma cells (A549)	198
	human lung cancer cells, A549.	192
Breast Cancer	human breast cancer cells (MCF-7 and MDA-MD-231 cells)	199
	ER-positive MCF-7 and ER-negative MDA-MB-231	200
	Human breast cancer MDA-MB-231, BT474,	201
Colon and Prostate cancer	human prostate cancer cells (PC-3, LNCaP, and C4-2)	202
	human prostate cancer cells CWR22rv1, LNCaP, PC-3 and DU145	203
	human colonic cancer cell lines, HT29 and HCT15	204
Cervical cancer	HeLa cells	205, 206
	human cervical cancer cell line (ME-180)	207
Liver cancer	hepatoma cell line HEPA-3B	205
	HepG2 cells	208
Melanoma	HaCaT keratinocytes	209, 210
	human melanoma A375.S2 cells	211
		212
Myeloma	Multiple myeloma cells	213
Ovarian cancer	BRCA1-blocked ER-positive BG-1 ovarian cancer cells	214
Anti-angiogenic	human umbilical vein endothelial cells (HUVECs)	215

Plumbagin was reported to induces apoptosis in MCF-7 cells with concomitant inactivation of Bcl-2 and the DNA binding activity of NF-κB [200]. Further, Manu et al recently reported that NF-κB inhibition by plumbagin causes a decrease in CXCR4 and other metastatic genes [201]. Aziz MH et al., reported that plumbagin at concentrations as low as 5 μM, inhibited in both cultured PCa cells and DU145 xenografts (a) the expression of PKCε, PI3K, phosphorylated AKT, phosphorylated JAK-2, and phosphorylated Stat3; (b) the DNA-binding activity of transcription factors activator protein-1, NF-κB, and Stat3; and (c) Bcl-xL, cdc25A, and cyclooxygenase-2 expression [203]. Based on these observations, we hypothesized that plumbagin may be able to modulate immune responses and might have significant application in prevention of inflammation associated disorders. To test this hypothesis, the immunomodulatory effects of plumbagin were studied in murine lymphocytes in vitro and in vivo.

CHAPTER - II

In vitro anti-inflammatory effects of plumbagin

This chapter summarizes the in vitro immunomodulatory properties of plumbagin as measured in terms of its ability to inhibit mitogen induced activation, cytokine secretion and proliferation of T cells. This chapter also describes effects of plumbagin on different lymphocyte subsets (CD4+, CD8+ and B cells) as well as on different activation markers and co-stimulatory molecules involved in activation of T cells and B cells.

2.1 MATERIALS AND METHODS

2.1.1 Reagents and Chemicals:

The following chemicals were obtained from Sigma Chemical Company, USA: Plumbagin,, Polyethyleneglycol (PEG), glycerol, Sulfanilamide, Naphthyl-ethylene-diamine-hydrochloride (NEDDH), penicillin, streptomycin, propidium iodide (PI), Lipopolysaccharide (LPS), sodium azide, TritonX-100, RPMI1640, Hoechst, tween 20, sodium chloride, sodium dihydrophosphate, disodiumhydrophosphate. Sodium citrate, trichloroacetic acid (TCA) and sodium hydroxide were procured from reputed local manufacturers. RPMI 1640, Fetal calf serum (FCS) was obtained GIBCO BRL. Concanavalin A (Con A) were purchased from Calbiochem, USA. CyQUANT cell proliferation assay kit and carboxy fluorescein diacetate succinimidyl ester (CFSE) were procured from Molecular Probes, Invitrogen.

2.1.2 Antibodies:

i. Following antibodies were obtained from BD Bioscience (USA): fluorochrome conjugated antibodies and respective isotype control against mouse CD69, CD25, CD54, CD71, CD80 and CD86. Antibodies against mouse CD3 and CD28.

ii. ELISA sets for detection of Interleukin-1β, Interleukin-2, Interleukin-4, Interleukin-6, Interferon-γ and TNF-α were obtained from BD Bioscience USA.

iii. Mouse CD4 and CD8 positive selection kit for enrichment and purification of CD4+ T helper cells and CD8+ cytotoxic T cells were obtained from Stem Cell Technologies.

2.1.3 Animal maintenance:

Eight to ten week old inbred C57BL/6 mice (MHC haplotype: H-2b) and Swiss albino (MHC haplotype: H2KdAbEbDbTlab) mice weighing approximately 20-25g were used [216]. They were reared in the animal house of Bhabha Atomic Research Centre. Mice were housed in plastic cages at constant temperature (23 ^{0}C) with a 12 / 12 hour light / dark cycle. All animals were given mouse chow and water *ad libitum*. Age and sex matched animals were used in different groups within any given experiment. The guidelines issued by the Institutional Animal Ethics Committee of Bhabha Atomic Research Centre, Government of India regarding the maintenance and dissection of small animals were strictly followed.

2.1.4 Maintenance of cell line:

RAW 264.7 cells obtained from National Centre for Cell Sciences (NCCS) were cultured in Dulbecco's modified Eagle's medium 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.

2.1.5 Treatment with plumbagin:

A 100mM solution of plumbagin was prepared in dimethyl sulfoxide (DMSO), stored as small aliquots at −20 °C, and then diluted as needed in cell culture medium. In all in vitro experiments, cells were treated with plumbagin for 4 h in 2-mercapto ethanol (ME) free RPMI medium and were further stimulated with Con A (10 µg/ml) without washing the cells. DMSO was used as vehicle control in vitro.

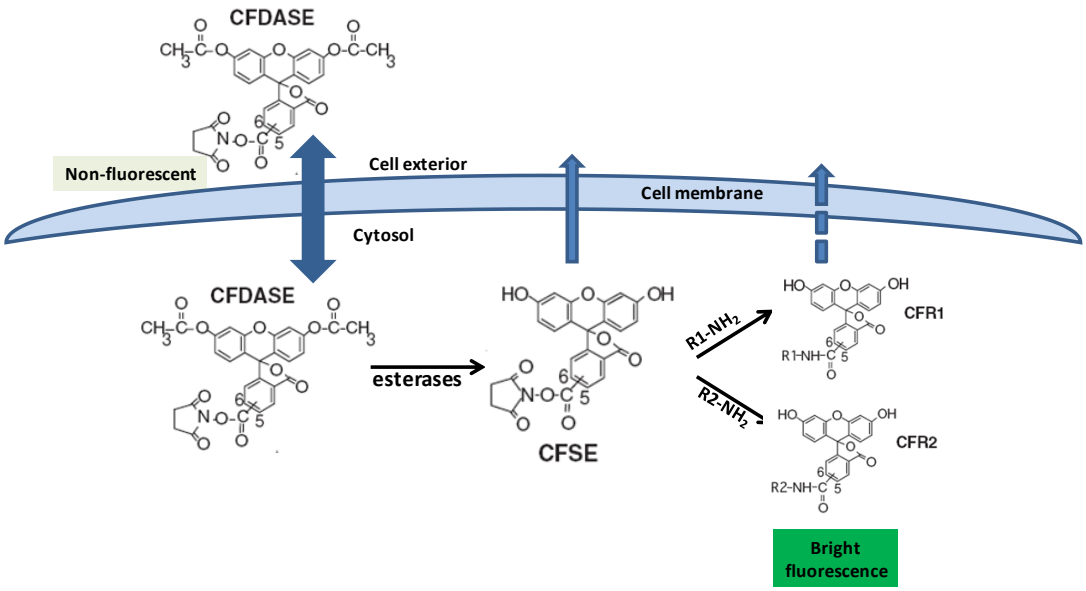
2.1.6 Splenic lymphocyte preparation:

Spleen is a major source of lymphocytes and accessory cells like dendritic cells, monocytes and macrophages in mice. In disease-free young and young-adult mice (6-8 weeks) majority of the lymphocytes are naïve and a very few lymphocytes show activated or memory phenotype. Spleen and lymph nodes were aseptically removed from these mice and placed in sterile petri dish containing RPMI 1640 medium. Single cell suspensions were prepared by gently squeezing the organs on a sterile wire mesh placed in the petri dish. Cell suspension was carefully transferred to 15 ml sterile tubes and centrifuged at 3000 rpm for 5 min. Red blood cells (RBC) were lysed by brief hypotonic shock using ice cold water (5ml, 10 sec) followed by ice cold 2X PBS (5 ml). The RBC membranes were allowed to pellet by centrifuging the tubes at 1000 rpm for 30 sec and lymphocytes were carefully decanted into a fresh tube. Lymphocytes were further centrifuged at 3000 rpm for 5 min and the cell pellet was resuspended in RPMI 1640 medium containing 10 % heat inactivated fetal bovine serum (Complete medium). Viable cells were counted in hemocytometer using a microscope after staining with trypan blue dye.

2.1.7 CFSE staining:

Proliferation of antigen specific lymphocytes in response to infections or tumors is very crucial to ensure efficient eradication of the pathogens and tumors. Cell proliferation can be measured by techniques that quantify bulk cell division. ^{3}H-thymidine incorporation and bromo-deoxy-uridine (BrDU) incorporation have been commonly used for estimating the cells in S phase of cell cycle in vitro and in vivo respectively. Other methods like MTT and XTT rely on mitochondrial dehydrogenase activity and are used to approximate the number of live cells in each treatment group as compared to that in control. Although these techniques give insight into general lymphocyte proliferation, novel fluorescence based methods have been developed in last two decades for estimation of individual cell proliferation. These techniques allow measurement of number of cell divisions for individual cells in vitro as well as in vivo.

CFSE is one such dye with a fluorescein molecule, two acetate moieties and one succinimidyl ester functional group (Lyons and Doherty; 1998). In this form it is membrane permeant and non-fluorescent. After diffusion into the cell, endogenous esterases remove the acetate groups, rendering the molecule highly fluorescent. The resultant carboxyfluorescein succinimidyl ester is highly fluorescent, and, due to its reduced lipophilicity, it exits from cells at a much slower rate. This slower exit rate also prolongs the time available for CFSE to covalently couple with amino groups, resulting in carboxyfluorescein (CF) being coupled, via a very stable amide bond, to the amino groups of intracellular molecules.



Scheme 2.1 Chemical reactions involved in labeling the cellular proteins with CFSE. The carbonyl group located between positions 5 and 6 on the benzene ring is to indicate that the dye is a mixture of 5- and 6-carbonyl structural isomers. Figure adapted with modifications from Parish et al (1999) [217].

It is these long-lived conjugates that allow stable labeling of cells with CFSE to be achieved [218] (Scheme 2.1).. The stability of CFSE-labeling allows monitoring of lymphocytes over a period of months in vivo. Cell division results in sequential halving of fluorescence, and up to 8 divisions can be monitored before the fluorescence is decreased to the background fluorescence of unstained cells. Since halving of fluorescence occurs in daughter cells, by calculating the proportion of cells in each division peak and dividing by the expected progeny at those divisions, the number of cells that have entered division can be calculated. This gives a precursor frequency estimate of responding cells in the cultures.

CFSE staining was used for assessment of lymphocyte proliferation in vitro. Spleen cells (100 X 10^6) suspended in RPMI 1640 (500µl) were mixed with equal volume of 20 µM CFSE and mixed by repeated pipetting followed by vortexing. Cells were incubated at 37°C for 5 min. The density of cells was kept very high to buffer any toxic effects of

CFSE. Cells were washed three times with ice cold RPMI 1640 medium supplemented with 10 % FCS to remove excess dye.

2.1.8 Estimation of T cell proliferation:

A number of agents can specifically or nonspecifically induce T cell activation, resulting in cytokine production, cytokine receptor expression, and ultimately proliferation of the activated T cells. Although proliferation is not a specific effector function of T lymphocytes in contrast to helper function for B lymphocytes or cytotoxicity; proliferation assays are reliable, simple, and easy to perform and have been widely used to assess the overall immuno-competence of an animal. Further, in vivo proliferation is an essential part of lymphocyte homeostasis. To monitor the proliferation in vitro, at the end of incubation periods indicated above, twenty thousand cells in each group were acquired in a flowcytometer (BD FACSAria or Partec Cyflo Space). The cell proliferation was measured by CFSE dye dilution. Per cent daughter cells that showed a decrease in CFSE fluorescence intensity were calculated using BD FACSDiva or FloMax® software.

2.1.9 Confocal Microscopy:

Confocal laser microscopy was used to study the entry of plumbagin into lymphocytes. Lymphocytes were incubated with or without plumbagin (10 µM) in medium containing 10% FBS. At the end of 24 h, the cells were centrifuged onto coverslips, fixed with paraformaldehyde, stained with Hoechst stain for nuclear staining, and mounted onto glass slides. Slides were examined using a LSM510 scanning module (Carl Zeiss Microscopy, Jena GmbH, Germany) with a krypton–argon laser, coupled to an Orthoplan Zeiss photomicroscope using a 488 nm laser line and a 510nm-band pass filter. Overlay images were recorded by superimposing simultaneous images from each channel.

2.1.10 Estimation of apoptosis by flowcytometry :

This method is based on reduced DNA stainability in apoptotic cells in which the peak DNA content is lower than the G1 peak. This is a consequence of progressive loss of DNA from the cells, due to activation of endonucleases and subsequent leakage of low molecular weight DNA products prior to measurement [219, 220]. The decrease of cellular DNA is apparent in flow cytometer, independent of the type of fluorochrome (propidium iodide, Hoechst, 7-aminoactinomycin). This method is quick, simple to perform and reliable and is applicable to all cell types. Propidium iodide is a DNA intercalating dye that binds to DNA in a stoichiometric manner. PI can be activated by 488nm laser and it emits fluorescence in red region (560-620 nm). Cells containing more DNA take up more PI than the cells containing less amount of DNA. Thus the relative fluorescence of apoptotic cells is lower than that of cells in G0/G1 phase of cell cycle [221].

2.1.11 Cell cycle analysis by flowcytometry:

The percentage of cells in different phases of cell cycle (G1, S/G2+M) was estimated from DNA content of individual cells stained with PI and analyzed in a flowcytometer [222]. Cells in G0/G1 phase of cell cycle have 2nDNA. The cells in G2 and M phases of cell cycle contain 4nDNA that is double of that in cells in G0/G1 phase. The cells in S phase show DNA higher than 2n and less than 4n.

One million splenocytes were stimulated with Con A in presence of plumbagin for 72 hr at 37^{o}C in 1 ml CM in a 5% CO_2 atmosphere. These cells were washed with 10 mM phosphate buffered saline (PBS) and incubated with 1 ml of staining solution containing 50 μg / ml propidium iodide, 0.1% sodium citrate and 0.1% triton X-100 overnight. A total of 20,000 cells were acquired in a flow cytometer (BD FACSAria or Partec Cyflo

Space) and were analyzed using BD FACSDiva or FloMax® software. Undivided cells were in G_1 phase of cell cycle (2n DNA content). The sub-G1 population represented the apoptotic cells. The cells containing more than 2n DNA represented S/G2+M phase of cells.

2.1.12 Flow cytometric analysis:

Flow cytometer allows simultaneous measurement of multiple physical characteristics of a single cell. These measurements are made on a per cell basis at routine rates of 500 to 4000 cells per second in a moving fluid stream. The information obtained on flow cytometer is the relative size of the cell (Forward scatter), its granularity (side scatter) and relative fluorescence intensities (FL1, FL2, FL3 and FL4). In the present studies fluorescence activated cell sorter (BD FACSAria or PARTEC Cyflospace) with 488 nm argon ion laser was used.

2.1.13 Measurement of cytokine secretion:

Cytokines production by stimulated lymphocytes was measured by sandwich ELISA technique. In this method the cytokine is captured between two antibodies specific for two different epitopes on the same protein (cytokine). Since the detection involves two antibodies, this process is more specific and 2 to 5 times more sensitive than those in which antigen is directly bound to the solid phase. To detect antigen, the wells of micro-titer plates are coated with specific (capture) antibody followed by incubation with test solutions containing antigen. Unbound antigen is washed out and a different antigen-specific antibody conjugated to enzyme (i.e., developing reagent) is added, followed by incubation. Unbound conjugate is washed out and substrate is added. The amount of substrate hydrolyzed is proportional to the amount of antigen in the test solution [223].

The concentration of IL-2, IL-4, IL-6 and IFN-γ in the supernatant of control unstimulated cells and cells stimulated with Con A or anti-CD3/CD28 in presence or absence of plumbagin for 24 hr or RAW cells stimulated with LPS in the presence or absence of plumbagin was estimated using cytokine ELISA sets (BD Pharmingen, USA). Cytokines induced by LPS in the culture supernatant of splenic adherent macrophage was also estimated. Splenocytes ($5x10^6$ cells/well) were incubated in a 24-well cell culture plate for 3 h at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The non-adherent cells were removed by aspiration. The adherent cells (macrophages) were then stimulated with LPS (50 μg/ml) in the presence or absence of plumbagin and further cultured for 6h or 24 h at 37°C. The concentration of IL-6 and TNF-α in the supernatant of LPS stimulated cells for 6 h and IL-1β for 24 h was estimated using cytokine ELISA sets (BD Pharmingen, USA).

The supernatants obtained from unstimulated cells and mitogen stimulated mouse splenocytes or RAW cells or splenic adherent macrophages were used as negative and positive control respectively. 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, 100 μl / well). The plates were sealed with parafilm to avoid evaporation and incubated at $4^{\circ}C$ overnight. Unbound antibody was aspirated and wells were washed three times with wash buffer (PBS with 0.05% Tween20). Wells were blocked with blocking buffer (PBS with 10% FCS) for one hr at $25^{\circ}C$ and washed three times with wash buffer (PBS with 0.05% Tween 20). 100μl culture supernatant was added to three wells in each group. To another set of wells, 100 μl serial dilutions of cytokine standards IL-2, IL-4, IL-6, IL1β, TNF-α and IFN-γ were prepared (as per manufacturer's protocol) and added in three replicates and plates were incubated at 25^0C for two hours. 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 and mixed in blocking buffer in 1:1 ratio and 100µl of freshly prepared mixture was added to each well. Plates were incubated at 25^0C for one hour. The wells were aspirated and washed five times to remove any unbound antibody. 100µl TMB (3,3',5,5'-Tetramethylbenzidine) substrate was added to each well and incubated at 25^0C for 15 min. Stop solution (1N HCl, 50µl) was added to inhibit further reaction and absorbance was taken at 450nm using an ELISA reader. Standard curves were generated from mean $\pm$ 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.

2.1.14 Purification of CD4+ T or CD+8 T cells:

Separation of cells based on the differential expression of cell-surface antigens is an important technique that has facilitated the study of specific subpopulations of lymphocytes. Cell separation can be a negative selection process, in which the isolated subpopulation lacks the selected cell-surface antigen, or it can involve positive selection, in which the isolated population has the selected antigen.

The panning technique can be used to both negatively and positively select a specific subpopulation when large numbers of cells are desired. The purity of specific cell populations at the end of one round of panning is routinely between 50-65%. Purity of cells can be increased up to 90% by repeating panning 3-4 times. Since one round of panning takes about 2 h, use of this method for obtaining high purity of lymphocyte subpopulations requires very long incubation times.

Alternatively, lymphocyte subsets can be isolated using a magnetic-isolation technique. This method is simple, quick, and reproducibly yields cells of high purity. In the present studies, magnetic sorting was used to purify CD4+ T or CD8+ T cells from total splenic lymphocytes using CD4 or CD8 positive selection kit (Stem Cell Technologies) in a Stem Cell Technologies Magnetic Cell Sorter. Cells were resuspended in RPMI medium (100 x 10^6 cells/ml) and CD4/CD8 PE labeling reagent (50µl/ml) was added. The cells were incubated at room temperature for 15 min in a 5 ml polypropylene tube (BD Falcon). After 15 min, PE selection cocktail (100 µl/ml) was added and these cells were incubated at room temperature for 15 min. Further, uniform suspension of magnetic nanoparticles was prepared by vigorous pipetting and added to these cells (50 µl/ml) followed by incubation at room temperature for 10 min. Total volume of cells was made to 2.5 ml by addition of ice-cold RMPI medium and cells were placed in a magnet. Magnetically labeled cells were allowed to adhere to the tube for 6-8 min and unbound cells were carefully decanted into a fresh tube. The residual cells were removed from the magnet and washed two times with RPMI to further remove the negative cells. For washing, the cells were resuspended in 2.5 ml RPMI medium. The tube was placed in the magnet and cells were allowed to adhere to the walls for 6-8 min. The unbound cells were carefully decanted. This step was repeated once again to further purify the CD4+ or CD8+ T cells. Since the positively selected cells have already been PE-labeled, the purity was assessed directly by flowcytometry. The purity of selected cells was CD4+ (purity: 93%) & CD8+ (purity: 92%) in T cells.

2.1.15 Antibody staining:

Direct immunofluorescence staining is a technique in which the cells (live or fixed) are incubated fluorochrome-conjugated antibody. This method was used for different

subpopulations in splenic leukocytes. Total splenocytes (0.5×10^6) were used for direct immunofluorescence staining. Cells were resuspended in 50µl buffer (PBS containing 10% goat serum, 0.1% sodium azide) and incubated on ice for 10 min for blocking Fc receptors. The cells were further incubated with 1µg of FITC or PE conjugated CD25 or CD69, CD54 or CD71 or CD80 or CD86 antibody in buffer for 30 min on ice in dark, washed three times with the buffer and 20,000 cells were acquired in the flowcytometer.

2.1.16 Measurement of nitric oxide in culture supernatant using Griess reagent:

Nitric oxide in the culture supernatant was estimated using Greiss reagent as described earlier [224, 225]. 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.

2.1.17 Statistical Analysis: The statistical significance of the differences in respect of all parameters studied between untreated and Con A/LPS/antioxidant treated cells in presence or absence of plumbagin in vitro was assessed by student's t-test. For comparing multiple groups, statistical analysis was done using ANOVA with Microcal Origin 6.0 software followed by post-hoc analysis using Schiffe's test.

2.2 RESULTS

2.2.1 Plumbagin uptake in lymphocytes:

Figure 2.1 shows the confocal images of lymphocytes treated with plumbagin for 24 h and stained with Hoechst. Splenic lymphocytes were incubated with or without plumbagin (10 µM) in medium containing 10% FBS. At the end of 24 h, the cells were centrifuged onto coverslips, fixed with paraformaldehyde, stained with Hoechst stain for nuclear staining. It was observed that plumbagin entered lymphocytes and majority of it was localized in the nucleus (Fig. 2.1).

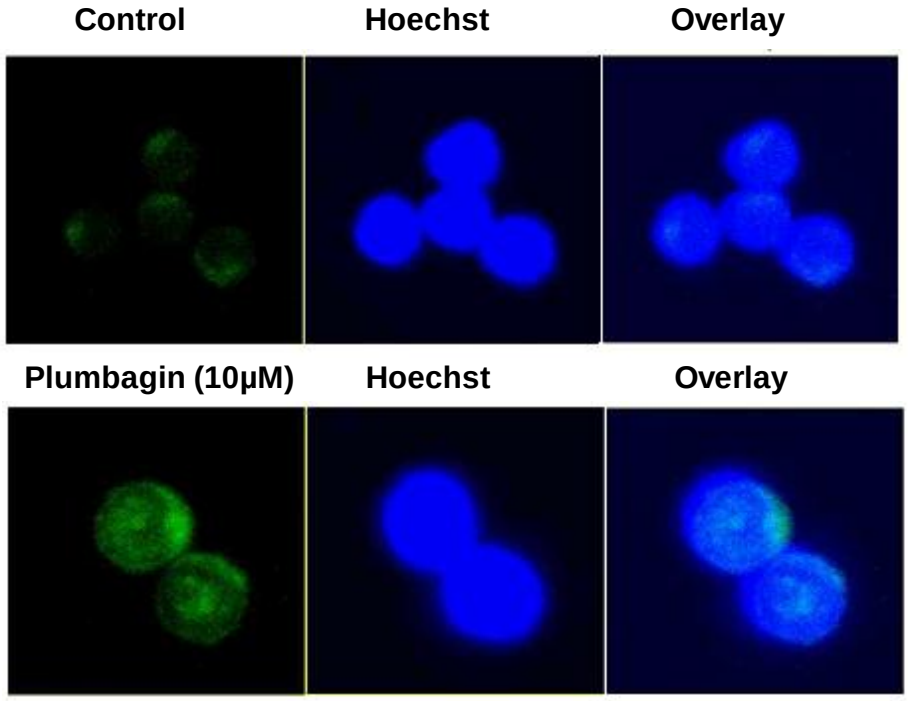


Fig 2.1: Images showing intracellular localization of plumbagin in lymphocytes: Lymphocytes were incubated with plumbagin for 24 h and fixed with paraformaldehyde. The cells were stained with Hoechst and observed under confocal microscope.

2.2.2 Plumbagin did not induce cell death in lymphocytes:

To estimate plumbagin induced cell death in naïve splenocytes, they were incubated with plumbagin (5µM) for 24 h and 72 h. These cells were harvested and stained with propidium iodide and acquired using a flowcytometer. Lymphocytes treated with plumbagin did not show any increase in apoptosis at 24h (preG1 peak) as compared to that in control cells (Figure 2.2).

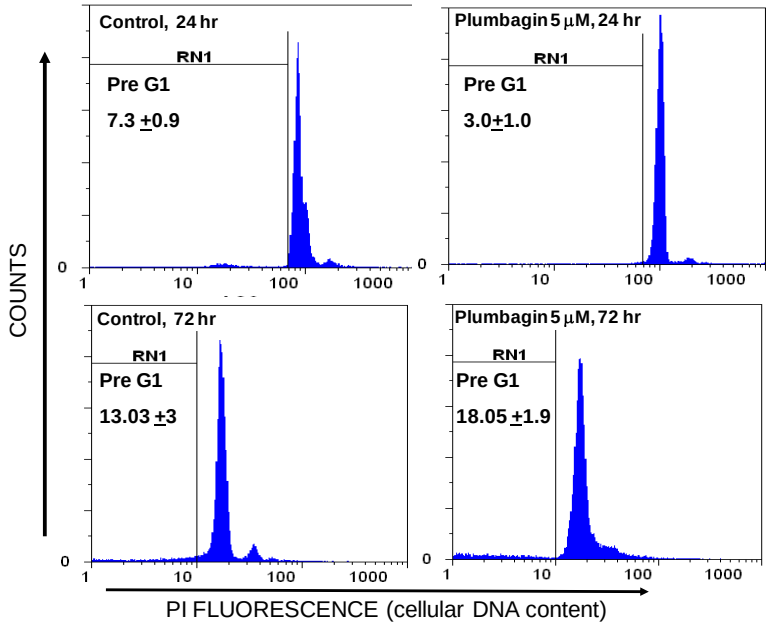
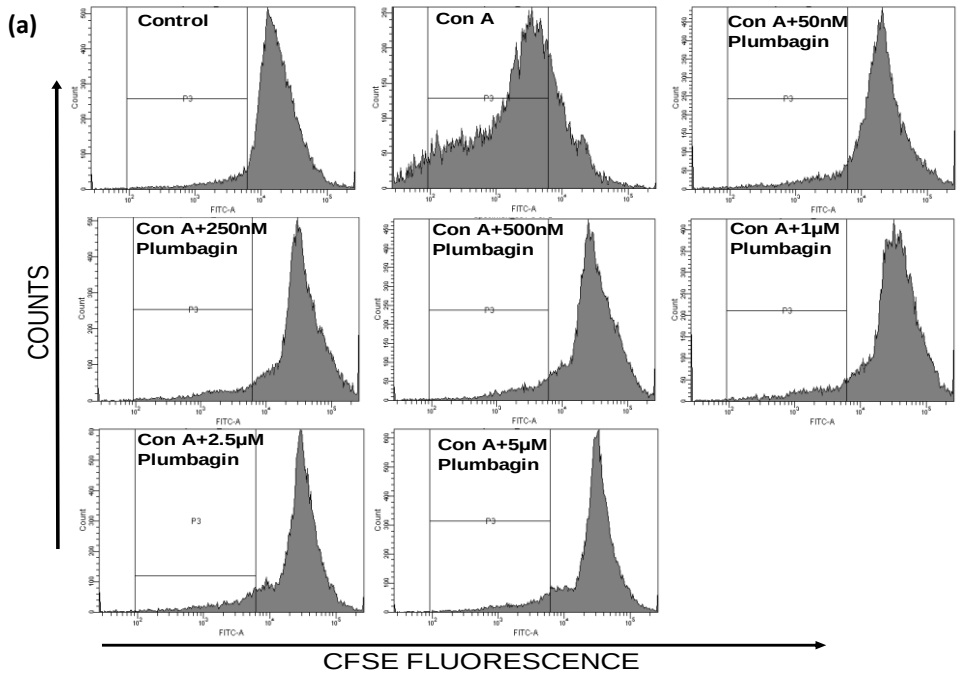


Fig. 2.2: Effect of plumbagin on viability of resting lymphocytes in vitro: Lymphocytes were treated with plumbagin (5 µM) at 37 °C in complete medium for 24 and 72 h and the cells were stained with propidium iodide solution and twenty thousand cells were acquired in a flowcytometer. Vehicle treated cells served as control. Percentage apoptosis (pre-G1 peak) in lymphocytes was estimated and is shown in the histograms. Two such independent experiments were carried out and results from one such experiment are shown. Data points represent mean±S.E.M. from three replicates. RN1 in flowcytometric histograms stands for Region 1 (hypodiploid/apoptotic cells).

2.2.3 Plumbagin inhibited Con A induced T cell proliferation:

Lymphocytes are known to proliferate in response to mitogens in vitro (Rocha et al., 1983). The effect of plumbagin on Con A induced lymphocyte proliferation was assessed by CFSE dye dilution. Two million CFSE labeled splenocytes were treated with plumbagin (50 nM to 5 µM, 4 h) and were stimulated with Con A (10 µg/ml) for 72 h at 37 °C in 2ml RPMI with 10% FCS in a 95% air/5% CO2 atmosphere. Vehicle treated cells served as a control. Fig. 2.3a 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 plumbagin. Frequency of daughter cells (Gate P3) increased significantly in Con A stimulated lymphocytes as compared to that in unstimulated cells (Fig. 2.3a). The bars represent percentage of daughter cells obtained after 72 h of Con A stimulation (Fig. 2.3b). Plumbagin completely inhibited Con A induced proliferation of lymphocytes (Fig. 2.3b).



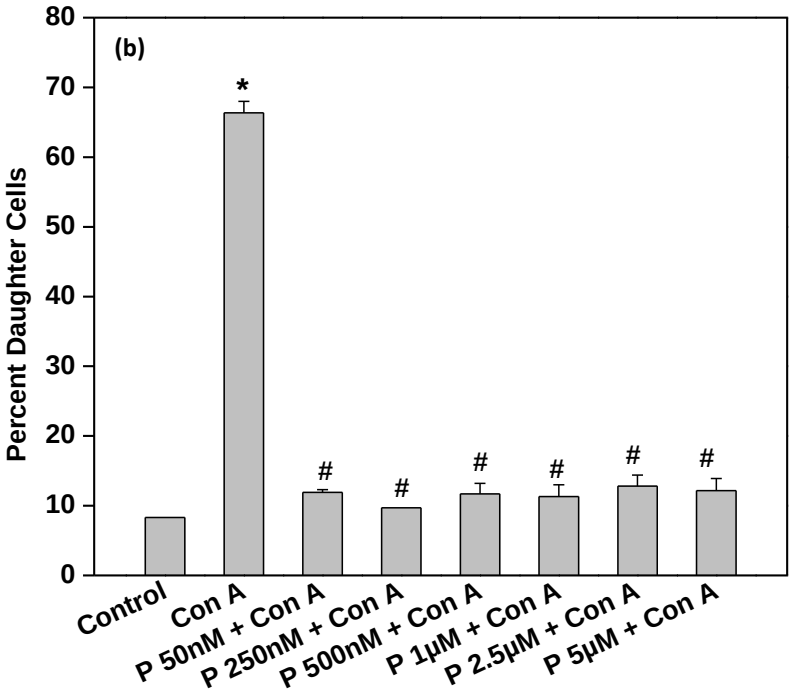
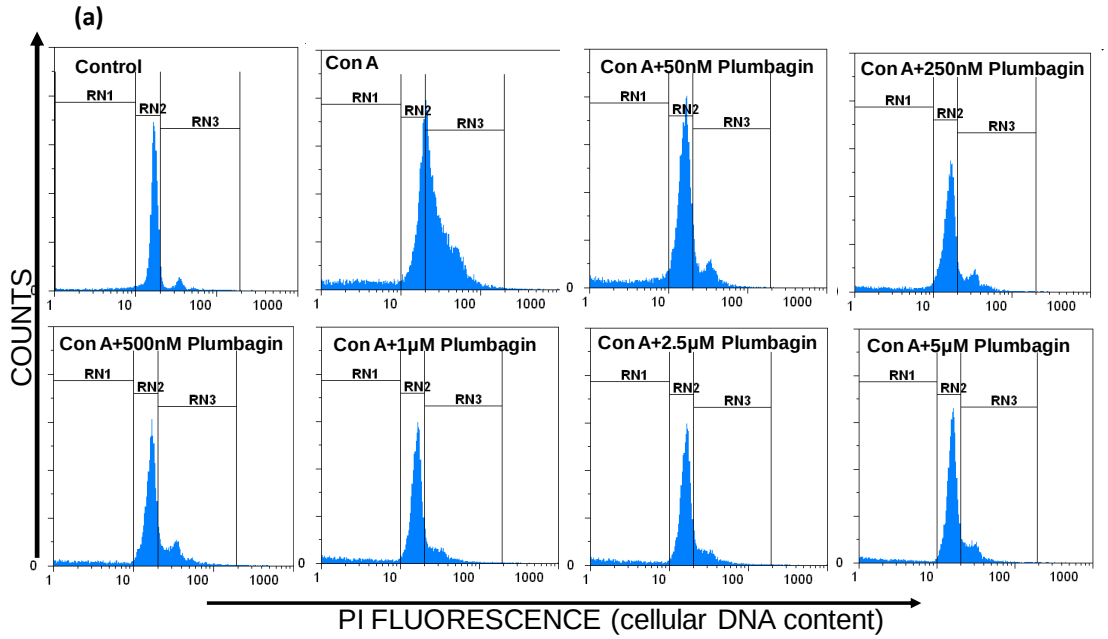


Fig. 2.3: Effect of plumbagin on Con A induced T cell proliferation in vitro: CFSE labeled lymphocytes were treated with plumbagin (50 nM to 5 µM, 4 h) and stimulated with the Con A for 72 h. Twenty thousand cells were acquired in a flowcytometer. Vehicle treated cells served as control. Percent daughter cells were calculated from decrease in mean fluorescence intensity. (a) Representative flowcytometric histograms of CFSE labeled cells representing plumbagin induced suppression of Con A induced proliferation. (b) Graph shows percentage of daughter cells in each treatment group. Each bar represents mean±S.E.M. from three replicates and three 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.

2.2.4 Plumbagin inhibited Con A induced cell cycle progression in lymphocytes:

Figure 2.4 shows the effect of plumbagin on cell cycle progression in Con A stimulated lymphocytes as assessed by PI staining. The bars represent percentage of cells in different phases of cell cycle 72 h after stimulation with Con A (10 µg/ml). Frequency of cells in S+G2/M phase of cell cycle increased significantly in Con A stimulated lymphocytes as compared to that in unstimulated cells (Fig. 2.4a). The fraction of cells in S+G2/M phase of cell cycle in plumbagin treated lymphocytes stimulated with Con A was significantly lower than that in lymphocytes stimulated with Con A alone (Fig. 2.4b). Also percentage of cells in G1 phase of cell cycle in plumbagin treated lymphocytes stimulated with Con A was significantly higher than that in lymphocytes stimulated with Con A alone indicating that plumbagin induced cell cycle arrest at G1 stage in activated T cells (Fig. 2.4b).



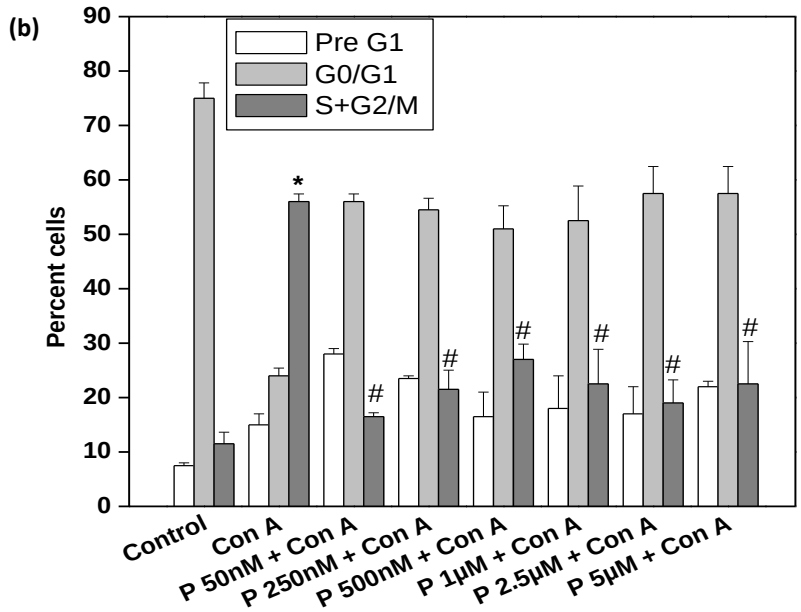


Fig. 2.4. Effect of plumbagin on Con A induced cell cycle progression: Lymphocytes were treated with plumbagin (50 nM to 5 µM, 4 h) and stimulated with the Con A (10 µg/ml) at 37 °C in complete medium for 72 h. The cells were stained with propidium iodide and twenty thousand cells were acquired in a flowcytometer. Vehicle treated cells served as control. (a) Representative flowcytometric histograms of PI labeled cells depicting plumbagin induced inhibition of cell cycle progression. RN1, RN2 and RN3 in flowcytometric histograms stand for Region 1 (hypodiploid/apoptotic cells), Region 2 (cells in G0/G1 phases of cell cycle) and Region 3 (Cells in S/G2/M phases of cell cycle) respectively. (a) The hollow bars represent percentage of cells containing less than 2n DNA (sub-G1/apoptotic cells), light gray bars show cells containing 2n DNA (in G1 phase) and the dark gray bars indicate the cells containing more than 2n DNA (in S+G2/M phase). Graph shows mean±S.E.M. from three replicates and two such experiments were carried out. *$p<0.01$, as compared to vehicle treated cells and #$p<0.01$, as compared to Con A stimulated cells.

2.2.5 Plumbagin inhibited Con A induced cytokine production:

Fig. 2.5 shows the concentration of IL-2 (Fig. 2.5a), IL-4 (Fig. 2.5b), IL-6 (Fig. 2.5c) and IFN-γ (Fig. 2.5d) cytokines in supernatants from plumbagin (50nM, 1µM and 5µM) treated cells stimulated with Con A (10µg/ml) as compared to that in the cells stimulated with Con A alone. Con A activated cells showed significantly higher secretion of IL-2, IL-4, IL-6 as well as IFN-γ as compared to that in control vehicle treated cells (Fig. 2.5a-d). Plumbagin (50nM and above) treated cells produced significantly lower amounts of IL-2, IL-6 and IFN-γ production at 50nM plumbagin (Fig. 2.5a-d). Complete inhibition of IL-2, IL-4, IL-6 and IFN-γ production was seen at 1µM plumbagin (Fig. 2.5a-d).

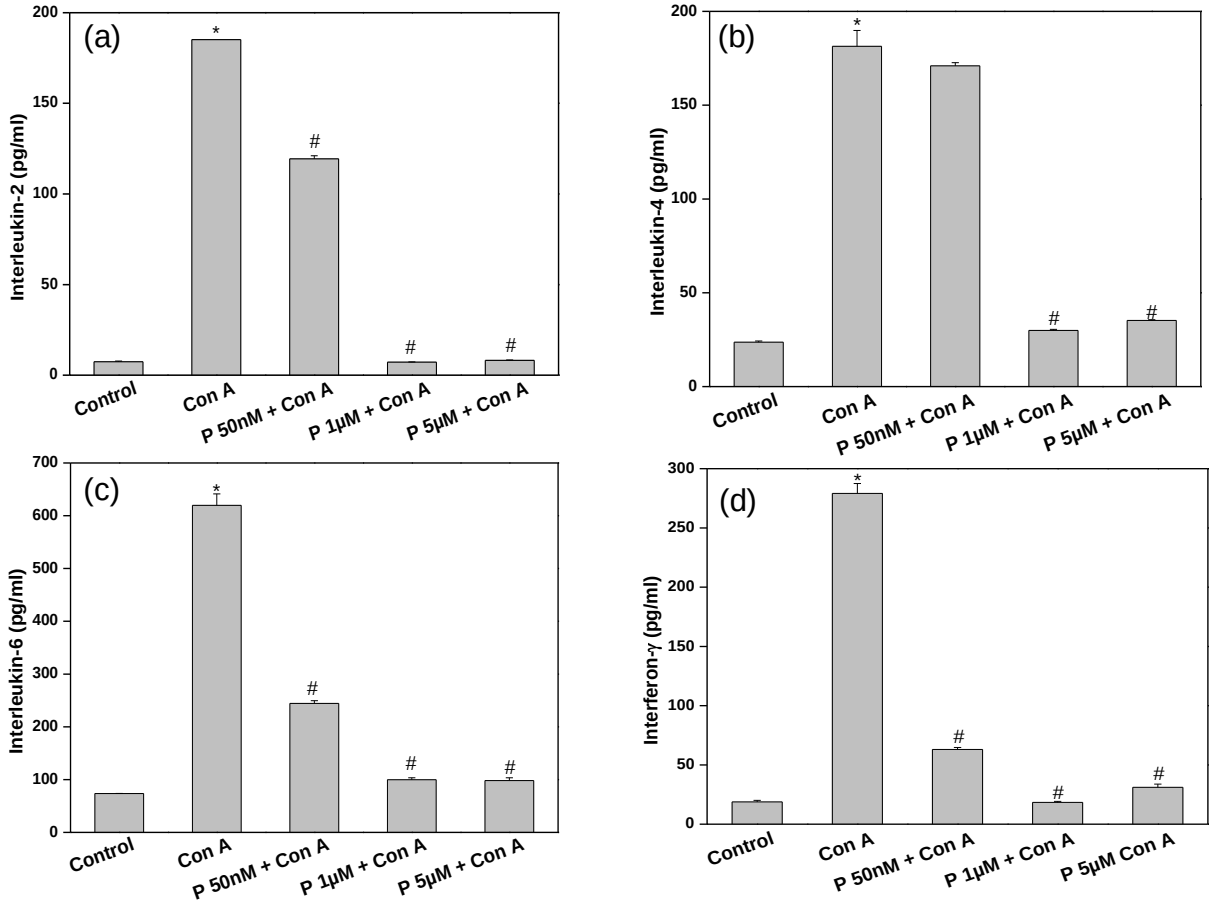
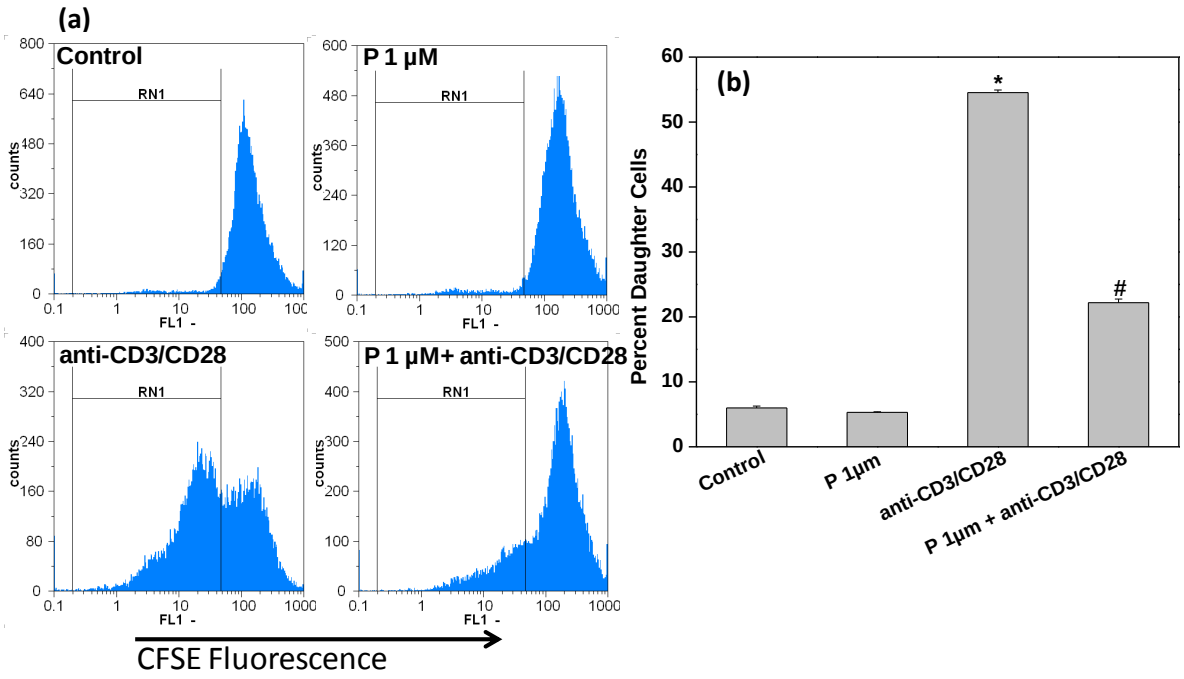


Fig. 2.5 Effect of plumbagin on Con A induced cytokine production in vitro: Lymphocytes were treated with different concentrations of plumbagin for 4 h before stimulation with Con A (10 µg/ml) for 24 h at 37 °C in complete medium. Vehicle treated cells served as control. The concentration of cytokines in the supernatant was estimated using ELISA. Graphs show concentration of (a) IL-2 (b) IL-4, (c) IL-6 and (d) IFN-γ. Each bar represents mean±S.E.M. from three replicates and three 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.

2.2.6 Plumbagin inhibited proliferation and cytokine secretion by CD4+ T cells in vitro: Immune response to any antigenic exposure is mediated via activation and proliferation of both CD4+ T cells and CD8+ T cells. Therefore, we studied whether plumbagin act on both these cell types or is specific to any particular cell type. CD4+ T cells were purified from splenic lymphocytes using immunomagnetic cell sorting kit. These cells were then labeled with CFSE and stimulated with plate bound anti-CD3 and soluble CD28 monoclonal antibodies in the presence or absence of plumbagin for 24 or 72h. As shown in Fig. 2.6a – e, plumbagin inhibited anti-CD3/CD28 mAb induced proliferation (Fig. 2.6a and b) and secretion of IL-2, IL-4 and IFN-γ cytokine by CD4+ T cells (Fig. 2.6c - e).



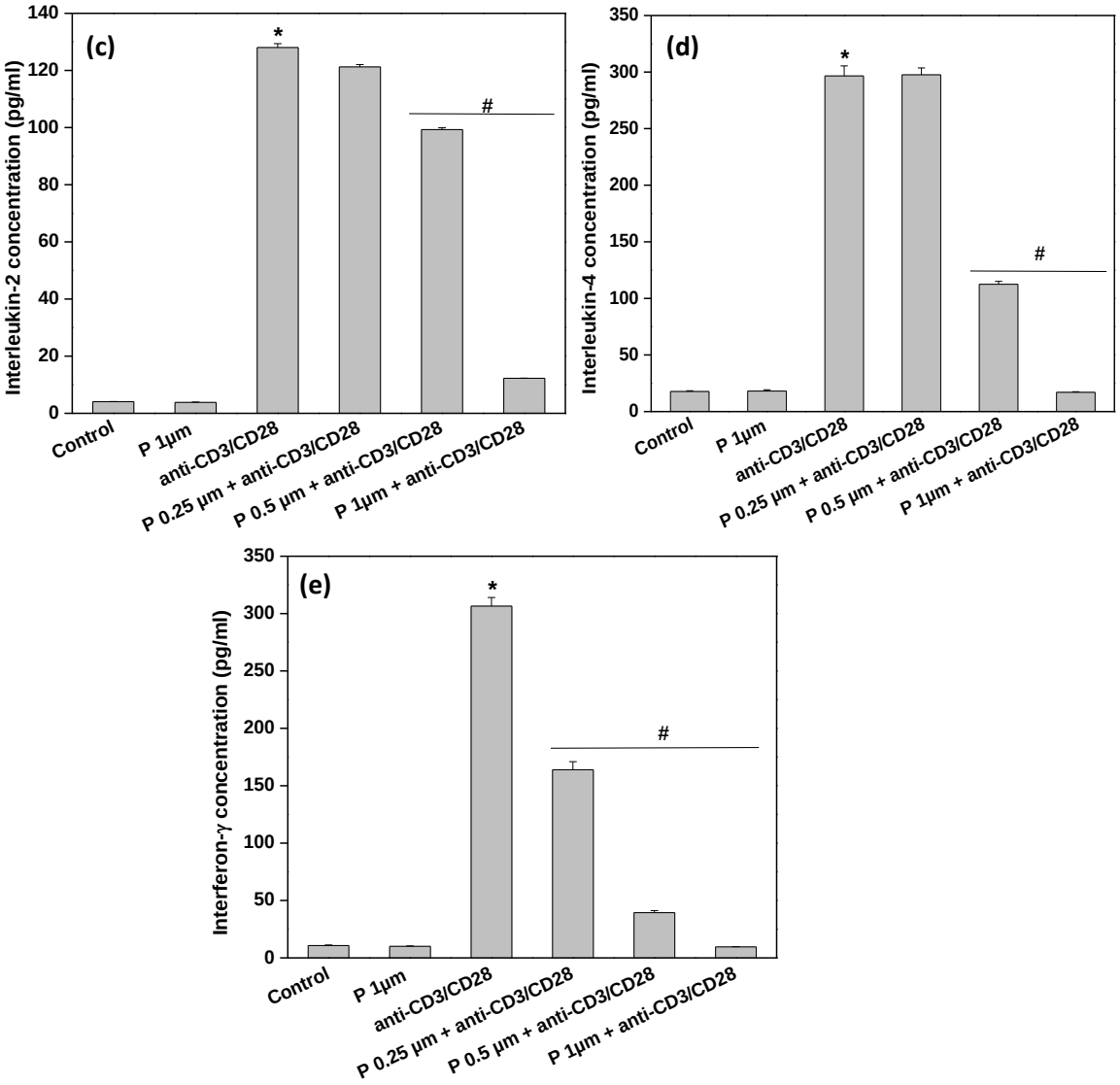
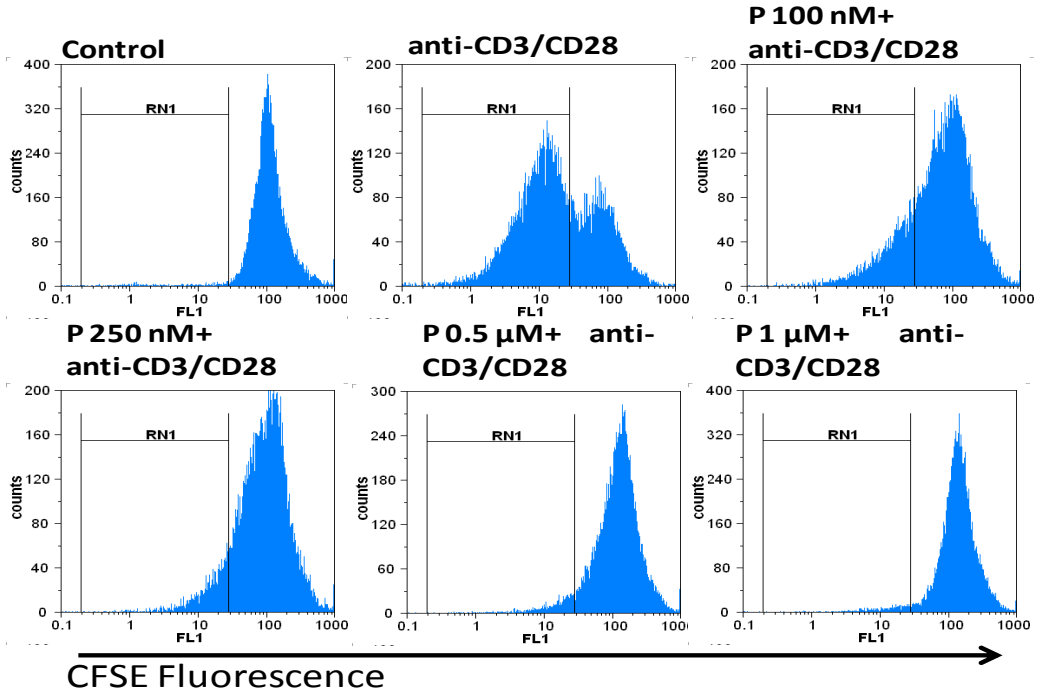


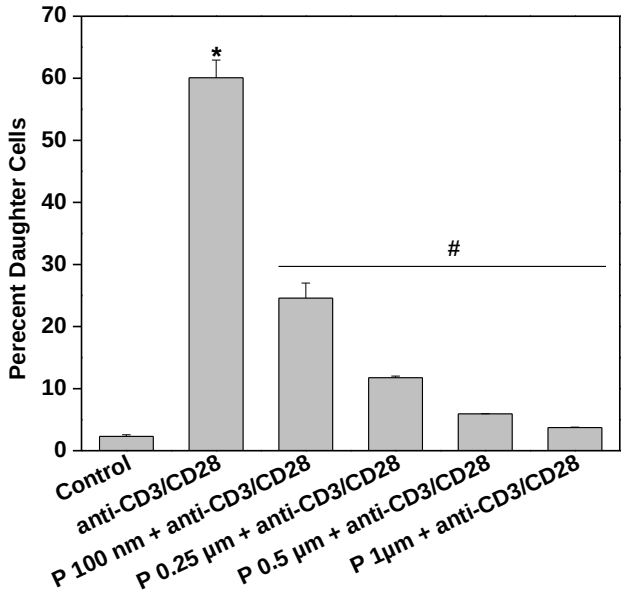
Fig. 2.6 Plumbagin inhibits anti-CD3/anti-CD28 induced proliferation and cytokine secretion by CD4+ T cells: CFSE labelled purified CD4+ T cells were pretreated with plumbagin before stimulation with plate bound anti-CD3mAb (1 µg/ml) and soluble anti CD28mAb (1 µg/ml) for 24 or 72h at 37 °C. (a) Representative flowcytometric histograms of CFSE labeled CD4+ T cells representing suppression of anti-CD3/anti-CD28 mAb induced proliferation by plumbagin. (b) Graphs show represents percentage of daughter cells from gate RN1. Percent daughter cells were calculated from decrease in mean fluorescence intensity. 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 #$p < 0.05$, as compared to anti-CD3/anti-CD28mAb stimulated cells. (c - e) The concentration of cytokines in the supernatant was estimated using ELISA. Graphs show concentration of (a) IL-2 (b) IL-4 and (c) IFN-γ. Each bar represents mean±S.E.M. from three replicates and three such independent experiments were carried out. *$p<0.01$, as compared to vehicle treated cells and #$p<0.01$, as compared to anti-CD3/anti-CD28 mAb stimulated cells.

2.2.7 Plumbagin inhibited proliferation of and cytokine secretion by CD8+ T cells in vitro: CD8+ T cells were purified from splenic lymphocytes using immunomagnetic cell sorting kit. The cells labeled with CFSE and stimulated with plate bound anti-CD3 and soluble CD28 monoclonal antibodies in the presence or absence of plumbagin for 24 or 72h. As shown in Fig. 2.7a – e, plumbagin inhibited anti-CD3/CD28 mAb induced proliferation (Fig. 2.7a and b) and secretion of IL-2, IL-6 and IFN-γ cytokine secretion by CD4+ T cells (Fig. 2.7 c - e).

(a)



(b)



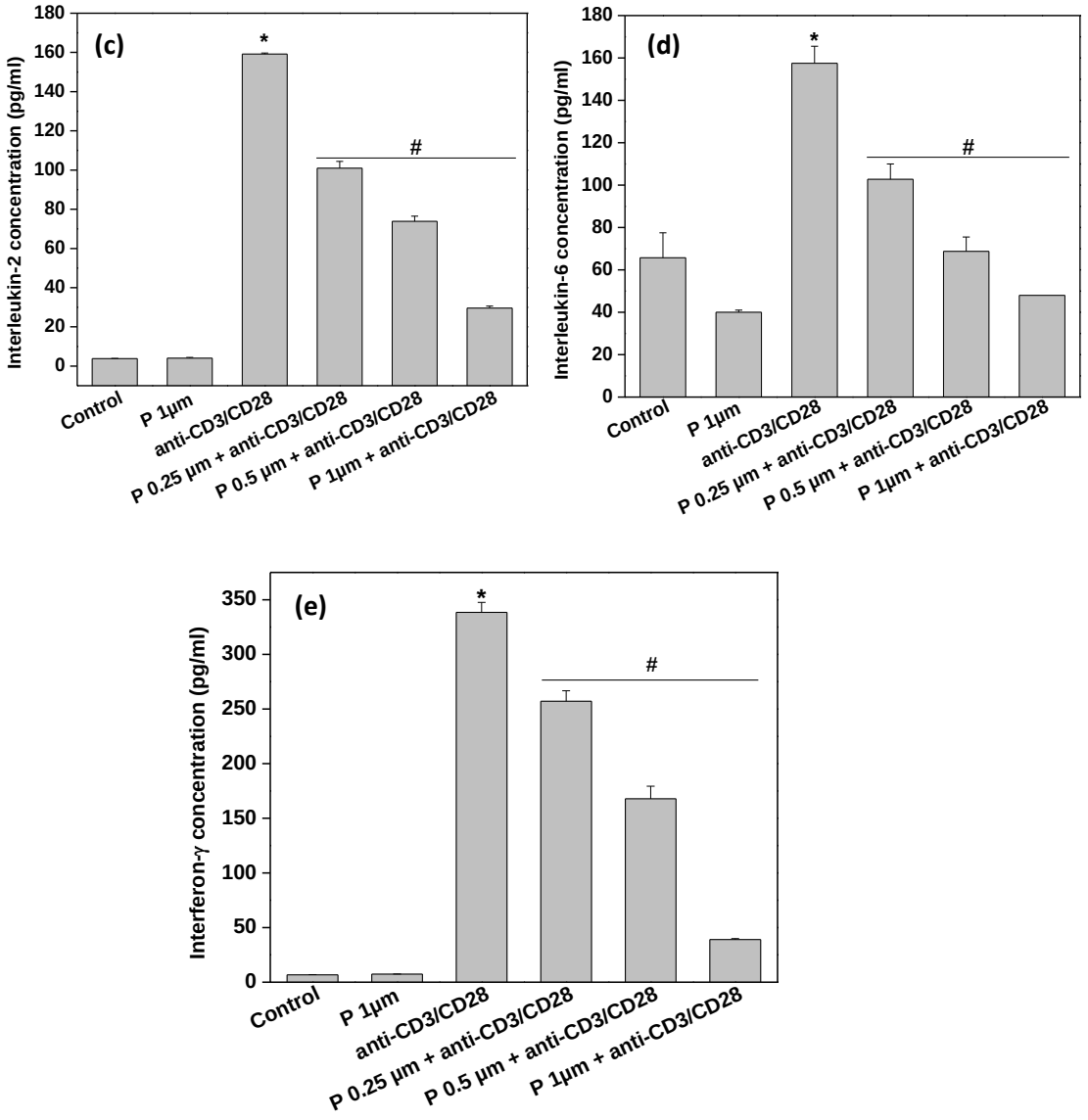


Fig. 2.7. Plumbagin inhibits anti-CD3/anti-CD28 induced proliferation and cytokine secretion by CD8+ T cells: CFSE labeled purified CD8+ T cells were pretreated with plumbagin before stimulation with plate bound anti-CD3mAb (1 mg/ml) and soluble anti CD28mAb (1 mg/ml) for 24 or 72h at 37 °C. (a) Representative flowcytometric histograms of CFSE labeled CD8+ T cells representing suppression of anti-CD3/anti-CD28 mAb induced proliferation by plumbagin. (b) Graphs show percentage of daughter cells in each treatment group from gate RN1. Percent daughter cells were calculated from decrease in mean fluorescence intensity. 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 #$p < 0.05$, as compared to anti-CD3/anti-CD28mAb stimulated cells. (c-e) The concentration of cytokines in the supernatant was estimated using ELISA. Graphs show concentration of (a) IL-2 (b) IL-6 and (c) IFN-γ. Each bar represents mean±S.E.M. from three replicates and three such independent experiments were carried out. *$p<0.01$, as compared to vehicle treated cells and #$p<0.01$, as compared to anti-CD3/anti-CD28mAb stimulated cells.

2.2.8 Plumbagin inhibited expression of Con A induced early T cell activation marker CD69:

Figure 2.8 shows the surface expression of early T cell activation markers on lymphocytes treated with plumbagin (2.5 and 5µM, 4 hr) and stimulated with Con A (10µg/ml) as compared to that in the cells stimulated with Con A alone. Con A activated cells showed significantly higher expression of CD69 as compared to that in control vehicle treated cells (Fig. 2.8 a and b). Plumbagin treatment prior to Con A stimulation lead to a complete suppression of CD69 on the surface of these cells (Fig. 2.8 a and b).

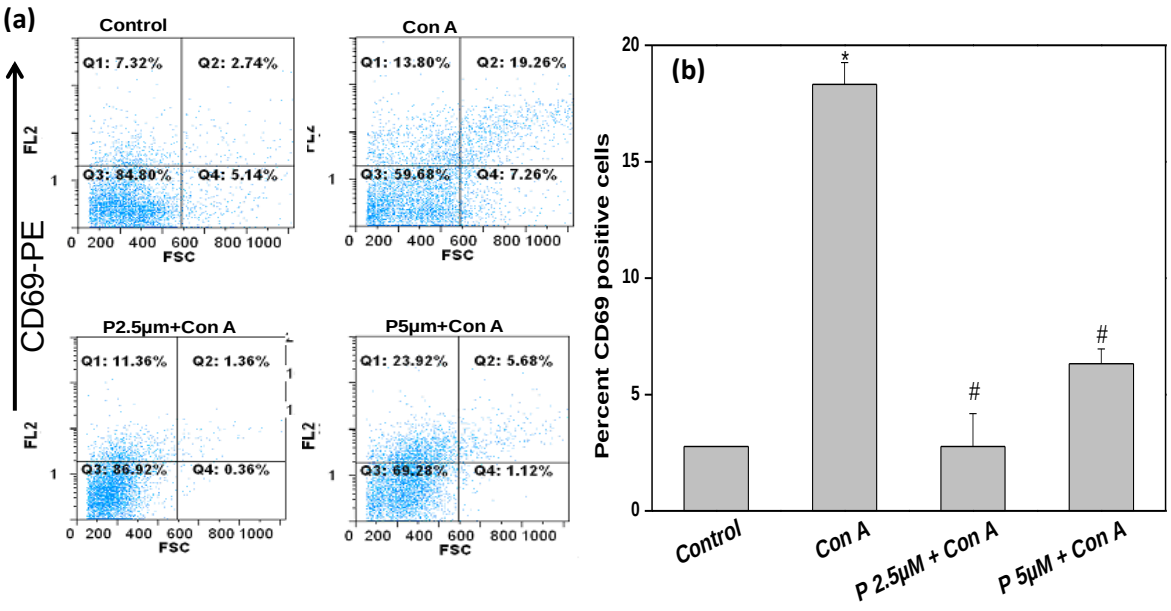


Fig. 2.8: Effect of plumbagin on CD69 surface expression on Con A stimulated lymphocytes in vitro: Lymphocytes were treated with plumbagin (2.5 and 5 µM, 4 h) and then stimulated with Con A (10 µg/ml) for 24 h at 37 °C in complete medium. Vehicle treated cells served as control. In each group, 0.5×10^6 cells were stained with PE conjugated anti-CD69 antibody. (a) Representative flowcytometric histograms of anti-CD69 labeled splenocytes representing plumbagin induced suppression of mitogen induced increase in expression of CD69. (b) Each bar represents frequency of CD69+ cells. Data points represent mean±S.E.M. from three replicates and two such experiments were carried out. *$p<0.01$, as compared to vehicle treated cells and #$p<0.01$, as compared to Con A stimulated cells.

2.2.9 Plumbagin inhibited expression of Con A induced T cell activation marker CD25 (Il-2 receptor α chain):

Fig. 2.9 shows the surface expression of early T cell activation marker on lymphocytes treated with plumbagin (2.5 and 5µM, 4 hr) and stimulated with Con A (10µg/ml) as compared to that in the cells stimulated with Con A alone. Con A activated cells showed significantly higher expression of CD25 as compared to that in control vehicle treated cells. Plumbagin treatment prior to Con A stimulation lead to a complete inhibition of CD25 on the surface of these cells (Fig. 2.9a and b).

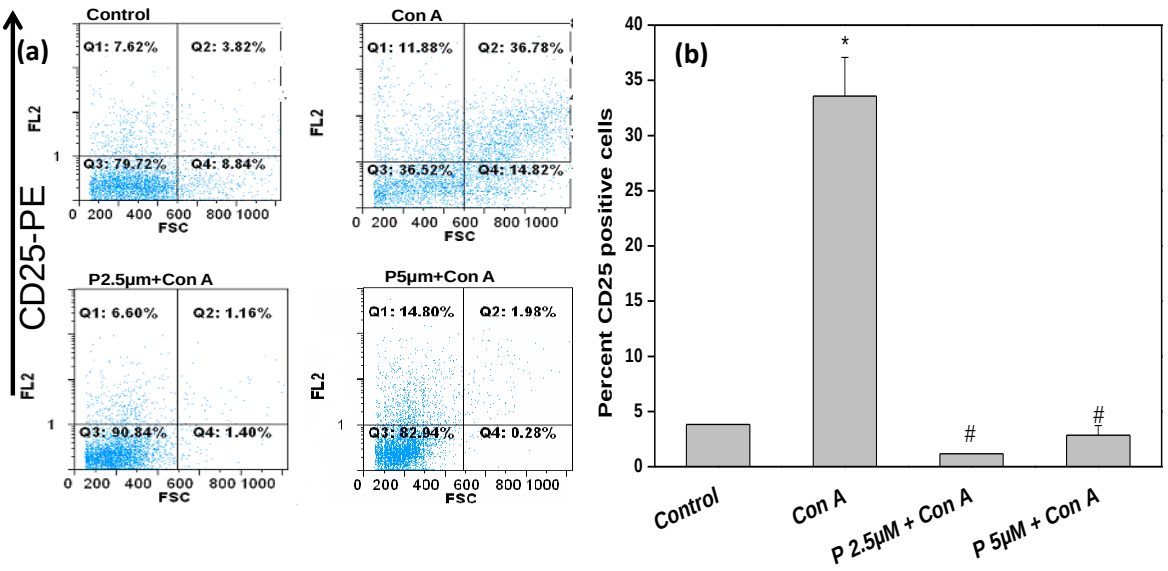


Fig. 2.9 Effect of plumbagin on CD25 surface expression on Con A stimulated lymphocytes in vitro. Lymphocytes were treated with plumbagin (2.5 and 5 µM, 4 h) and then stimulated with Con A (10 µg/ml) for 24 h at 37 °C in complete medium. Vehicle treated cells served as control. In each group, 0.5×10^6 cells were stained with PE conjugated anti0CD25 antibody. (a) Representative flowcytometric histograms of anti-CD25 labeled splenocytes representing plumbagin induced suppression of mitogen induced increase in expression of CD25. (b) Each bar represents frequency of CD69+ cells. Data points represent mean±S.E.M. from three replicates and two such experiments were carried out. *$p<0.01$, as compared to vehicle treated cells and #$p<0.01$, as compared to Con A stimulated cells.

2.2.10 Plumbagin inhibited mitogen induced expression of intercellular adhesion molecule-1 (ICAM-1) on splenocytes:

Fig. 2.10 shows the surface expression of ICAM-1 on splenic lymphocytes treated with plumbagin (1µM, 4 hr) and stimulated with Con A (10µg/ml) as compared to that in the cells stimulated with Con A alone. Con A activated cells showed significantly higher expression of ICAM-1 as compared to that in control vehicle treated cells. Plumbagin treatment prior to Con A stimulation lead to a complete inhibition of ICAM-1 on the surface of these cells (Fig. 2.10a and b).

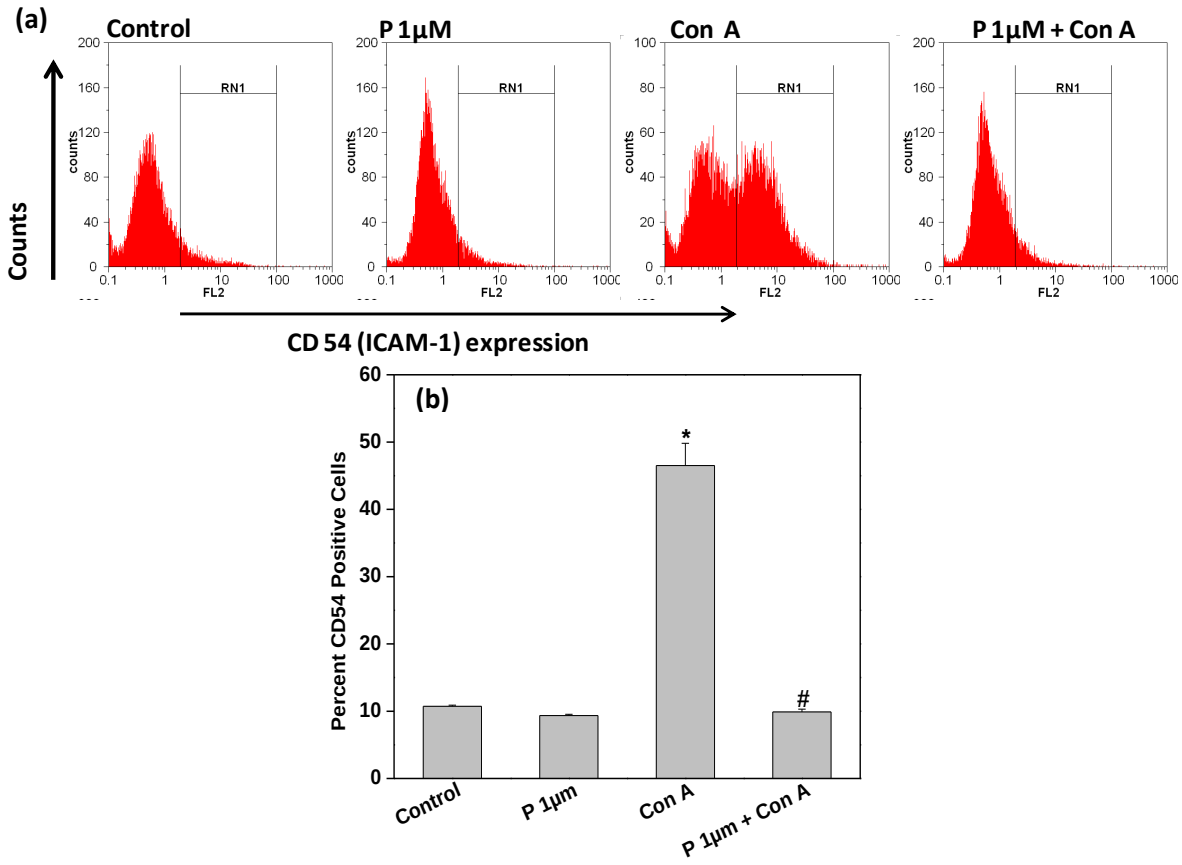


Fig. 2.10: Effect of plumbagin on ICAM-1 surface expression on Con A stimulated lymphocytes in vitro: Lymphocytes were treated with plumbagin (1 µM, 4 h) and then stimulated with Con A (10 µg/ml) for 24 h at 37 °C in complete medium. Vehicle treated cells served as control. In each group, 0.5×10^6 cells were stained with PE conjugated anit-CD54 antibody. (a) Representative flowcytometric histograms of anti-CD54 labeled splenocytes representing plumbagin induced suppression of mitogen induced increase in expression of CD54. (b) Each bar represents frequency of CD54+ cells. Data points represent mean±S.E.M. from three replicates and two such experiments were carried out. *$p<0.01$, as compared to vehicle treated cells and #$p<0.01$, as compared to Con A stimulated cells.

2.2.11 Plumbagin inhibited mitogen induced expression of CD71 (transferrin receptor) on splenocytes:

CD71, the transferrin receptor, mediates iron uptake and is critical for growth of activated lymphocytes. Fig. 2.11 shows the surface expression of CD71 on splenic lymphocytes treated with plumbagin (1µM, 4 hr) and stimulated with Con A (10µg/ml) as compared to that in the cells stimulated with Con A alone. Plumbagin treatment prior to Con A stimulation lead to a complete inhibition of mitogen induced increase in the expression of CD71on the surface of these cells (Fig. 2.11a and b).

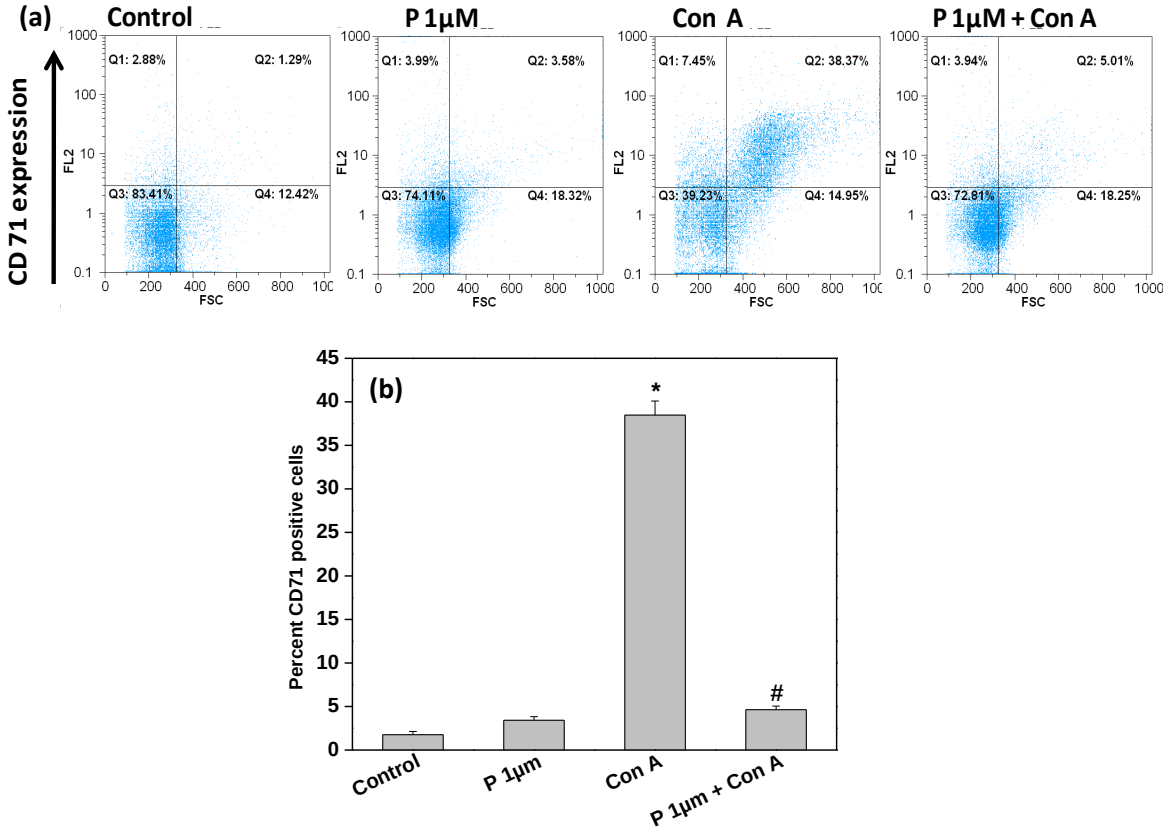


Fig. 2.11: Effect of plumbagin on CD71 surface expression on Con A stimulated lymphocytes in vitro: Lymphocytes were treated with plumbagin (1 µM, 4 h) and then stimulated with Con A (10 µg/ml) for 24 h at 37 °C in complete medium. Vehicle treated cells served as control. In each group, 0.5×10^6 cells were stained with PE conjugated anit-CD54 antibody. (a) Representative flowcytometric histograms of anti-CD54 labeled splenocytes representing plumbagin induced suppression of mitogen induced increase in expression of CD54. (b) Each bar represents frequency of CD54+ cells. Data points represent mean±S.E.M. from three replicates and two such experiments were carried out. *p<0.01, as compared to vehicle treated cells and #p<0.01, as compared to Con A stimulated cells.

2.2.12 Plumbagin inhibited LPS induced proliferation B cells in vitro:

The effect of plumbagin on LPS induced B cell proliferation was assessed by CFSE dye dilution. Two million CFSE labeled splenocytes were treated with plumbagin (1 µM, 4 h) and were stimulated with LPS (50 µg/ml) for 72 h at 37 °C in 2ml RPMI with 10% FCS. Vehicle treated cells served as a control. Fig. 2.12a shows the representative flow cytometric histograms of CFSE labeled splenic lymphocytes stimulated with LPS for 72h in vitro in the presence of concentrations of plumbagin. Frequency of daughter cells (Gate RN1) increased significantly in LPS stimulated lymphocytes as compared to that in unstimulated cells (Fig. 2.12a). The bars represent percentage of daughter cells obtained after 72 h of Con A stimulation (Fig. 2.12b). Plumbagin was able to completely inhibited LPS induced proliferation of B cells (Fig. 2.12b).

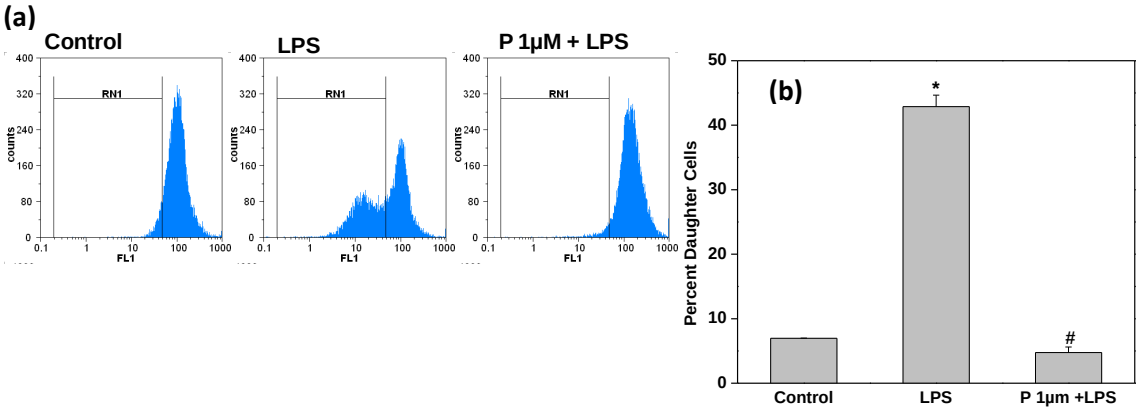


Fig. 2.12: Effect of plumbagin on LPS induced B cell proliferation in vitro: CFSE labeled lymphocytes were treated with plumbagin (1 µM, 4 h) and stimulated with the LPS for 72 h. Twenty thousand cells were acquired in a flowcytometer. Vehicle treated cells served as control. Percent daughter cells were calculated from decrease in mean fluorescence intensity. (a) Representative flowcytometric histograms of CFSE labeled cells representing plumbagin induced suppression of LPS induced proliferation. (b) Each bar represents percentage of daughter cells in each treatment group. Each bar represents mean±S.E.M. from three replicates and two such experiments were carried out. *$p<0.01$, as compared to vehicle treated cells and #$p<0.01$, as compared to Con A stimulated cells.

2.2.13 Plumbagin inhibited expression of LPS induced expression of B cell co-stimulatory marker CD80 (B7.1):

Fig. 2.13 shows the surface expression of B cell co-stimulatory marker CD80 on lymphocytes treated with plumbagin (1µM, 4 hr) and stimulated with LPS (50µg/ml) as compared to that in the cells stimulated with LPS alone. LPS activated cells showed significantly higher expression of CD80 as compared to that in control vehicle treated cells. Plumbagin treatment prior to LPS stimulation leads to a complete inhibition of CD80 on the surface of these cells (Fig. 2.13a and b).

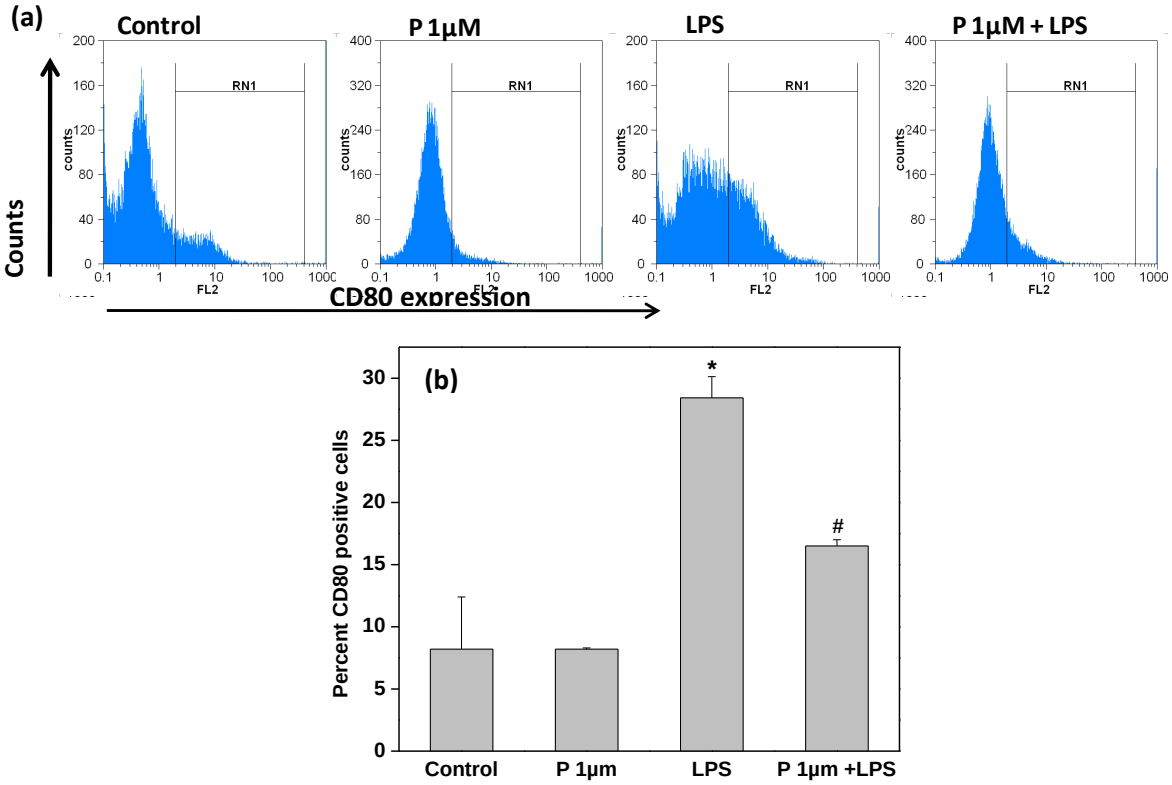


Fig. 2.13: Effect of plumbagin on CD80 surface expression on LPS stimulated lymphocytes in vitro: Lymphocytes were treated with plumbagin (1 µM, 4 h) and then stimulated with LPS (50 µg/ml) for 48 h at 37 °C in complete medium. Vehicle treated cells served as control. In each group, 0.5×10^6 cells were stained with PE conjugated anti-CD80 antibody. (a) Representative flowcytometric histograms of anti-CD80 labeled splenocytes representing plumbagin induced suppression of mitogen induced increase in expression of CD80. (b) Each bar represents frequency of CD80+ cells. Data points represent mean±S.E.M. from three replicates and two such experiments were carried out. *p<0.01, as compared to vehicle treated cells and #p<0.01, as compared to LPS stimulated cells.

2.2.14 Plumbagin inhibited expression of LPS induced expression of B cell co-stimulatory marker CD86 (B7.2): Fig. 2.14 shows the surface expression of B cell co-stimulatory marker CD86 on lymphocytes treated with plumbagin (1µM, 4 hr) and stimulated with LPS (50µg/ml) as compared to that in the cells stimulated with LPS alone. LPS activated cells showed significantly higher expression of CD86 as compared to that in control vehicle treated cells. Plumbagin treatment prior to LPS stimulation leads to a complete inhibition of CD80 on the surface of these cells (Fig. 2.14a and b).

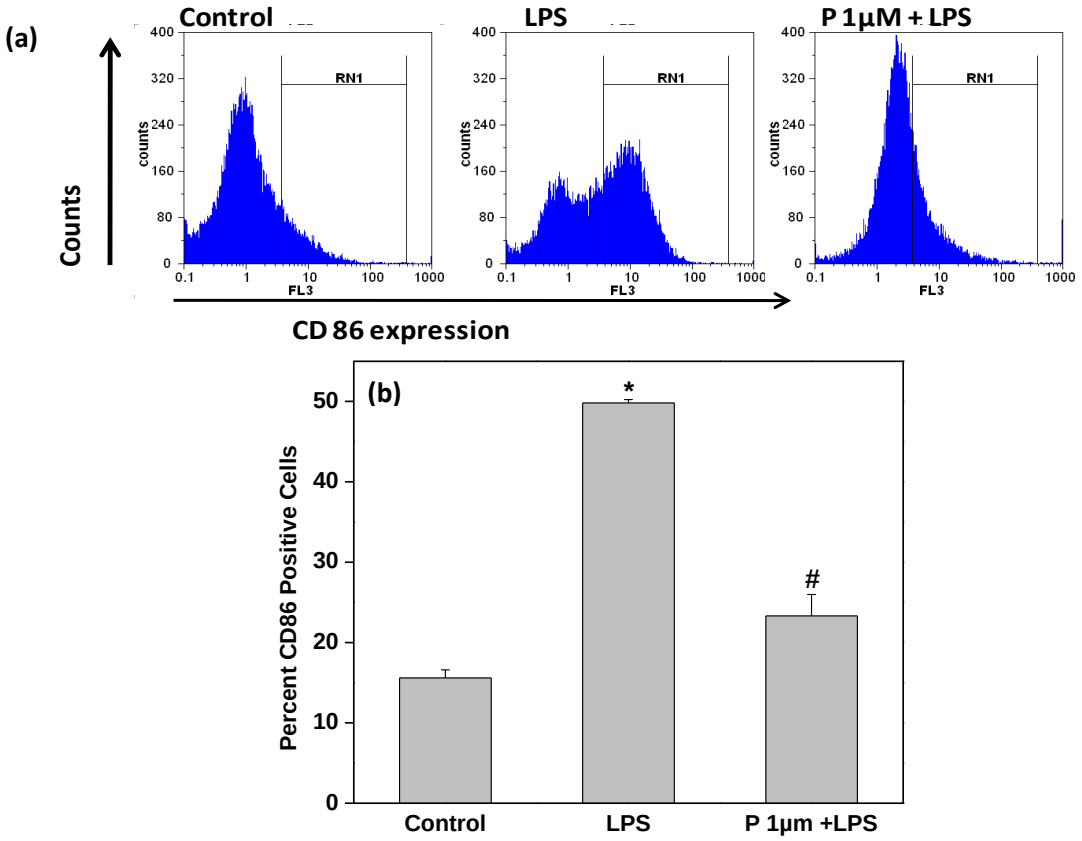


Fig. 2.14: Effect of plumbagin on CD86 surface expression on LPS stimulated lymphocytes in vitro: Lymphocytes were treated with plumbagin (1 µM, 4 h) and then stimulated with LPS (50 µg/ml) for 48 h at 37 °C in complete medium. Vehicle treated cells served as control. In each group, 0.5×10^6 cells were stained with PE conjugated anti-CD86 antibody. (a) Representative flowcytometric histograms of anti-CD86 labeled splenocytes representing plumbagin induced suppression of mitogen induced increase in expression of CD86. (b) Each bar represents frequency of CD86+ cells. Data points represent mean±S.E.M. from three replicates and two such experiments were carried out. *p<0.01, as compared to vehicle treated cells and #p<0.01, as compared to LPS stimulated cells.

2.2.15 Plumbagin exhibited therapeutic potential by inhibiting cytokine secretion when added post-mitogenic stimulation:

To explore its therapeutic potential, plumbagin (0.5 and 1μM) was added to lymphocytes post-mitogenic stimulation at different time points. It was observed that plumbagin was able to completely suppress Con A induced cytokine secretion in murine splenic lymphocytes even when added up to 6 h after mitogenic stimulation (Fig. 2.15).

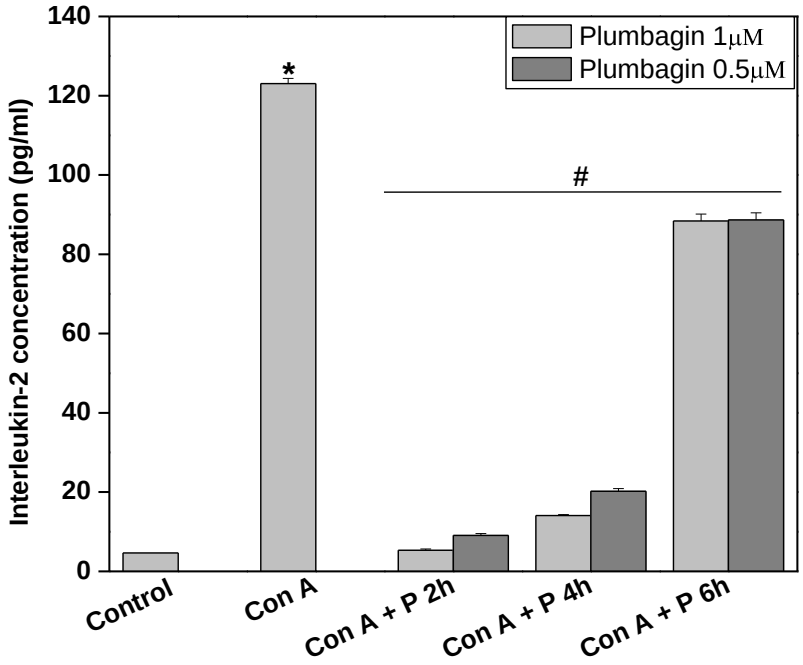


Fig.2.15: **Plumbagin inhibits cytokine production in activated lymphocytes:** Lymphocytes were stimulated with Con A (10 mg/ml) following which plumbagin (0.5 and 1 μM) were added at the indicated time points and the cells were further cultured for 24 h at 37 °C. The concentration of IL-2 in the culture supernatant was estimated using ELISA. Each bar represents concentration of IL-2. 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.

2.2.16 Immunosuppressive effects of plumbagin are long lasting:

Experiments were carried out to determine whether the immunosuppressive effects of plumbagin are transient or long lasting. Lymphocytes were treated with plumbagin (1µM) for 4h and these cells were then washed and rested in complete medium for 24 or 48 or 72h and then stimulated with Con A. At the end of 24h, supernatant was harvested and IL-2 secretion by these cells was estimated. Plumbagin was able to completely suppress IL-2 secretion by these cells even when they were rested for 72h after treatment with plumbagin indicating that the amount of plumbagin that had entered the cells during the initial 4h treatment was sufficient to induce hyporesponsiveness in these cells against mitogenic stimuli (Fig. 2.16).

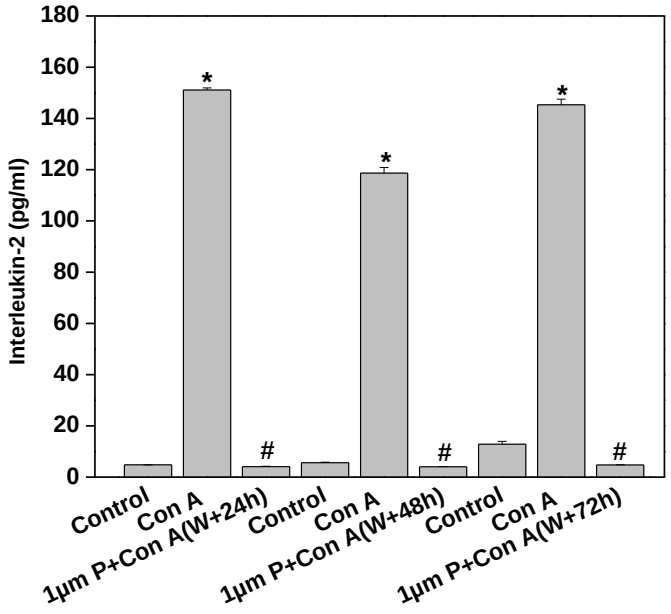
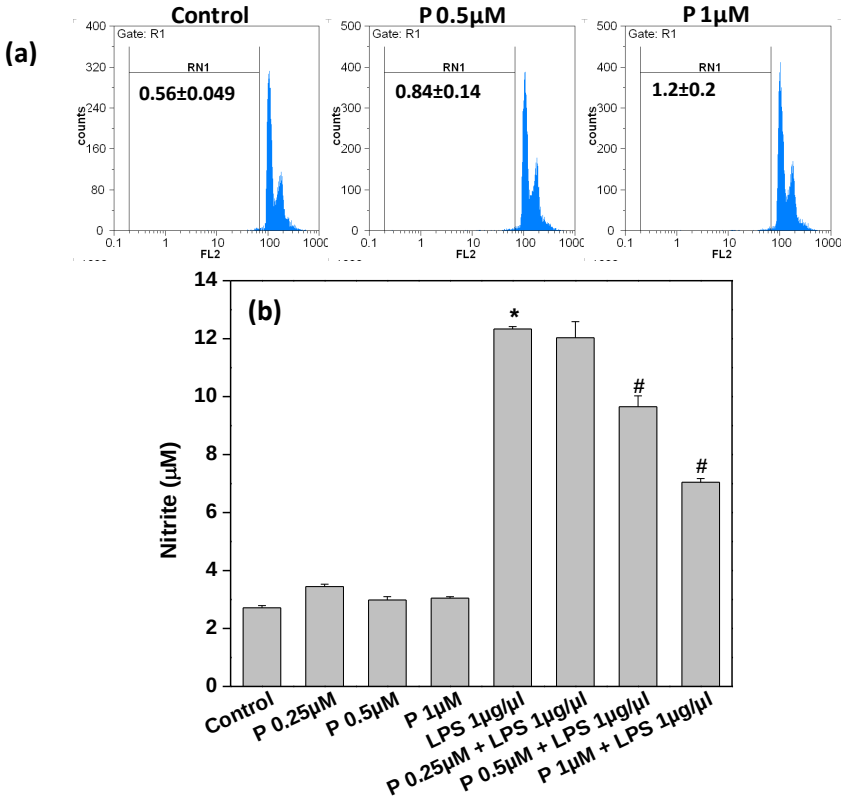


Fig. 2.16: Immunosuppressive effects of plumbagin are long lasting: Splenic **l**ymphocytes were treated with plumbagin (1µM) for 4 hr, washed twice with complete medium and rested for 24 or 48 or 72h. They were then stimulated with Con A (10 mg/ml) and further cultured for 24 h at 37 °C. The concentration of IL-2 in the culture supernatant was estimated using ELISA. Each bar represents concentration of IL-2. Each bar represents mean±S.E.M. from three replicates and three 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.

2.2.17 Plumbagin significantly inhibited LPS induced nitric oxide release and production of TNF-α, IL-6 and Prostaglandin-E2 by RAW cells:

Macrophages play a critical role in both non-specific (innate immunity) as well as specific immune responses mechanisms (adaptive immunity). Their functions include phagocytosis of cellular debris and pathogens, either as stationary or as mobile cells and also to stimulate lymphocytes and other immune cells to respond to pathogens. Experiments were carried out to study the effects of plumbagin on effector functions of activated macrophages using a mouse macrophage cell line (RAW264.7 cells). Initially experiments were carried out to ascertain if plumbagin induces cell death in RAW cells. It was observed that plumbagin upto 1µM did not induce any significant increase in the cell death of RAW cells (Fig. 2.17a). Further, plumbagin was able to inhibit LPS induced production of nitric oxide as well as secretion of TNF-α and IL-6 in a dose dependent manner (Fig. 2.17b - d). Plumbagin also inhibited LPS induced production of intracellular PGE2 in RAW cells (Fig. 2.17e).



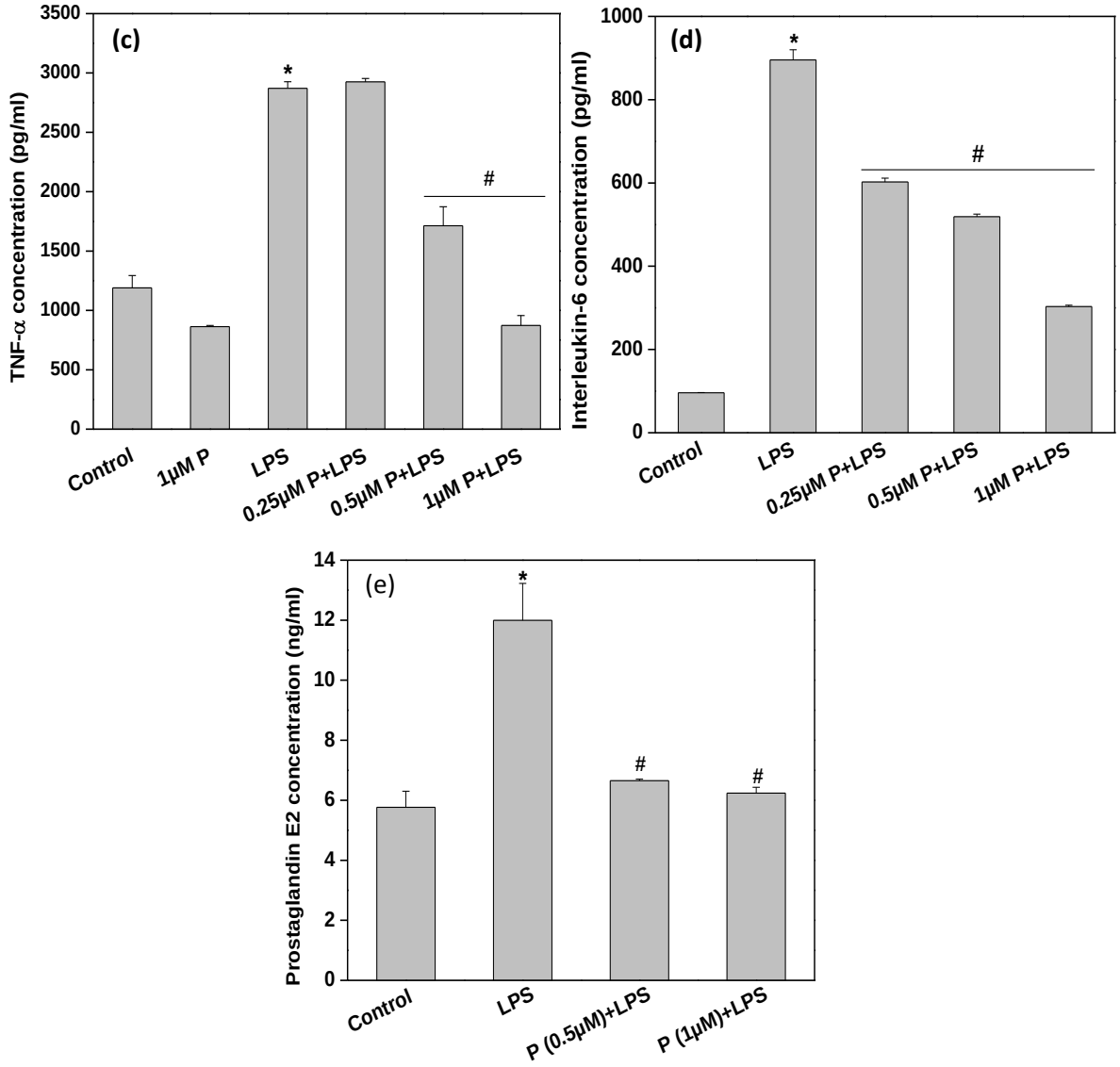


Fig. 2.17: Effect of plumbagin on LPS induced nitric oxide release and TNF-α, IL-6 and PGE2 production by RAW macrophages: (a) Plumbagin did nit induce cell death in RAW cells: RAW cells were incubated with indicated concentration of plumbagin for 24h and then stained with PI followed by flowcytometric analysis to enumerate cell death. (b) RAW cells were pre-treated with different concentrations of plumbagin for 4 h before stimulation with LPS (1 µg/ml) for 24 h at 37 °C in complete medium. Nitrite levels in the culture supernatant was measured using Greiss reagent. Vehicle treated cells served as control. Each bar represents mean ± SD of 3 replicates and 2 such experiments were carried out. (c and d) TNF-α, IL-6 cytokine levels in the supernatant was estimated by ELISA. (e) After 24 h of incubation intracellular PGE2 production was measured. 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.

2.2.18 Plumbagin significantly inhibited LPS induced production of TNF-α, IL-6 and IL-1β by splenic adherent macrophages: Splenocytes ($5x10^6$ cells/well) were incubated in a 24-well cell culture plate for 3h at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The non-adherent cells were removed by aspiration. The adherent cells (macrophages) were incubated with plumbagin (1 µM for 4 h) and then stimulated with LPS (50 µg/ml) and further cultured for 24h at 37 °C. Plumbagin was able to inhibit LPS induced secretion of TNF-α, IL-6 and IL-1β (Fig. 2.18 a – c).

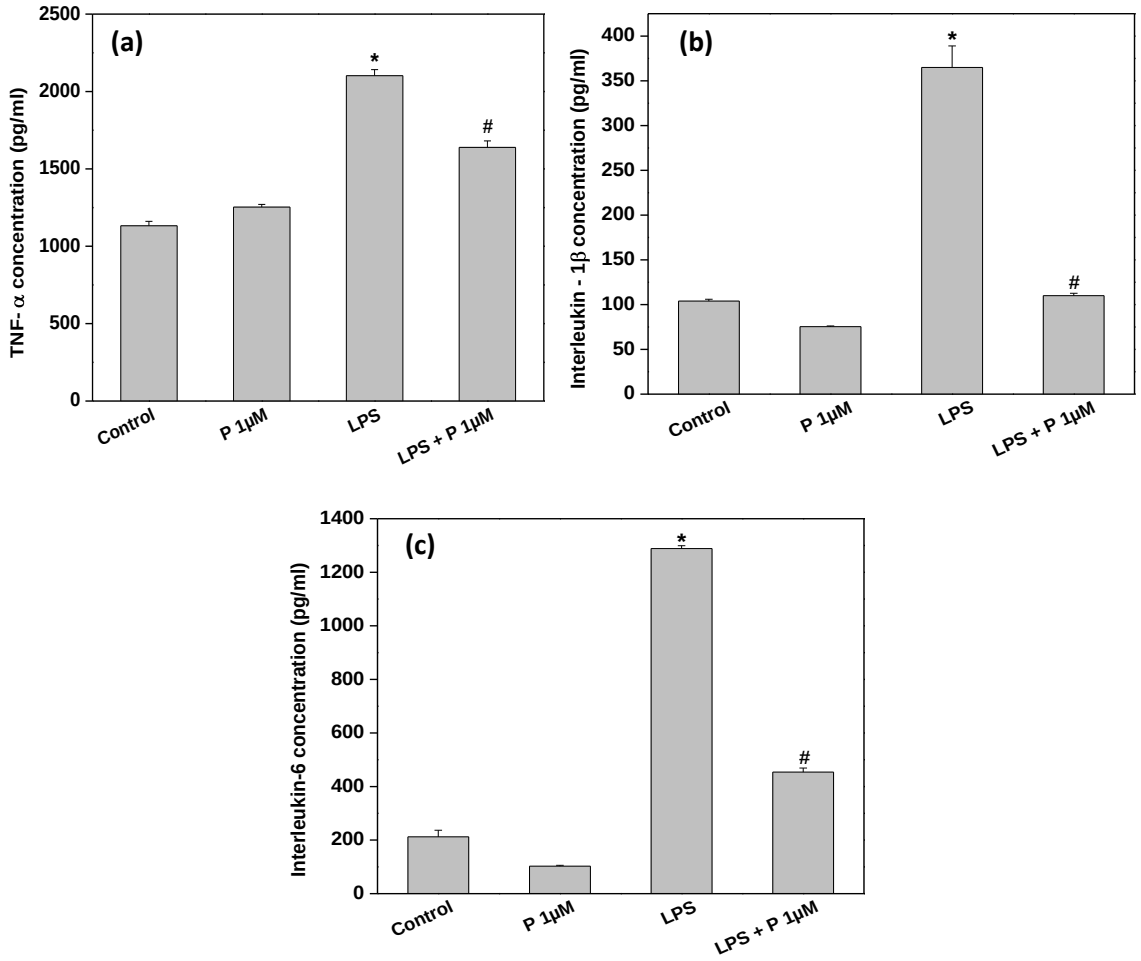


Fig. 2.18 Effect of plumbagin on LPS induced TNF-α, IL-6 and IL-1β production by splenic macrophages: Splenic adherent macrophages were pre-treated with different concentrations of plumbagin for 4 h before stimulation with LPS (1 µg/ml) for 24 h at 37 °C in complete medium. (a – c) TNF-α, IL-6 and IL-1β cytokine levels in the supernatant was 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 and #$p<0.01$, as compared to LPS stimulated cells.

CHAPTER - III

Mechanism of action of plumbagin

This chapter summarizes the studies that were carried out to elucidate the mechanism of immunomodulatory effects of plumbagin in murine splenic lymphocytes. This chapter details the identification of possible molecular targets of plumbagin in resting and activated lymphocytes. The biochemical and signaling mechanisms responsible for the novel anti-inflammatory action of plumbagin are described in this chapter.

3.1 MATERIALS AND METHODS

3.1.1 Reagents and Chemicals:

The following chemicals were obtained from Sigma Chemical Company, USA: Plumbagin,, HEPES (hydroxyethyl piperazineethanesulfonic acid), ethylenediaminetetraacetic (EDTA), bovine serum albumin (BSA), ethylene glycol tetraacetic acid (EGTA), phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, benzamidine, nonidet P-40, propidium iodide (PI), Hoechst, monochlorobimane (MCB), dihydroethidiun (DHE), maleic acid diethyl ester (DEM), diphenyliodonium (DPI), rotenone (ROT), allopurinol (AP), Glutathione (GSH), N-acetly cysteine (NAC), Dithiothretol (DTT), Dimethylsulfoxide (DMSO), Protein G immunoprecipitation kit, glycerol, penicillin, streptomycin, TritonX-100, tween20, sodium chloride, sodium dihydrophosphate, disodiumhydrophosphate. RPMI 1640, Fetal calf serum (FCS) was obtained GIBCO BRL. Rapamycin, Ly294002, concanavalin A (con A), Mn (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP), Trolox, catalase assay kit and superoxide dismutase enzyme assay kit were purchased from Calbiochem (USA). Hydroxyphenly fluorescein (HPF), 5-(and-6)-carboxy-2,7-dichlorofluorescein diacetate (DCF-DA), fluoresceindiacetate (FDA), streptavidin agarose, biotin C2-iodoacetamide, glutathione

ethyl ester biotin amide (Bio-GEE) and carboxy fluorescein diacetate succinimidyl ester (CFSE) were procured from Molecular Probes, Invitrogen.

3.1.2 Antibodies:

iv. Following primary antibodies were purchased from Cell Signaling Technologies: mouse: P65, p-ERK, ERK, p-IKKα/β, IKKα, IKKβ, IκB-α, p-P38, P38, p-JNK, JNK, p-AKT, and AKT. FITC labeled anti-mouse IgG and antibodies against, Bcl-2, Bcl-xl, Cyclin A, and β-actin were purchased from Sigma Chemical Co. (USA). Anti-GSH antibody was purchased from Abcam.

v. ELISA sets for detection of interleukin-2, interleukin-4, interleukin-6 and interferon-γ were obtained from BD Bioscience USA.

3.1.3 Animal maintenance:

As described in chapter 2, section 2.1.3

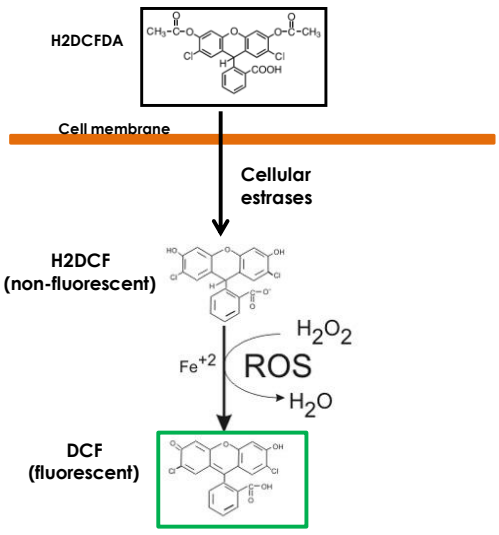
3.1.4 Splenic lymphocyte preparation:

As described in chapter 2, section 2.1.6

3.1.5 Pro-oxidant measurements

2',7'-dichlorofluorescein diacetate (H_2DCFDA) and dihydroethidium (DHE), have been used extensively in tissue culture experiments to evaluate reactive oxygen species (ROS) production. H2DCF is known to be relatively more specific towards hydrogen peroxide production. DHE, by virtue of its ability to freely permeate cell membranes is used extensively to monitor superoxide production and is retained well by cells [226, 227]. This technique for detecting intracellular ROS, particularly hydrogen peroxide, depends on

oxidation of the non-fluorescent substrate 2',7'-dichlorofluorescein (H_2DCF) to a green fluorescent product [228], As cell membranes are permeable to esterified forms of H_2DCF, they can enter cells freely where, as a result of deacetylation by intracellular esterases, they become trapped intracellularly. Depending on the nature of oxidative stress being employed, the rate of oxidation may be monitored by a fluorimeter, fluorescence microscopy or by flow cytometry. Hydroxyphenyl fluorescein (HPF) is a highly selective probe for the detection of highly reactive oxygen species (hROS). It is a cell permeable highly sensitive fuorescent probe for hydroxyl radical (OH•), and peroxynitrite (ONOO-) detection. It has little reactivity towards other hROS such as: hypochlorite (-OCl), singlet oxygen (0_2^1), superoxide ($02^{-\bullet}$), hydrogen peroxide (H_20_2), nitric oxide (NO•) and alkyl peroxide (RO2•). To detect intracellular ROS, lymphocytes were preincubated with 20 µM H_2DCF-DA or FDA (20 µM) or DHE (5 µM) or HPF (5 µM) for 30 min at 37 °C for 25 min at 37°C before being treated with various concentrations of plumbagin. The oxidized form of the dye (DCF) acts as a control for changes in uptake, ester cleavage, and efflux [229]. After 1 h of incubation, the increase in fluorescence resulting from oxidation of H_2DCF to DCF was measured using a spectrofluorimeter.



Scheme 3.1 Chemical reactions involved in the measurement of intracellular ROS using H_2DCFDA dye.

3.1.6 Intracellular GSH assay:

A widely used method for determining GSH in living cells is by adding monochlorobimane (MCB) to cell culture medium and allowing intracellular glutathione-S-transferases to form GSH–MCB adducts that can be measured fluorometrically. The membrane permeant MCB has been used extensively to examine GSH content in living cell populations [230-232]. To measure intracellular GSH, lymphocytes were incubated with the indicated concentrations of DEM or treated with plumbagin for 4 h at 37°C. Monochlorobimane (final concentration, 40 μM, 30min at 37°C) was loaded into cells [233]. Fluorescence emission from cellular sulfhydryl-reacted monochlorobimane was measured using a spectrofluorimeter. Monochlorobimane is also known to react with small-molecular weight thiols other than GSH but GSH forms the major monochlorobimane reactive thiol. Hence, MCB fluorescence is referred to as GSH levels. There are several reports in the literature measuring GSH levels using this dye [234-236].

3.1.7 Measurement of catalase and superoxide dismutase enzyme activities:

Catalase is a common enzyme found in nearly all living organisms and catalyzes the decomposition of hydrogen peroxide to water and oxygen [237]. Hydrogen peroxide is a harmful by-product of many normal metabolic processes and to prevent damage to cells and tissues, it must be quickly converted into other, less dangerous substances. This function is performed by catalase which rapidly catalyzes the decomposition of hydrogen peroxide into less reactive gaseous oxygen and water molecules.

The reaction of catalase in the decomposition of hydrogen peroxide is:

$$2\ H_2O_2 \xrightarrow{\text{catalase}} 2\ H_2O + O_2$$

Superoxide dismutases (SOD) are enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide [238]. They play an important role in the antioxidant defense against the potentially damaging reactions of superoxide radical ($O_2^{-\bullet}$) in cells exposed to oxidative stress. This enzyme catalyzes the reaction

$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

To measure enzyme activity of intracellular catalase and SOD, lymphocytes were incubated with the indicated concentrations of plumbagin for 4 h and the activity of catalase and SOD enzymes was measured according to manufacturer's protocol.

3.1.8 CFSE staining:

As described in chapter 2, section 2.1.7

3.1.9 Estimation of T cell proliferation:

As described in chapter 2, section 2.1.8

3.1.10 Measurement of cytokine secretion:

As described in chapter 2, section 2.1.13

3.1.11 Purification of CD4+ T cells:

As described in chapter 2, section 2.1.14

3.1.12 Western blot analysis:

Splenocytes were treated with plumbagin (5 µM, 4 h) and were stimulated with Con A (10 µg/ml) for 1h or 24h at 37 °C and cytosolic extract prepared as explained in [14]. Vehicle

treated cells served as a control. Cells were washed with ice-cold PBS and suspended in 0.1ml 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 15 minutes, after which 25µl of 10% NP40 was added and tubes were vortexed once for 60 seconds, incubated on ice for 5 minutes and again vortexed three times for 25 seconds each with intermittent incubation on ice for 5 minute each. The supernatants were collected by centrifuging the cells at 8,000 rpm for 6 minute at 4°C and used as cytosolic fraction and the nuclear pellet was used to isolate nuclear proteins as described below in the next section. The protein content of the cytosolic fraction was determined using Bradford reagent (BioRad Protein assay kit). Equal amounts of protein (30 µg) were resolved by (sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10%) and transferred to nitro cellulose membrane. After the membrane was blocked in 5% nonfat powdered milk, the membrane was incubated overnight with the primary antibody specific to IκB-α or phospho-ERK or phospho-IKKα/β or phospho-P38 or phospho-JNK or phospho–AKT for 1h and Bcl-2 or Bcl-xl or cyclin A for 24h samples, washed three times with Tris-buffer saline containing 0.05% Tween 20 (TBST) and further incubated with horseradish peroxidase-labeled secondary antibody for 1 h. The membranes were washed, and specific bands were visualized on X-ray films using Enhanced Chemiluminiscence Kit (Roche, Germany). The membrane was stripped and reprobed with actin β or ERK or IKK-α or IKK-β or P38 or JNK or AKT antibody.

3.1.13 Electrophoretic mobility shift assay:

The splenocytes were treated with plumbagin (5 µM, 4 h) and were stimulated with Con A (10 µg/ml) for 1 h at 37 °C and nuclear extracts were prepared [14]. The nuclear pellets were

resuspended in 25 µl of ice-cold nuclear 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), and the tubes were incubated on ice for 15 min with intermittent agitation. This nuclear extract was microcentrifuged for 5 min at 12,000 rpm, and the supernatant was collected in fresh tubes and frozen at −70 °C. EMSA was performed by incubating 15 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**GGGACTTTCCGCTGGGGACTTTCC**AGGGAGGCGTGG-3′; boldface indicates NF-κB binding sites) in the presence of 0.5 µg of poly (2′-deoxyinosinic-2′-deoxycytidylic acid) (poly (dI–dC)) in binding buffer (25mM HEPES, pH 7.9, 0.5mM EDTA, 0.5mM DTT,1% Nonidet P 40, 5% glycerol, and 50mM NaCl) for 30min at 37 °C. Poly (dI–dC) is a synthetic polynucleotide used as a nonspecific competitor DNA to reduce the background. The DNA–protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels using buffer containing 50 mM Tris, 200 mM glycine, and 1 mM EDTA, pH8.5. The dried gel was exposed on phosphorimage plate and the radioactive bands were visualized using a phosphorimage plate scanner (Amersham Biosciences,USA).

3.1.14 Immunocytochemistry and confocal microscopy for localization of P65:

CD4+ T cells were purified as described in chapter 2, section 2.1.11 and were incubated with or without plumbagin (1 µM) in medium containing 10% FBS for 4h. At the end of 4h these cells were stimulated with plate bound mouse anti-CD3 antibody and soluble anti-CD28 antibody for 1h. Further, these cells were centrifuged onto coverslips, fixed with 4% paraformaldehyde (10 min), washed in PBS (three times for 5 min);

permeabilized with 0.02% (v/v) Tween 20 in PBS (3 washes for 5 minutes), washed in PBS (three times for 5 min) and blocked with 5% BSA in PBS for 1–2 h at room temperature. Antibody to P65 were diluted 1/100 in 1% BSA in PBS and applied overnight at 4°C, followed by washing in PBS (three times for 5 min) and incubation for 2h at room temperature with FITC-conjugated anti-mouse IgG diluted 1/300 in 1% BSA in PBS. Coverslips were washed with (three times for 5 min), stained with PI for nuclear staining, and mounted onto glass slides. Slides were examined using a LSM510 scanning module (Carl Zeiss Microscopy, Jena GmbH, Germany) with a krypton–argon laser, coupled to an Orthoplan Zeiss photomicroscope using a 488 nm laser line. Overlay images were recorded by superimposing simultaneous images from each channel.

3.1.15 Absorption spectroscopy:

Absorbance spectra of plumbagin with or without GSH were determined using absorption spectroscopy. Plumbagin (100 µM) was mixed with 10 mM of GSH in a total volume of 1 ml and incubated at 37°C for 1 h. Absorption spectra of the samples were recorded using a spectrophotometer.

3.1.16 HPLC separation of products of reaction of plumbagin with GSH:

A Waters (Milford, MA) Millenium32 chromatographic system equipped with model 510 pumps, a U6K injector, a system interface module, and a diode array detector was used for sample analyses. Separations were achieved on Waters Spherisorb S3 ODS2 reverse phase column (4.6m x 150mm). The column was equilibrated with HPLC grade water at a flow rate of 1ml/min prior to injecting 25 ml of the reaction mixture. Elution was carried out using a gradient of acetonitrile from 0% to 100% over 20 min, beginning 2 min after

injection of the sample. Absorbance of the effluent was measured using a diode array detector.

3.1.17 Liquid chromatography–mass spectrometry:

LC–MS was performed using a Varian ProStar 410 AutoSampler in combination with Varian 1200 L LC–MS equipment triple quadrupole mass (QqQ) spectrometer with ESI source (Varian, Inc., USA). The column was 3 µm C18, 100mm x 2.1mm (Phenomex). A gradient mobile phase was used at 0.2 ml/min. The ESI source operated in positive ionization mode.

3.1.18 Labeling, pull down, and detection of protein thiolates:

To detect protein thiolate anions, lymphocytes were preincubated with plumbagin for 4 h after which the cells were harvested and whole cell lysates were prepared. The lysates were incubated with BIAM (0.2 mM) for 30 min at 37°C and then incubated with streptavidin agarose beads (20µl/mg of protein) for 1 h at 4°C. The agarose beads were separated by centrifugation, washed four times with RIPA buffer (1% NP-40, 0.1% SDS, 0.5mg/ml sodium deoxycholate, 150mM NaCl, and 50mM Tris–HCl, pH 7.5) and boiled in SDS sample buffer. Proteins in the eluent were resolved by SDS–PAGE and detected by Coomassie blue staining [239].

3.1.19 Purification of putative s-glutathionylated proteins using BIO-GEE:

Lymphocytes were incubated with Bio-GEE (250 µM, 1 h) prior to the addition of plumbagin at the concentrations indicated for 4 h with cycloheximide (1 µg/ml). Incubations were terminated, and a soluble protein extract was obtained as described above. The soluble proteins were then incubated with streptavidin agarose beads (20µl/mg of protein) for 30min at 4°C. The agarose beads were separated by centrifugation, washed

four times with RIPA buffer, and boiled in SDS sample buffer. Proteins in the eluent were resolved by SDS–PAGE and detected by Coomassie blue staining [240].

A second portion of the supernatant was subjected to SDS–PAGE, and the separated proteins were transferred to a nitrocellulose membrane. The membrane was probed with anti-GSH antibody and glutathionylated proteins were detected with HRP-conjugated streptavidin secondary antibody. The membranes were washed and specific bands were visualized on X-ray films using Enhanced Chemiluminiscence Kit (Roche, Germany).

3.1.20 Immunoprecipitation:

Protein cell lysates (500 µg) were cleared of abundant proteins by preincubation for 1 h with Protein G-agarose. The supernatant was then collected and incubated with anti-GSH antibody for 1 h at 4°C. Protein G-agarose was then added overnight at 4°C and the proteins were then immunoprecipitated using Protein G immunoprecipitation kit. The immunoprecipitated proteins were eluted, subjected to SDS–polyacrylamide gel electrophoresis, and the separated proteins were transferred to a nitrocellulose membrane. After the membrane was blocked in 5% nonfat powdered milk, the membrane was incubated overnight with the primary antibody specific to P65 washed three times with Tris-buffer saline containing 0.05% Tween 20 (TBST) and further incubated with horseradish peroxidase-labeled secondary antibody for 1 h. The membranes were washed, and specific bands were visualized on X-ray films using Enhanced Chemiluminiscence Kit (Roche, Germany).

3.1.21 Statistical Analysis: The statistical significance of the differences in respect of all parameters studied between untreated and Con A/LPS treated cells in presence or absence of plumbagin in vitro was assessed by student's t-test.

3.2 RESULTS

3.2.1 Plumbagin modulates basal cellular reactive oxygen species levels:

Figure 3.1 shows the levels of ROS in control and plumbagin-treated lymphocytes. To detect intracellular ROS, lymphocytes were preincubated with DCF-DA (relatively specific for hydrogen peroxide) or dihydroethidium (specific for superoxide radicals) or hydroxyphenyl fluorescein (specific for hydroxyl radicals) for 25 min at 37 °C before being treated with various concentrations of plumbagin for 1h. Plumbagin induced a significant increase in basal ROS levels in lymphocytes which was further augmented in presence of diethymaleate, a GSH depleting agent (Fig. 3.1a). In order to rule out the possibility that the increase in fluorescence intensity of DCF observed could also be due to direct oxidation of H_2DCF by plumbagin, we used oxidation insensitive analogue of H_2DCF-DA, fluorescein diacetate (FDA) to monitor the changes in uptake, ester cleavage, or efflux. Plumbagin did not increase the fluorescence of oxidation insensitive dye fluorescein diacetate suggesting that increased fluorescence intensity seen with H_2DCF-DA could be attributed to its oxidation due to increase in basal ROS levels in the presence of plumbagin (Fig. 3.1b). Further, the effect of plumbagin on basal superoxide levels was estimated using dihydroethidium. It was observed that plumbagin induced a significant increase in basal levels of superoxide radicals as evident from an increase in the levels of DHE oxidation product (2-hydroxyethidium) in plumbagin treated lymphocytes (Fig. 3.1c). The effect of plumbagin on basal hydroxyl radical levels was estimated using hydroxyphenylfluorescein. It was observed that treatment of lymphocytes with plumbagin did not induce any changes in the basal levels of hydroxyl radicals (Fig. 3.1d).

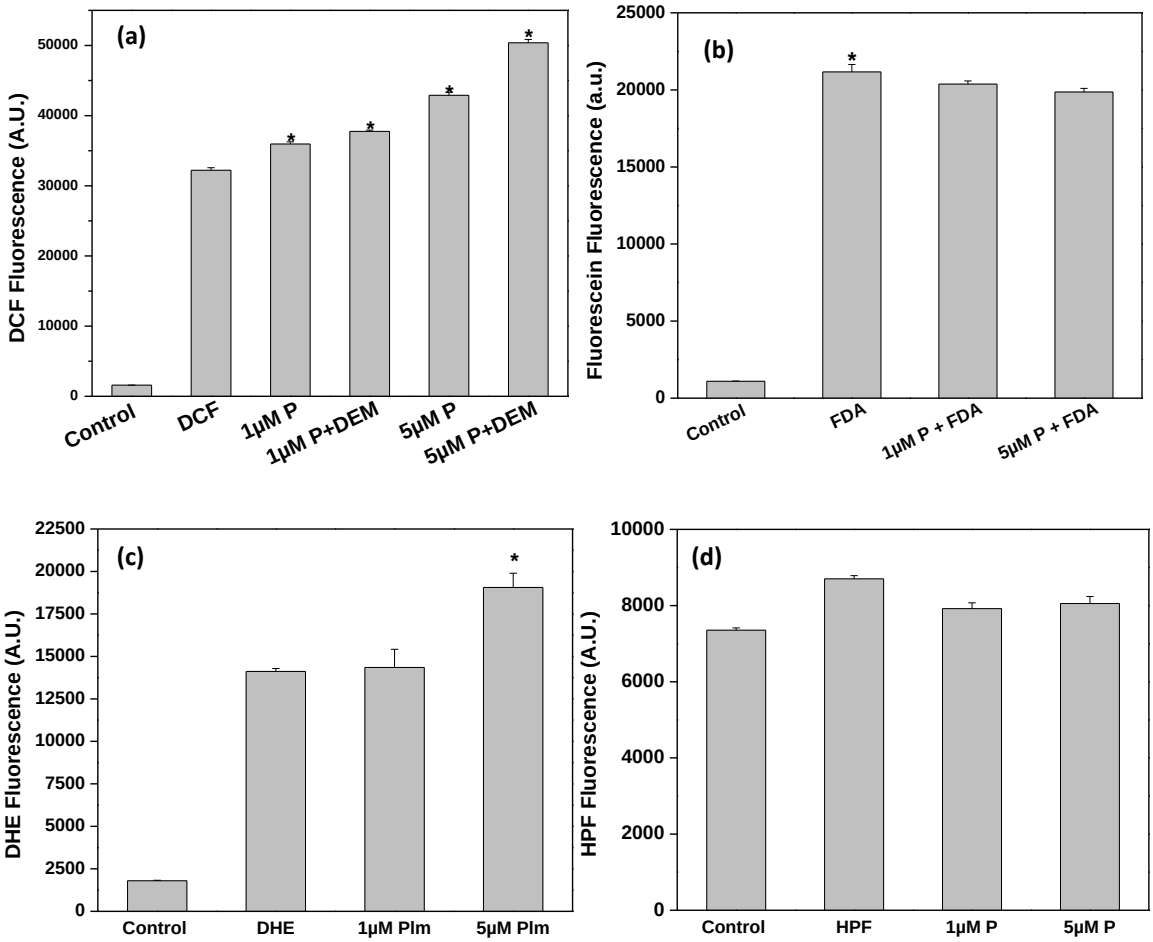


Fig. 3.1 Plumbagin modulates basal ROS levels in lymphocytes: Lymphocytes were stained with H_2DCF-DA (20 µM) or FDA (20 µM) or DHE (5 µM) or HPF (5 µM) for 30 min at 37°C and were treated with indicated concentration of plumbagin or diethylmaleate (90µM). Fluorescence emission was measured at 520 nm following excitation at 480 nm (a) Each bar shows mean DCF fluorescence ± SEM from four replicates and three such independent experiments were carried out. (b) Each bar shows mean fluorescein fluorescence ± SEM from four replicates and three such independent experiments were carried out. (c) Each bar shows mean hydroxyethidium fluorescence ± SEM from four replicates and three such independent experiments were carried out. (a) Each bar shows mean HPF fluorescence ± SEM from four replicates and three such independent experiments were carried out.*P<0.01 as compared to cells stained with respective dyes.

3.2.2 Plumbagin depleted basal intracellular glutathione levels in lymphocytes:

GSH, ubiquitously distributed in biological systems, is known to be an important molecule in defense against oxidative stress [241]. GSH scavenges reactive oxygen species and is thus often decreased during oxidative stress. To estimate the effect of plumbagin on intracellular GSH levels, lymphocytes were incubated with plumbagin or DEM for 4h and the intracellular GSH levels were measured using monochlorobimane. Plumbagin-treated cells showed significantly lower levels of intracellular GSH as compared to untreated cells (Fig. 3.2a). The positive control, DEM showed depletion of GSH to a similar extent as plumbagin (Fig. 3.2a).

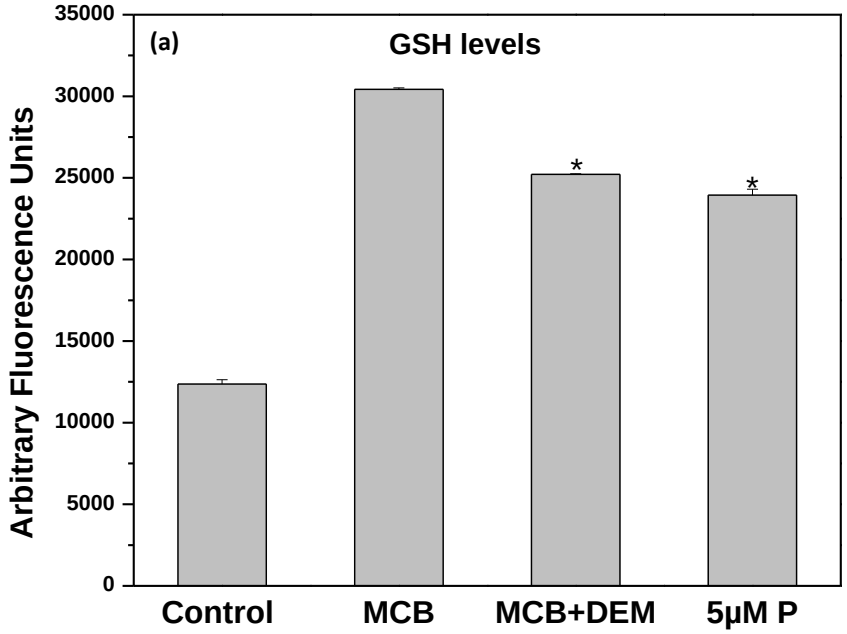


Fig 3.2a: Plumbagin depleted intracellular thiols: Lymphocytes were treated with plumbagin or DEM (90 μM) for 4 h at 37 °C and stained with MCB (final concentration 40 μM) for 30min. Fluorescence emission was measured at 490 nm following excitation at 394 nm. Each bar shows mean MCB–GSH adduct fluorescence ± SEM from four replicates and three such independent experiments were carried out. *P<0.01 as compared to untreated cells stained with MCB.

Further, to identify the intracellular source of ROS in plumbagin-treated cells, lymphocytes were incubated with inhibitors of xanthine oxidase (allopurinol, AP, 1mM), NADPH oxidase (DPI, 50 μM), or mitochondrial complex I inhibitor (rotenone, 10 μM).

These inhibitors did not abrogate the plumbagin-induced increase in ROS levels in lymphocytes (Fig. 3.2b). Incubation of lymphocytes with NAC (10mM) prior to addition of plumbagin, on the other hand, led to decrease in ROS production (Fig. 3.2b). All the inhibitors of ROS generation used in our experiment reduced the DCF fluorescence suggesting that basal ROS levels decreased upon addition of these inhibitors to lymphocytes (Fig. 3.2c).

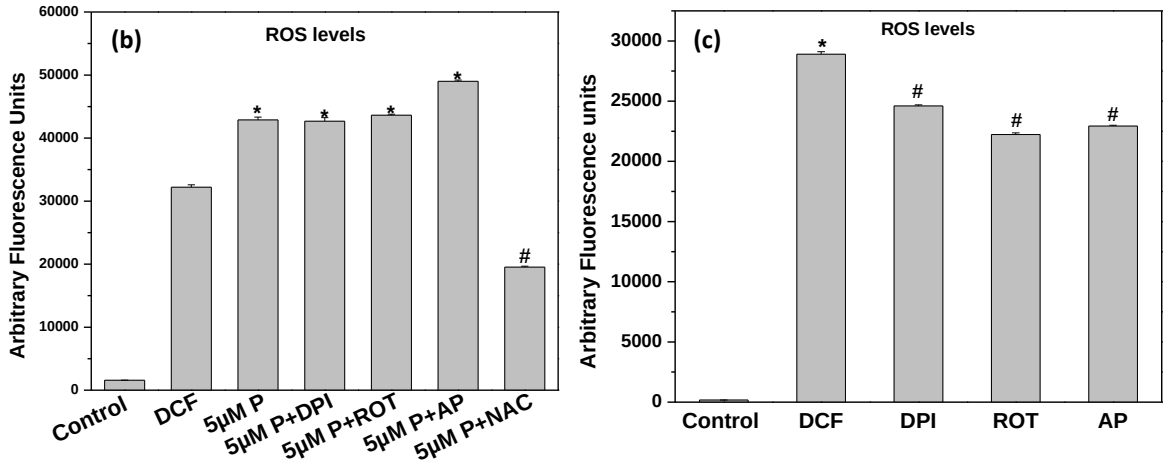


Fig3.2b and c: Effect of different inhibitors of ROS generation or N-acetylcysteine (NAC, 10mM) on plumbagin-mediated increase in ROS: (b) Lymphocytes were incubated with inhibitors of NADPH oxidase, xanthine oxidase, or mitochondrial complex I (diphenyliodonium, DPI, 50 µM or allopurinol, AP, 1mM or rotenone, ROT, 10 µM, respectively) or NAC (10mM) for 2 h and stained with DCF-DA. The cells were treated with plumbagin for 1 h at 37 °C and fluorescence emission was measured at 520 nm following excitation at 488 nm. Each bar shows mean DCF fluorescence±SEM from four replicates and two such independent experiments were carried out.*$p<0.01$ as compared to untreated cells stained with DCF-DA. # $p<0.01$ as compared to plumbagin treated cells. (c) Effect of different inhibitors of ROS generation on basal ROS levels: Lymphocytes were incubated with inhibitors of NADPH oxidase, xanthine oxidase, or mitochondrial complex I (diphenyliodonium, DPI, 50 µM or allopurinol, AP, 1mM or rotenone, ROT, 10 µM, respectively) for 2 h and stained with DCF-DA. Fluorescence emission was measured at 520 nm following excitation at 488 nm. Each bar shows mean DCF fluorescence±SEM from four replicates and two such independent experiments were carried out. *$p<0.01$ as compared to untreated control cells. #$p<0.01$ as compared untreated cells stained with DCF-DA.

3.3.3 Plumbagin modulates intracellular anti-oxidant enzyme levels in lymphocytes:

Since plumbagin induced increase in basal ROS levels, the activities of antioxidant enzymes like catalase and SOD in lymphocytes treated with plumbagin were measured. Plumbagin-treated cells showed significantly higher catalase activity as compared to untreated cells (Fig. 3.3a and b) without altering the SOD activity in lymphocytes (Fig. 3a and b).

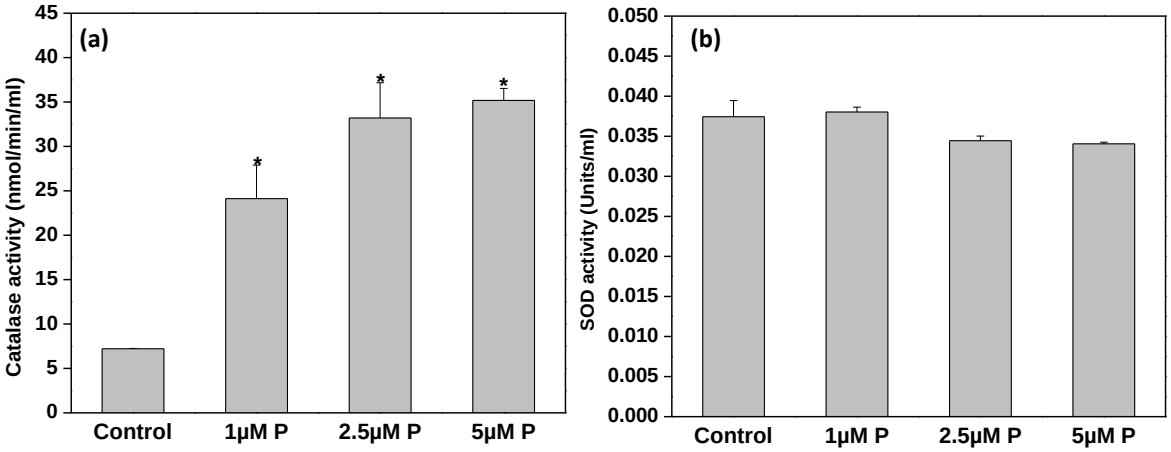
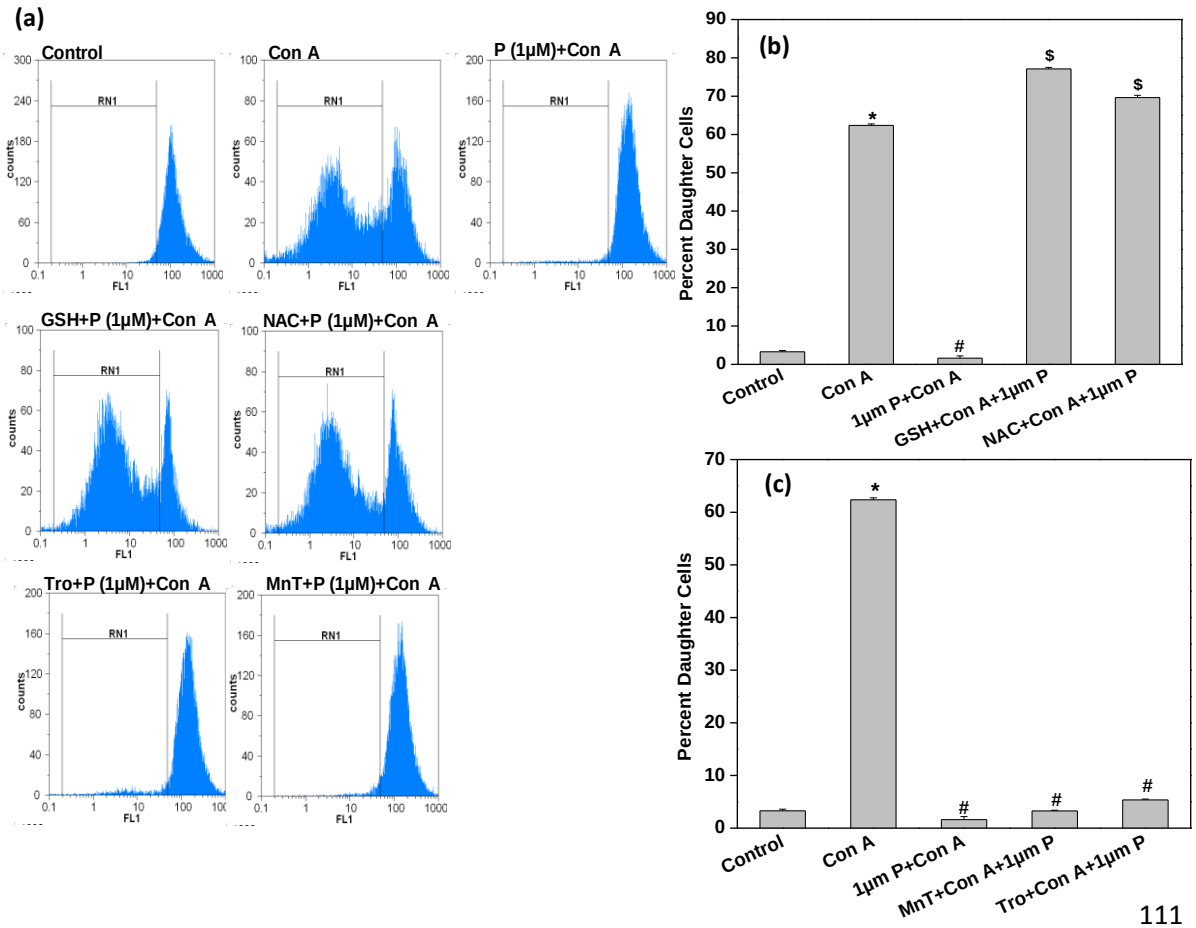


Fig 3.3a and b: Plumbagin enhanced catalase activity but not SOD activity: Lymphocytes were incubated with plumbagin for 4 h and the activity of catalase (a) and SOD (b) enzymes was measured according to manufacturer's protocol. Each bar represents catalase activity (a) and SOD activity (b). * $P<0.01$ as compared to vehicle-treated cells. Each bar shows mean±SEM from three replicates and two such independent experiments were carried out.

3.3.4 Immunosuppressive effects of plumbagin in lymphocytes were abrogated by thiol antioxidants: Figure 3.4 shows the modulation of immunosuppressive effects of plumbagin by different antioxidants. Thiol-containing antioxidants (GSH, and NAC) abrogated the anti-proliferative effect of plumbagin in mitogen-activated lymphocytes (Fig. 3.4a and b). However, antioxidants that do not contain a thiol group (MnTBAP and trolox) did not restrain the antiproliferative action of plumbagin (Fig. 3.4c). The control group in Figure 3.4b and c is from the same experiment. Similarly, anti-inflammatory action of plumbagin as seen by suppression of mitogen-induced cytokines was also curbed by thiol-containing antioxidants, GSH and NAC, (Fig. 3.4d - g) but not by non-thiol antioxidants (Fig. 3.4h). Similar results were obtained with another thiol-containing anti-oxidant DTT. Both thiol and non-thiol antioxidants per se did not affect con A induced IL-2 secretion (Fig. 3F)



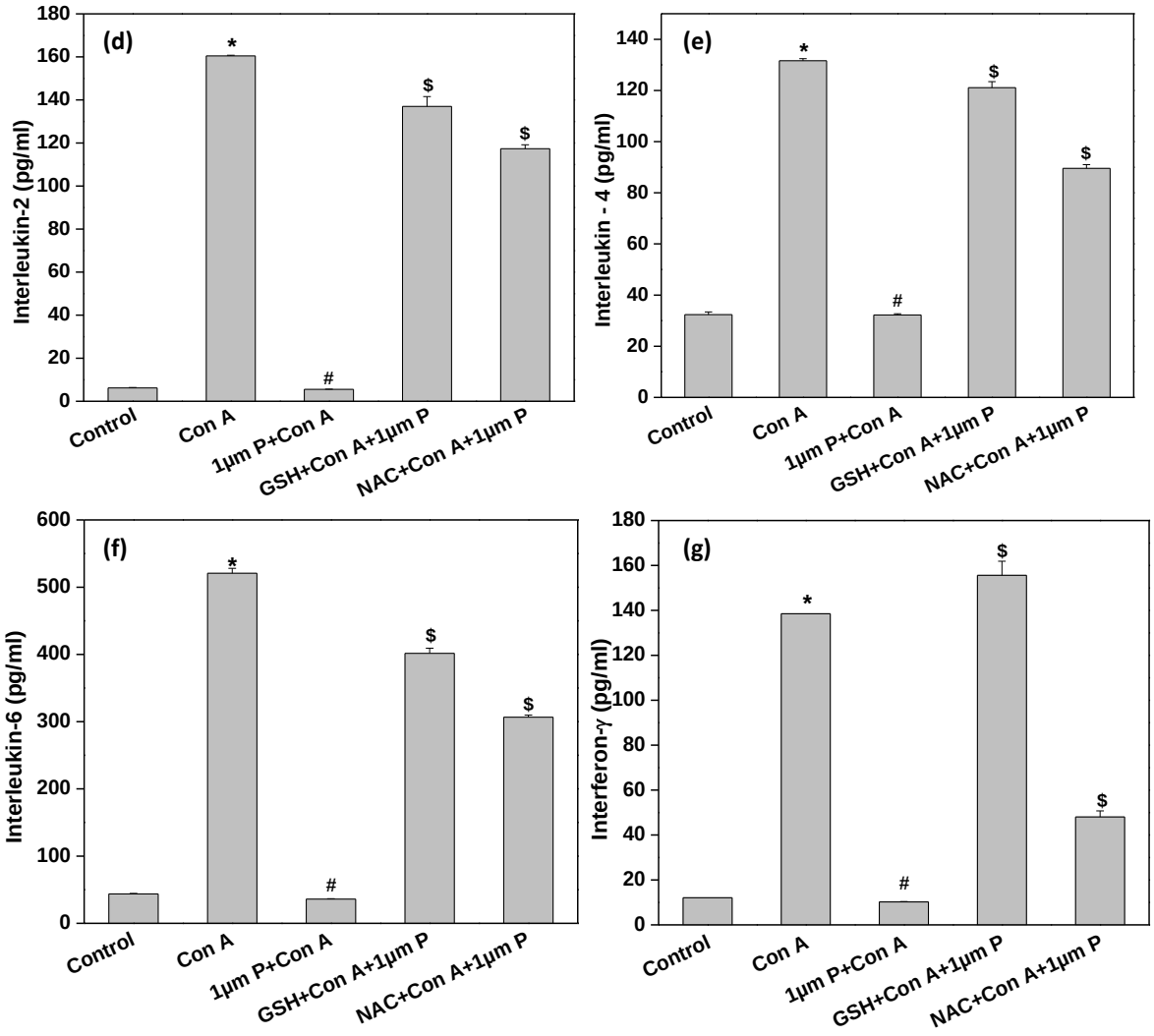


Fig.3.4 Immunosuppressive effects of plumbagin were abrogated by thiol-containing antioxidants: Lymphocytes were stained with CFSE and were incubated with different antioxidants (GSH 10mM or NAC 10mM or MnTBAP 100 µM or trolox 100 µM) for 2 h. The cells were stimulated with Con A in presence or absence of plumbagin for 72 h at 37 °C in a 5%CO2/99% air atmosphere. Cell proliferation was measured from CFSE dye dilution using a flowcytometer. (a) Representative flowcytometric histograms showing the effect of plumbagin on lymphocyte proliferation and its modulation by thiol-containing antioxidants and non-thiol antioxidants. (b and c) Each bar represents percentage of daughter cells in each treatment group. (d – g) Effect of thiol-containing antioxidants on cytokine production in plumbagin-treated lymphocytes stimulated with con A. Lymphocytes were incubated with different antioxidants (GSH or NAC or) for 2 h. The cells were stimulated with con A in presence or absence of plumbagin for 24 h at 378C in a 5%CO2/95% air atmosphere. The concentration of IL-2 (A), IL-4 (B), IL-6 (C), and IFN-γ (D) in the culture supernatant was estimated by ELISA. Each bar shows mean±SEM from three replicates and three such independent experiments were carried out. *$p<0.01$ as compared to vehicle treated cells and #$p<0.01$ as compared to untreated cells stimulated with con A. $ $p<0.01$ as compared to plumbagin treated cells stimulated with Con A.

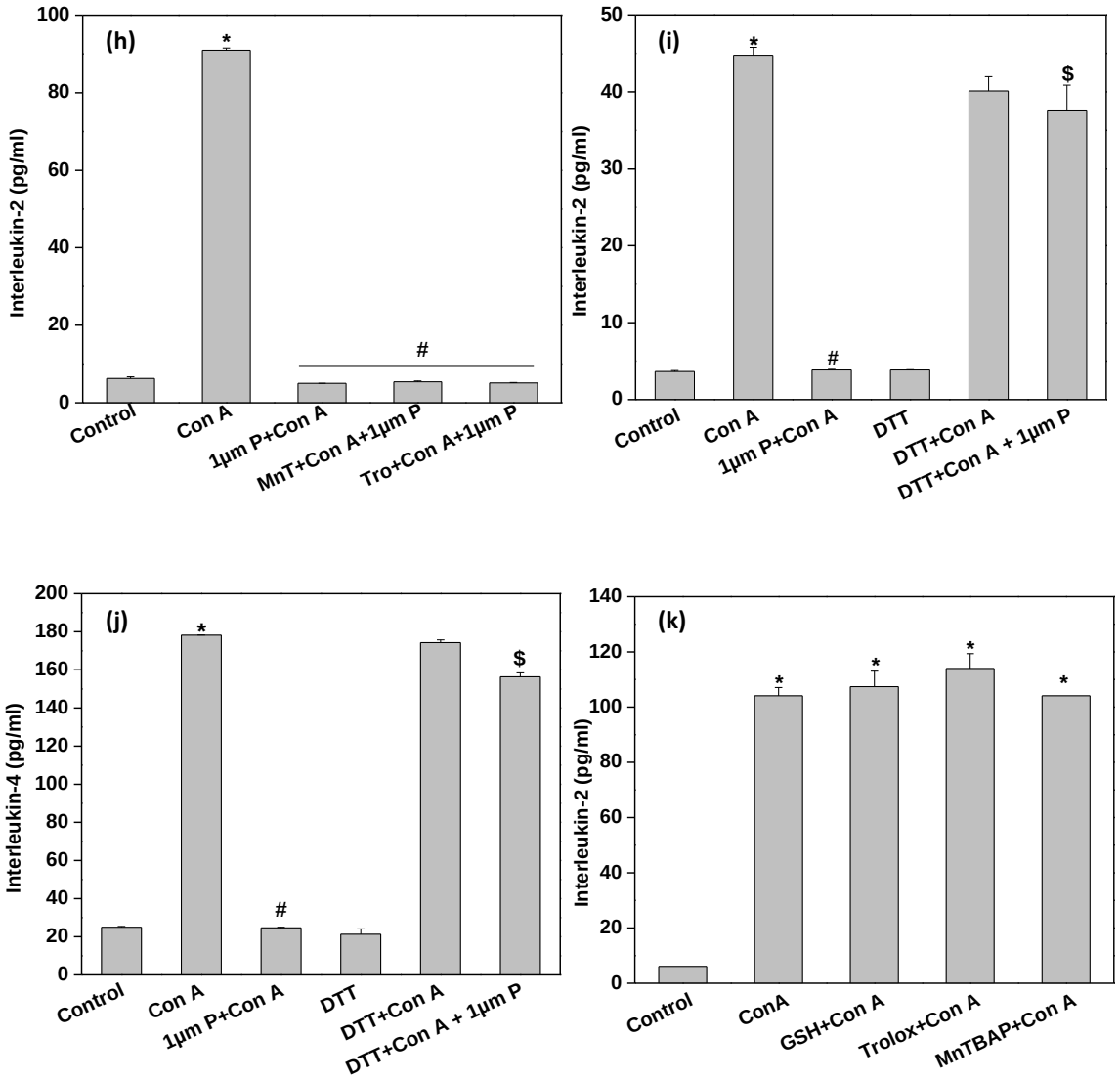


Fig. 3.4 h – j: (h) Non-thiol antioxidants could not abrogate the immunosuppressive effects of plumbagin: Lymphocytes were incubated with different antioxidants (Trolox or MnTBAP) for 2 h. The cells were stimulated with Con A in presence or absence of plumbagin for 24 h at 37°C in a 5%CO2/95% air atmosphere. The concentration of IL-2 in the culture supernatant was estimated by ELISA. Each bar shows mean±SEM from three replicates and three such independent experiments were carried out. *p<0.01 as compared to vehicle treated cells and #p<0.01 as compared to untreated cells stimulated with con A. (i and j) Immunosuppressive effects of plumbagin were abrogated by thiol-containing antioxidant DTT: Lymphocytes were incubated with DTT 100 µM for 2 h. The cells were stimulated with Con A in presence or absence of plumbagin for 24 h at 37 °C in a 5%CO2/99% air atmosphere. The concentration of IL-2 (i) and IL-4 (j) in the culture supernatant was estimated by ELISA. Each bar shows mean±SEM from three replicates and three such independent experiments were carried out. *p<0.01 as compared to vehicle treated cells and #p<0.01 as compared to untreated cells stimulated with con A. $ p<0.01 as compared to plumbagin treated cells stimulated with Con A.(k) Effect of thiol and non-thiol antioxidants on Con A induced IL-2 production as measured by ELISA. Each bar represents mean±SEM from three replicates and two such independent experiments were carried out.*p<0.01 as compared to control untreated cells.

3.3.5. Effect of antioxidants on immunosuppressive effects of classical immunomodulatory drugs:

Experiments were carried out to study the effects of thiol and non-thiol anti-oxidants on the immunosuppressive effects of well known immunosuppressive drugs like rapamycin (mTOR inhibitor) and Ly294002 (PI3kinase inhibitor). It was observed that the anti-inflammatory action of rapamycin and Ly294002 could not be abrogated by thiol or non-thiol anti-oxidants indicating a novel mechanism of action of plumbagin as compared to the existing immunomodulatory agents (Figure 3.5).

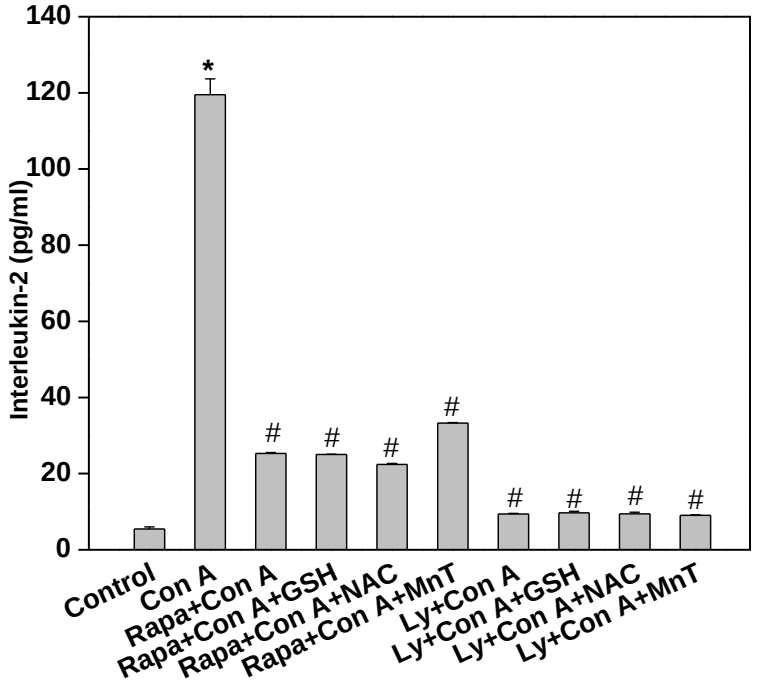
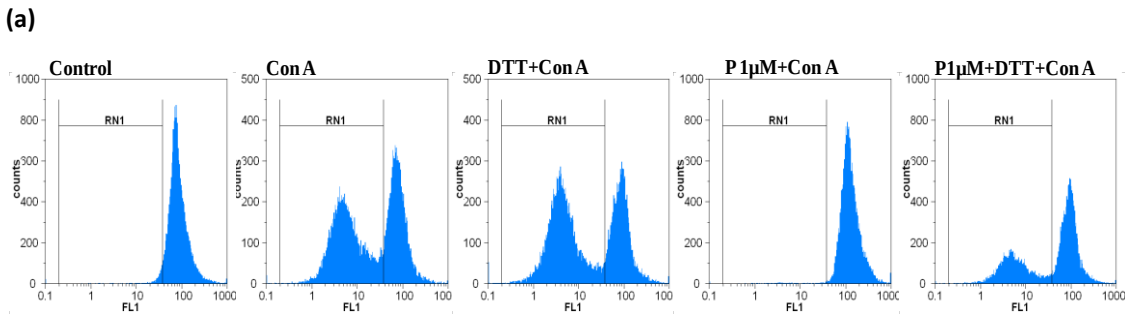
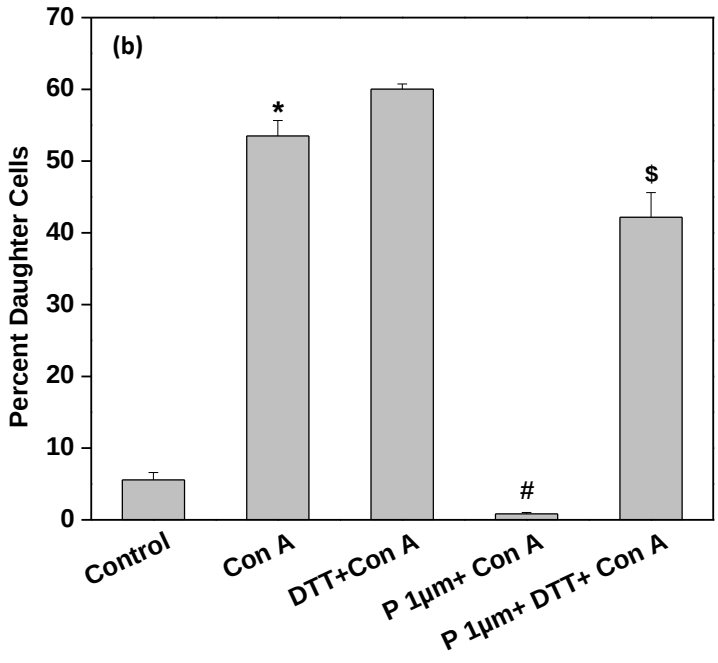


Fig 3.5 Suppression of IL-2 secretion by rapamycin and Ly294002 were not abrogated by thiol or non-thiol antioxidant: Lymphocytes were incubated with different antioxidants like GSH (10 mM) or NAC (10 mM) or MnTBAP (100 μM) for 2 h. The cells were stimulated with Con A in the presence or absence of rapamycin of Ly294002 for 24 h at 37 °C. IL-2 secretion was measured in the culture supernatant. Each bar represents mean±SEM 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.

3.3.6 DTT was able to reverse the immunosuppressive effects of plumbagin in lymphocytes: Experiments were carried out to study whether plumbagin induced immunosuppression could be reversed by thiol anti-oxidants. DTT was added after treatment of lymphocytes with plumbagin and these cells were then stimulated with Con A. It was observed that DTT was able to significantly reverse plumbagin induced suppression of lymphocyte proliferation (Fig 3.6 a and b) and cytokine secretion (Fig3.6 c - f).





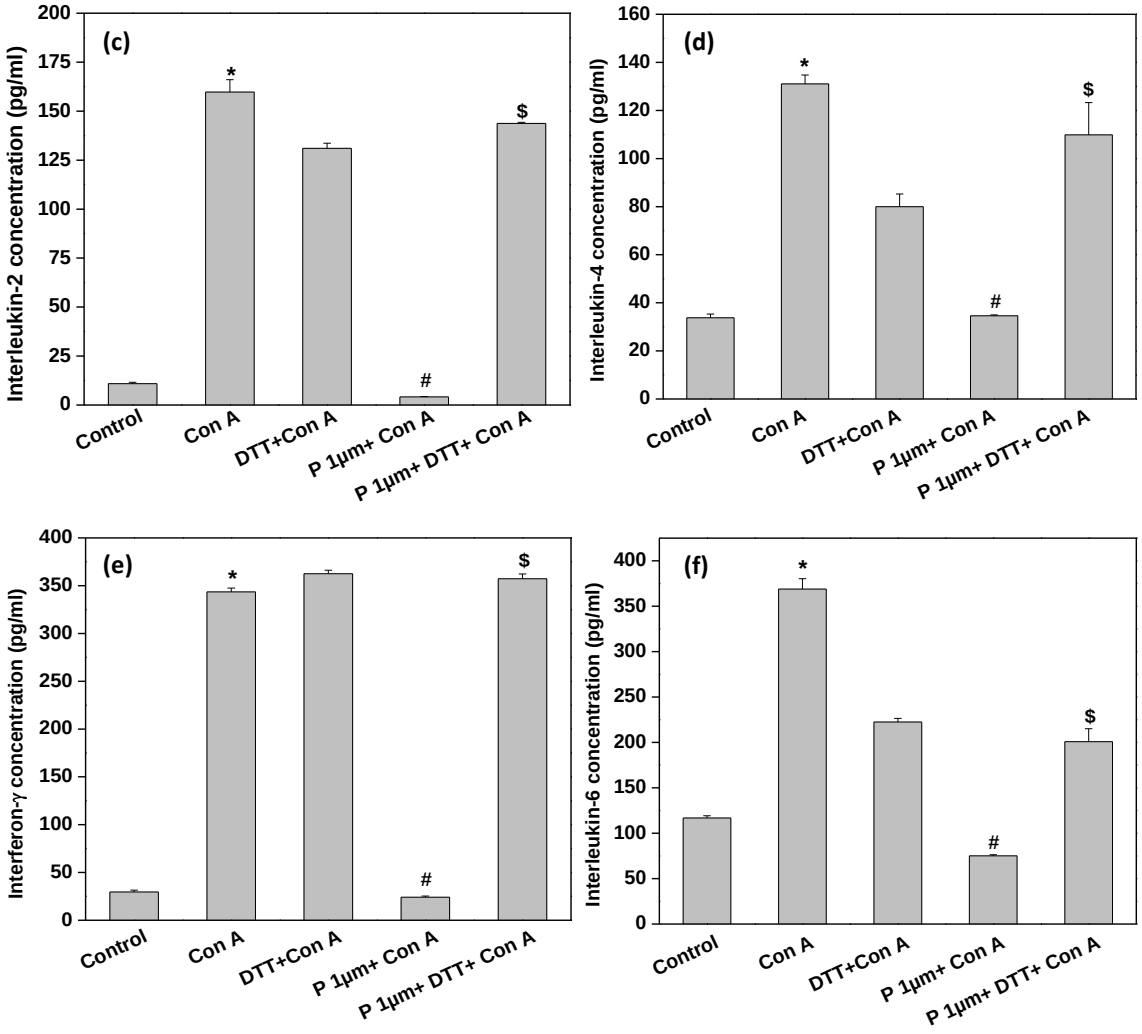
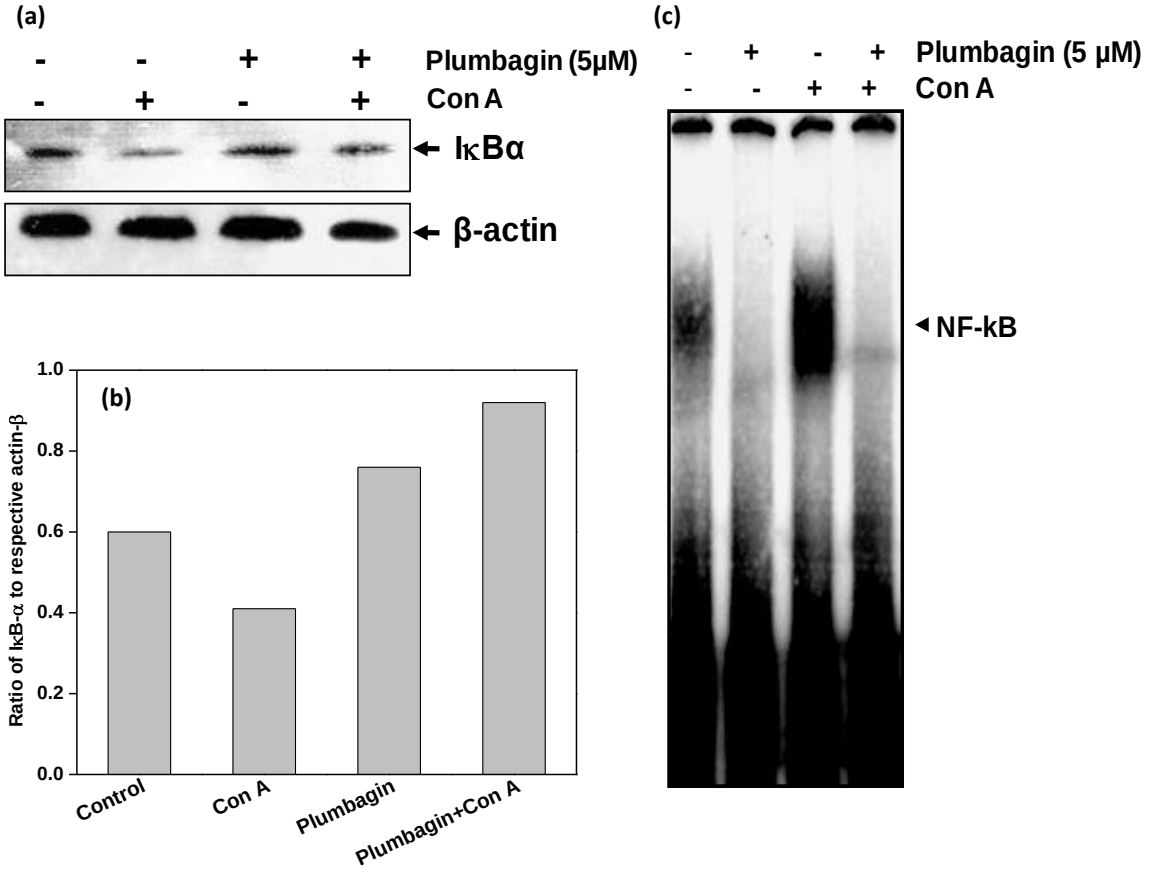


Fig 3.6: Immunosuppressive effects of plumbagin could be reversed by DTT: Lymphocytes were treated with plumbagin (1 µM) for 2 h and then incubated with DTT (1µM) for 24h.. These cells were stimulated with Con A for 24 or 72h to study proliferation and cytokine secretion respectively. (a) Suppression of Con A induced proliferation of lymphocytes by plumbagin was reversed by DTT treatment. Representative flowcytometric histograms showing the effect of DTT on immuosuppressive effects of plumbagin. (b) Each bar represents percentage of daughter cells in each treatment group. (c – f) The concentration of IL-2 (c), IL-4 (d), IL-6 (e), and IFN-γ (f) in the culture supernatant was estimated by ELISA. Each bar represents mean±SEM 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 and \$ $p<0.01$, as compared to vehicle treated cells.

3.3.7. Plumbagin suppressed Con A induced NF-κB activation in splenic lymphocytes: Figure 3.7 shows the effect of plumbagin on Con A induced NF-κB activation in lymphocytes. Con A (10 µg/ml) stimulated cells showed degradation of IκB-α in the cytosolic fraction and NF-κB activation in the nuclear fraction as compared to that in vehicle treated control cells (Fig 3.7a and c). However, cells treated with plumbagin followed by stimulation with Con A did not show IκB-α degradation (Fig. 3.7a) and NF-κB activation (Fig. 3.7c). Similar suppression of NF-κB nuclear translocation was obtained when CD4+ T cells were stimulated with anti-CD3/CD28 antibodies in the presence or absence of plumbagin indicating plumbagin treatment was able to abrogate anti-CD3/CD28 induced activation of CD4+ T cell via inhibition of NF-κB nuclear translocation (Fig. 3.7d).



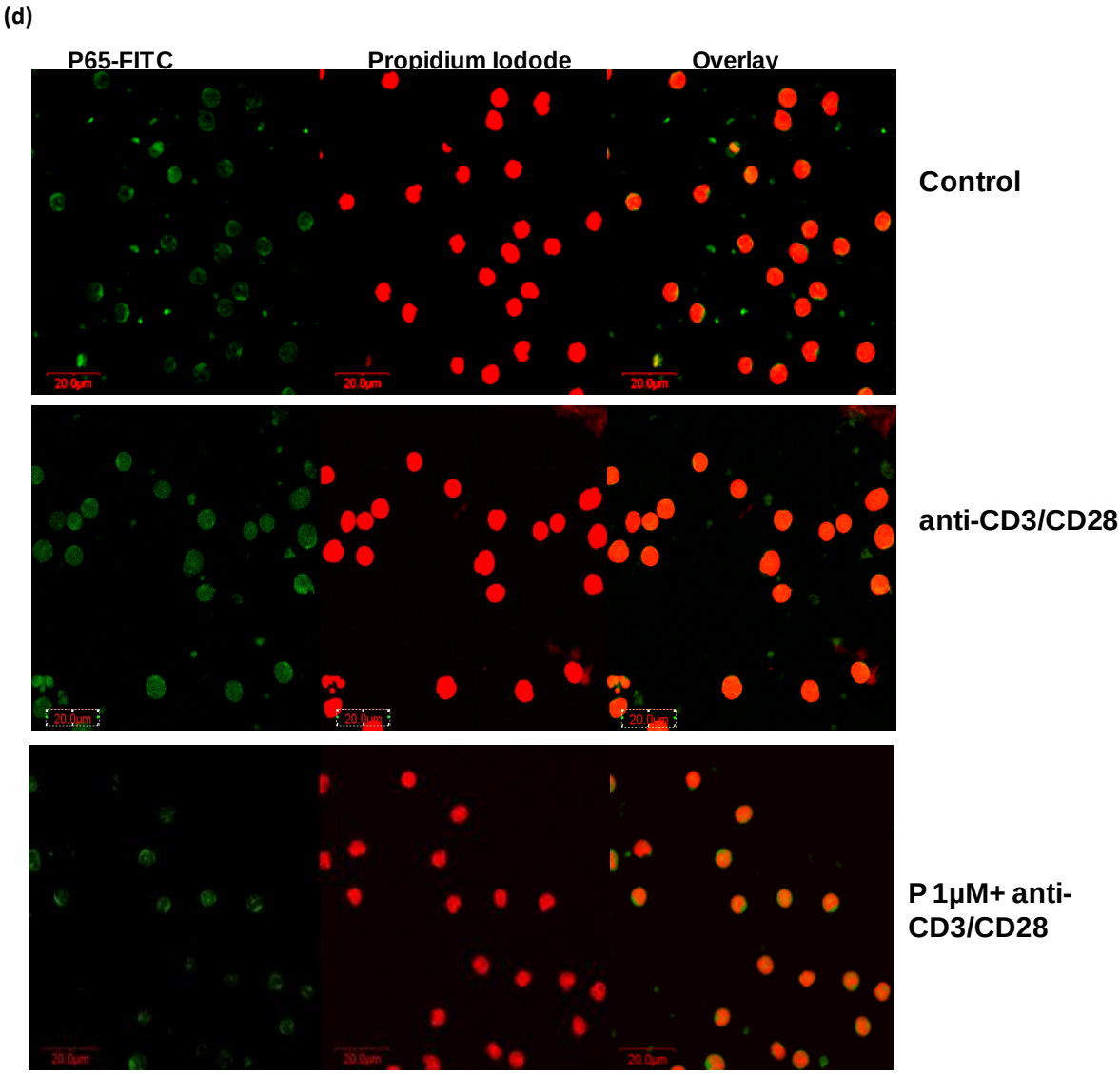
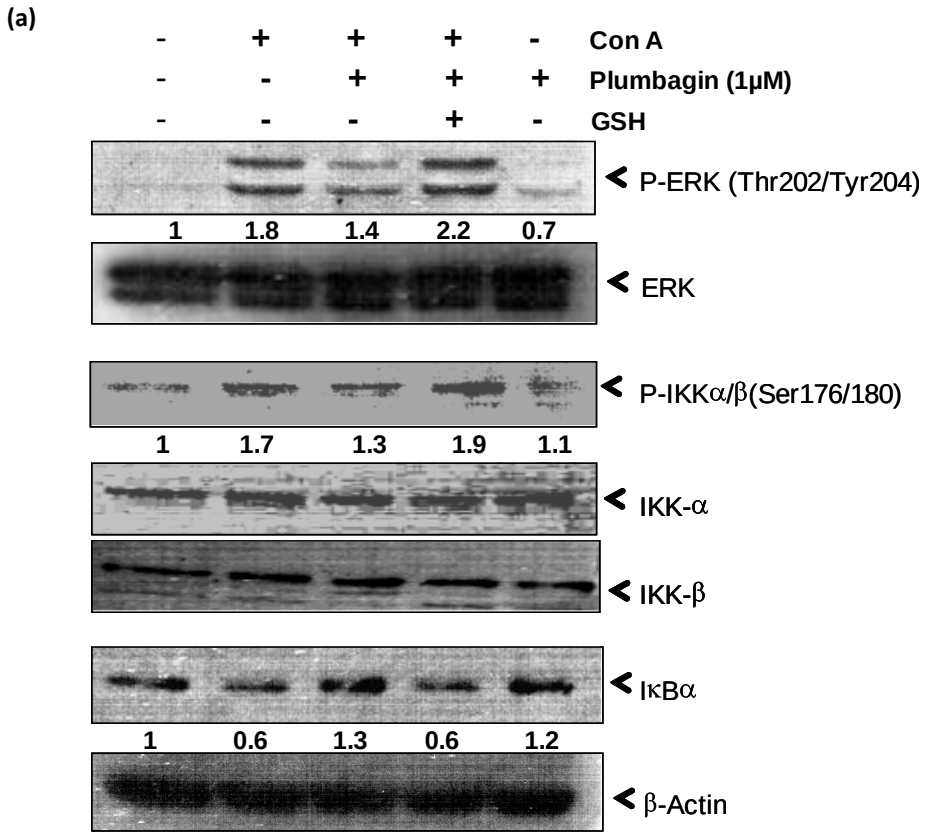


Fig. 3.7. Effect of plumbagin on Con A induced IκBα degradation and NF-κB activation: (a) Lymphocytes were incubated with 5 µM plumbagin for 4 h and further stimulated with Con A (10 µg/ml) for 1 h. Cytoplasmic extracts were prepared and equal amount of protein in each group was fractionated on 10% SDS-PAGE and electro-transferred onto nitrocellulose membrane. Western blot analysis was performed using IκBα antibody. β-actin expression was used as loading control. (b) The ratio of intensity of each band with that of respective β-actin band is shown. (c) Lymphocytes were incubated with 5 µM plumbagin for 4 h and stimulated with Con A (10 µg/ml) for 1 h. The nuclear extracts were prepared and analysed for NF-κB activation by EMSA. (d) Plumbagin inhibited NF-κB activation in CD4+ T cells: Lymphocytes were stimulated with anti-CD3/CD28 for 1 h in the presence or absence of plumbagin. Cells were harvested, fixed, permeabilized and stained with antibody against p65 followed by FITC-labeled secondary antibody staining. Nuclear staining was performed by propidium iodide. FITC/PI overlay is shown. Two independent experiments were carried out and representative figure from one such experiment is shown.

3.3.8 Suppressive effects of plumbagin on mitogen-induced signaling events were sensitive to GSH:

Figure 3.8a shows the activation of ERK and IKK and degradation of IκBα in mitogen-activated lymphocytes as compared to unstimulated cells. Plumbagin inhibited mitogen-induced activation of ERK and IKK and degradation of IκBα in lymphocytes. Plumbagin also down-modulated the Con A induced expression of NF-κB dependent genes like Bcl-2, Bcl-xl, and cyclin A (Fig. 3.8b). The suppressive action of plumbagin in activated lymphocytes was completely abrogated by GSH (Fig. 3.8a and b). At the same time, plumbagin did not suppress mitogen-induced activation of redox-sensitive molecule c-Jun N-terminal kinase (JNK) (Fig. 3.8c). The mitogen-induced activation of P38MAPkinase and AKT was also not suppressed by plumbagin as shown in Figure 3.8c suggesting that it has specific targets in cells.



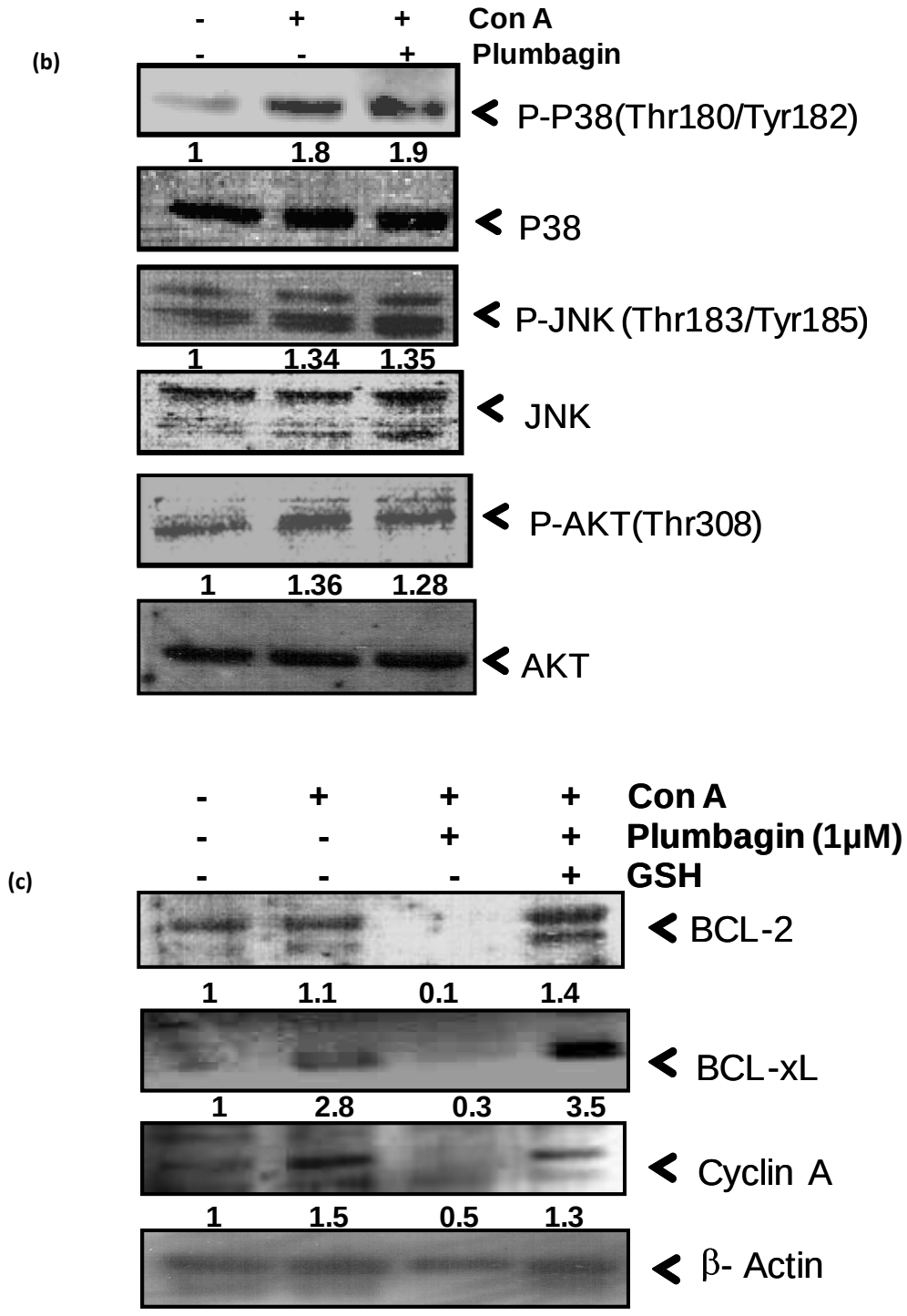
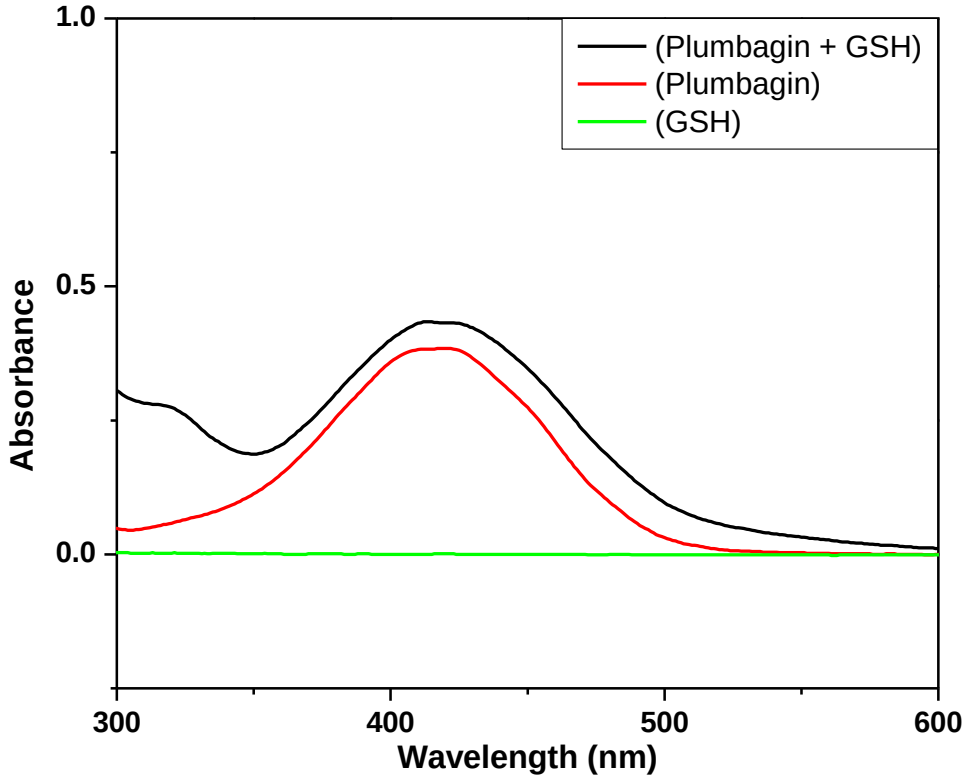


Fig. 3.8: Inhibition of proliferation/survival associated signaling molecules by plumbagin and their modulation by GSH in activated lymphocytes: Lymphocytes were incubated with plumbagin (1 µM, 4 h) in presence or absence of GSH and were stimulated with Con A for 1 h (a and c) or 24 h (b).Cytosolic extracts were prepared, fractionated on 10%SDS–PAGE, and electrotransferred to nitrocellulose membrane. Western blot analysis was performed using different antibodies specific for (a) p-ERK, ERK, p-IKK-α/β, IKK-α, IKK-β, IκBα, (b) Bcl-2, Bcl-xl, Cyclin A, and (c) p-P38, p-38, p-JNK, JNK, p-AKT, AKT. β-Actin was used as loading control. Two such independent experiments were carried out.

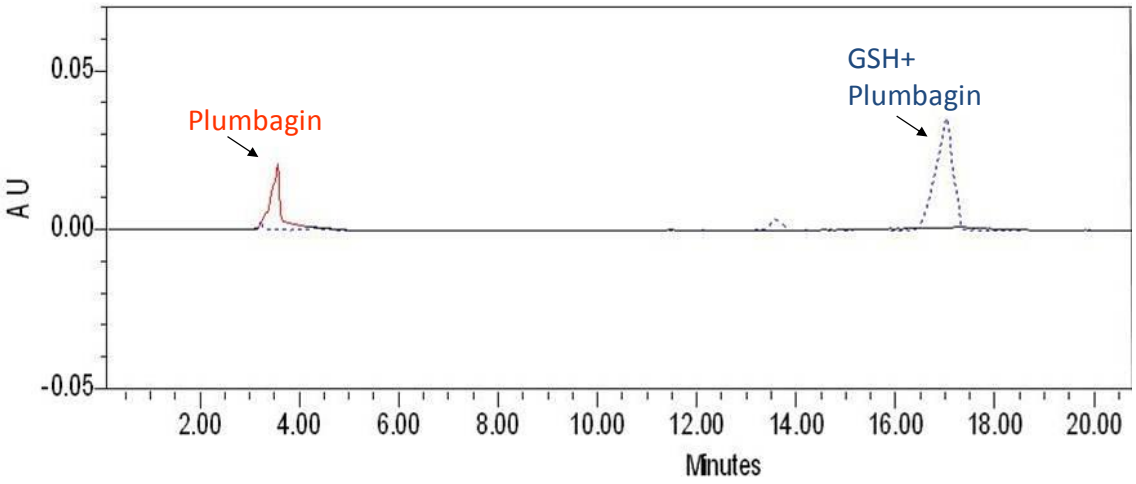
3.3.9 Plumbagin interacts with GSH:

Since anti-proliferative and anti-inflammatory effects of plumbagin were sensitive to presence of thiol antioxidants, experiments were carried out to determine whether plumbagin physically interacted with thiol groups. Interacted of plumbagin with GSH was studied using absorption spectroscopy and significant changes in absorption spectra of plumbagin were observed in presence of GSH as compared to plumbagin alone indicating a possible interaction of plumbagin with GSH (Fig. 3.9a). To further confirm and characterize this interaction, plumbagin was incubated with GSH and subjected to HPLC separation. Retention time of plumbagin on C18 column was 3.5min (Fig. 3.9b, red line). This peak disappeared when plumbagin was pre-incubated with GSH and a new peak appeared at 17.5min (Fig. 3.9b, blue dotted line). A minor peak also appeared at 13.5min. These results indicated that the reaction of plumbagin with GSH formed a single major product which could be adduct of plumbagin with GSH. To characterize this adduct, LC–MS analysis was carried out. Molecular mass analysis revealed that the major peak corresponded to plumbagin–GSH adduct (M/Z: 494) (Fig. 3.9c, upper panel). In addition to plumbagin–GSH complex, plumbagin also induced GSH to GSSG conversion. A peak corresponding to molecular mass 204 also appeared which may be hydroxylated plumbagin (Fig. 3.9c, lower panel). There were other minor peaks appearing at different molecular masses, which could not be characterized. However, these products did not appear in HPLC separation. This could be due to similar polarity of the products making them separate at the same retention time.

(a)


(Plumbagin + GSH)
(Plumbagin)
(GSH)
Absorbance
1.0
0.5
0.0
300
400
500
600
Wavelength (nm)

(b)


Plumbagin
GSH+
Plumbagin
A U
0.05
0.00
-0.05
2.00
4.00
6.00
8.00
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Minutes

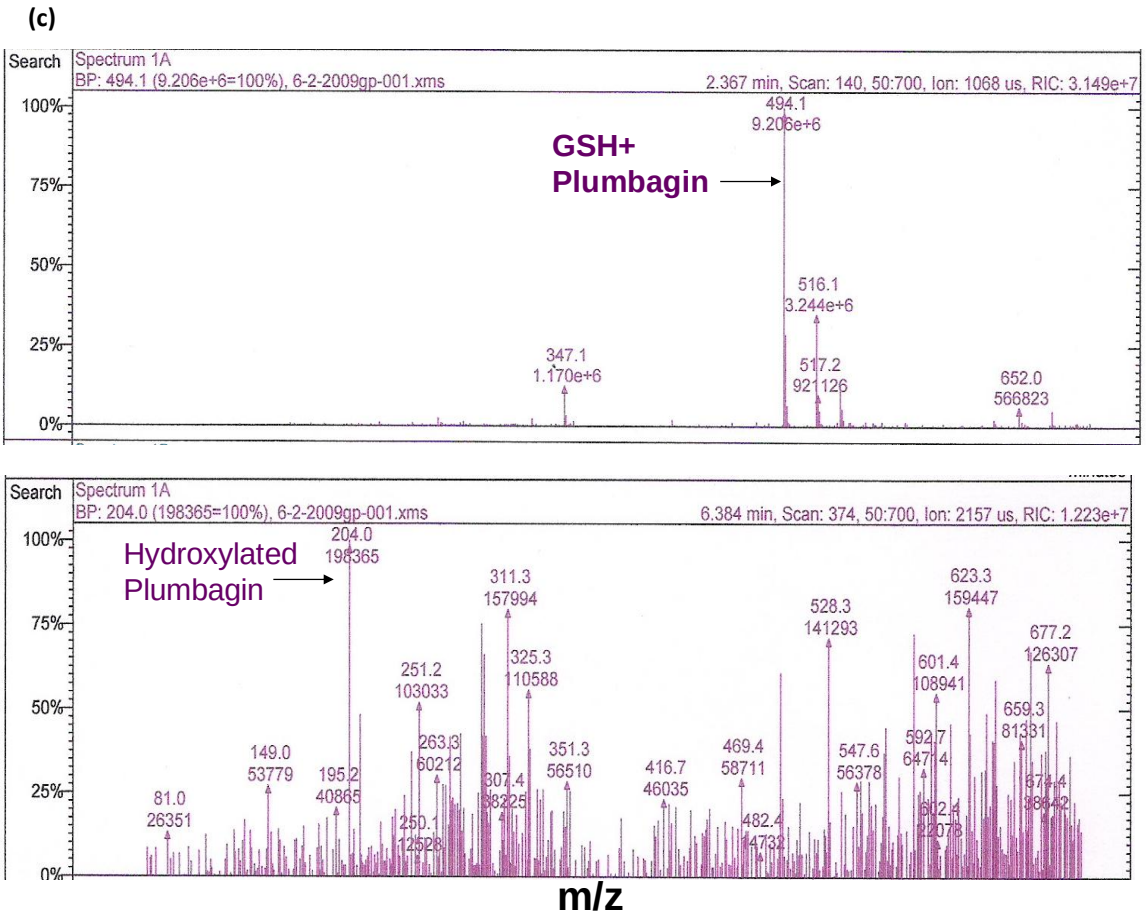


Fig. 3.9: Direct interaction of plumbagin with GSH. (a) To monitor the interaction, changes in absorption spectra of plumbagin was observed in presence or absence of GSH.(b) HPLC separation of products of reaction of plumbagin with GSH. Plumbagin (100 µM) was mixed with GSH (10mM) in 10mM potassium phosphate buffer. After 1 h incubation at 37°C, 25 µl of each sample was subjected to HPLC. Effluent was monitored using a diode array detector. Red line (solid) indicates plumbagin alone and blue (dotted) line indicates reaction product(s) of plumbagin with GSH. (c and d) Identification of products of reaction of plumbagin with GSH by mass spectrometry. Sample containing 100 µM plumbagin in 10mM GSH was subjected to LC–MS analysis using ESI ion trap detector. Two major peaks corresponding to plumbagin–GSH adduct (mol. Wt 494, upper panel) and hydroxylated product of plumbagin (mol. wt 204, lower panel) were observed following ESI-MS analysis.

3.3.10 Plumbagin induced glutathionylation of proteins in lymphocytes:

Since plumbagin reacted with GSH and also induced conversion of GSH to GSSG in cell-free systems and also depleted cellular GSH levels, experiments were carried out to determine whether plumbagin induces protein glutathionylation in cells. Lymphocytes were treated with plumbagin and cell lysates were incubated with BIAM. Using equal amount of protein in each group, non-glutathionylated proteins conjugated with BIAM were pulled down using streptavidin agarose beads and were resolved on SDS– PAGE. Lymphocytes treated with plumbagin showed marked decrease in free thiol groups on proteins (Fig. 3.10a). To assess the effect of plumbagin on protein glutathionylation, cells were incubated with cell permeable biotinylated-glutathione-ethyl ester (Bio-GEE). Bio-GEE loaded cells were treated with vehicle or plumbagin and cell lysates were prepared. Equal amount of protein in each group was used for precipitation of glutathionylated proteins using streptavidin agarose beads and resolved on SDS–PAGE. Coomassie staining showed a dose-dependent increase in specific glutathionylated proteins in plumbagin-treated samples as compared to that in untreated control cells (Fig. 3.10b).

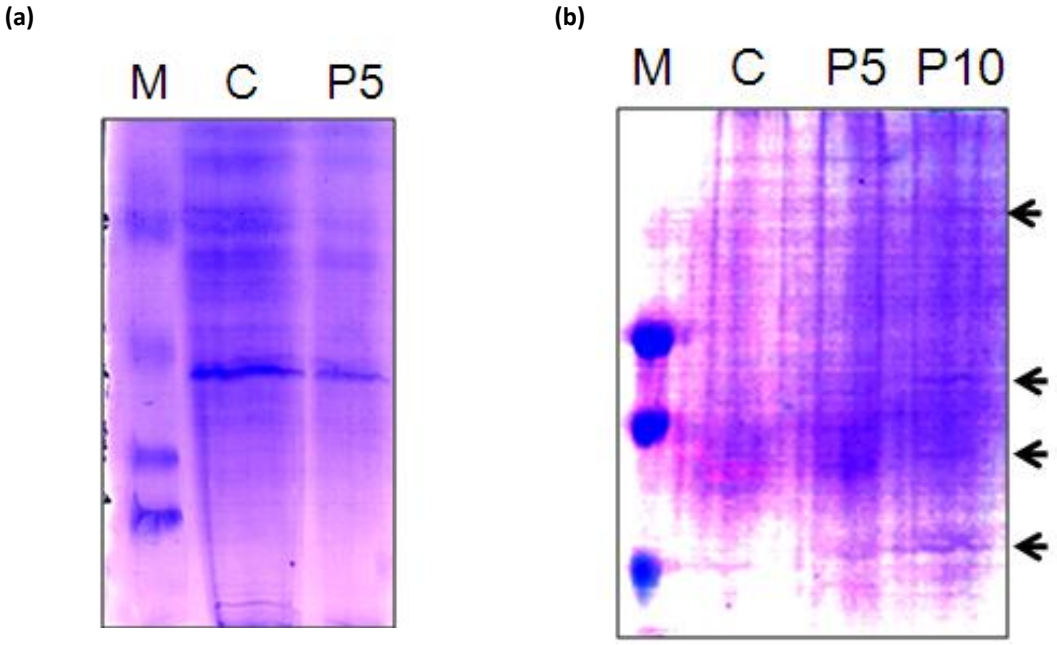


Fig.3.10. Plumbagin induced protein glutathionylation: (a) Enrichment of iodoacetamide reactive sulfhydryl groups on proteins. Iodoacetamide (IAM) binds selectively to protein thiolate anions. The lysates of lymphocytes incubated with 5 µM plumbagin for 4 h were treated with BIAM (0.2mM) for 30min at 37°C. Streptavidin agarose beads (20 ml/mg of protein) were added to the lysates and incubated for 1 h at 48C. The agarose beads were separated by centrifugation, washed four times with RIPA buffer, and boiled in SDS sample buffer. Proteins in the eluent were resolved by SDS–PAGE and detected by Coomassie blue staining. C: Purification of putative S-glutathionylated proteins. Protein extract was obtained as described under the Materials and Methods Section from Bio-GEE loaded lymphocytes incubated with plumbagin (0 or 5 or 10 mM, 4 h). Biotin-labeled proteins were extracted from total protein using streptavidin agarose beads. The beads were washed with RIPA buffer and boiled in SDS sample buffer. The eluted proteins were resolved by SDS–PAGE and were detected by Coomassie blue staining of the gel. Two such independent experiments were carried out.

3.3.11 Plumbagin induced glutathionylation of NF-κB in lymphocytes:

Lymphocytes were treated with plumbagin (5µM) for 4h, cell lysates were prepared, fractionated on 10%SDS–PAGE (non-reducing), electrotransferred to nitrocellulose membrane and probed with anti-GSH antibody. It was observed that plumbagin treatment induced a significant increase in protein glutationylation (Fig. 3.11a). Further, glutathionylated proteins were immunoprecipitated using anti-GSH antibody and probed with anti-p65 antibody by western blotting to identify any changes in glutathionylation of NF-κB. It was observed that plumbagin treatment induced glutathionylation of p65 in lymphocytes (Fig. 3.11b).

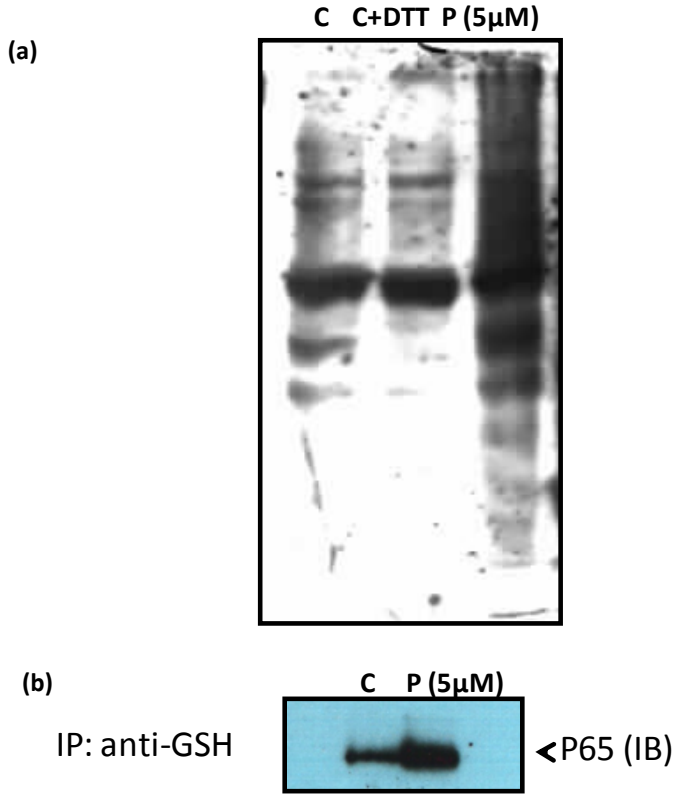
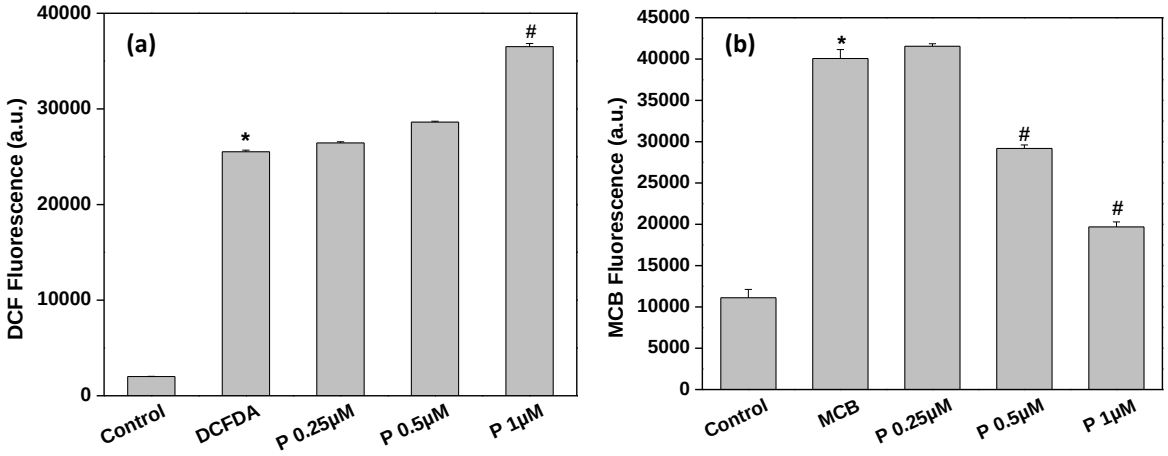


Fig. 3.11 Induction of NF-κB glutathionylation in lymphocytes by plumbagin: (a) Lymphocytes were incubated with plumbagin (1 M, 4 h), cell lysates were prepared, fractionated on SDS–PAGE (non-reducing) and electrotransferred to nitrocellulose membrane. Western blot analysis was performed using anti-GSH antibody (b) Lymphocytes were incubated with plumbagin (1 M, 4 h), cell lysates were prepared and immunprecipitated using anti-GSH antibody, fractionated on 10%SDS–PAGE, and electrotransferred to nitrocellulose membrane. Western blot analysis was performed using P65 antibody.

3.3.12 Plumbagin modulated cellular redox status in RAW cells and inhibited LPS induced MAPKinase and NF-κB activation in these cells: Since plumbagin was able to inhibit LPS induced secretion of TNF-α, IL-6 and IL-1β in RAW cells, experiments were carried out to elucidate the mechanism of action of plumbagin in these cells. It was observed that similar to the results obtained in lymphocytes, plumbagin increased basal ROS levels and depleted intracellular GSH levels in RAW cells. Further, plumbagin was also able to suppress mitogen induced activation of MAPkinase and NF-κB in RAW cells.



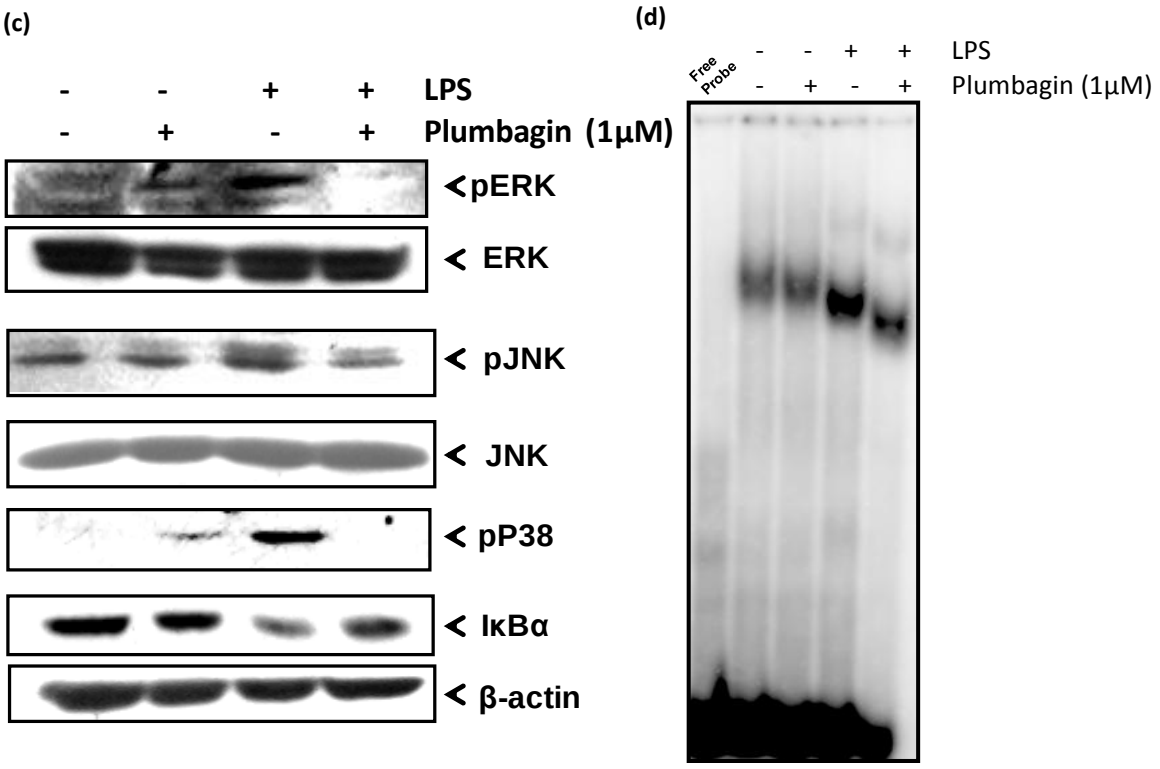


Fig. 3.12 Plumbagin modulates cellular redox status and abrogates LPS induced MAPKinase and NF-κB activation in RAW cells: (a) Lymphocytes were stained with H_2DCF-DA (20 µM) for 30 min at 37°C and were treated with indicated concentration of plumbagin. Fluorescence emission was measured at 520 nm following excitation at 480 nm. Each bar shows mean DCF fluorescence ± SEM from four replicates and two such independent experiments were carried out. *p<0.01 as compared to untreated cells. #p<0.01 as compared to untreated cells stained with DCFDA. (b) Plumbagin depleted intracellular GSH levels: RAW cells were treated with different concentrations of plumbagin for 4 h at 37°C and stained with MCB (final concentration 40 mM) for 30 min. Fluorescence emission was measured at 490 nm following excitation at 394 nm. Each bar shows mean MCB–GSH adduct fluorescence ± SEM from four replicates and two such independent experiments were carried out.*p<0.01 as compared to untreated cells. #p<0.01 as compared to untreated cells stained with MCB. (c) Inhibition of MAPKinase and NF-κB activation in RAW cells: Lymphocytes were incubated with plumbagin (1 µM, 4 h) and stimulated with LPS for 1h. Cytosolic extracts were prepared, fractionated on 10%SDS–PAGE, and electrotransferred to nitrocellulose membrane. Western blot analysis was performed using different antibodies specific for P-ERK, ERK, IκBα, P-P38, P-JNK, JNK. β-Actin was used as loading control. (d) RAW cells were incubated with 1 µM plumbagin for 4 h and stimulated with LPS for 1 h. The nuclear extracts were prepared and analysed for NF-κB activation by EMSA. Two such independent experiments were carried out.

CHAPTER - IV

In vivo anti-inflammatory efficacy of plumbagin

This chapter details the studies carried out to establish the in vivo anti-inflammatory efficacy of plumbagin using mouse models of graft-versus-host disease (GVHD), allograft transplantation and lipopolysaccharide induced septic shock. The mechanism of in vivo action of plumbagin was also elucidated in detail using LPS induced septic shock model and is described in this chapter.

4.1 MATERIALS AND METHODS

4.1.1 Reagents and Chemicals:

The following chemicals were obtained from Sigma Chemical Company, USA: Plumbagin, HEPES, lipopolysaccharide (3×10^6 endotoxin units/mg from E. coli serotype 026:B6), ethylenediaminetetraacetic (EDTA), bovine serum albumin (BSA), Polyethylene glycol tetraacetic acid (EGTA), phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, benzamidine, nonidet P-40, NXTRACT CelLytic nuclear extraction kit, penicillin, streptomycin, sodium chloride, sodium dihydrophosphate, disodiumhydrophosphate. RPMI 1640, Fetal calf serum (FCS) was obtained GIBCO BRL. 5-(and-6)-carboxy-2,7-dichlorofluorescein diacetate (DCF-DA) and CyQuant-NF cell proliferation assay kit was procured from Molecular Probes, Invitrogen.

4.1.2 Antibodies:

As described in chapter 3, section 3.1.2

4.1.3 Animal maintenance:

As described in chapter 2, section 2.1.3

4.1.4 Plumbagin administration to mice:

In in vivo experiments, mice were injected i.p. with plumbagin in 25% PEG (2mg/kg body weight) or Phosphate buffered saline (PBS). The mice in control group were treated with an equal volume of vehicle (25% PEG in saline or PBS). Plumbagin was dissolved in 25% PEG or PBS and different amounts of plumbagin (2-5 mg / kg body weight (kgbw) were injected i.p. to Swiss mice using 26.5 gauze size needle. The total volume to be injected was filled in a 1 ml syringe and air bubbles were removed. The mouse was restrained with left hand in an upside down position and abdominal surface was cleaned with 50% dettol solution to sterilize the surface. The needle was inserted into the lower left quadrant of the abdomen, avoiding the abdominal midline and fluid was injected with moderate pressure. Abdominal surface was again cleaned with 50% dettol swab and mice were placed back in the cage.

4.1.5 Splenic lymphocyte preparation:

As described in chapter 2, section 2.1.6

4.1.6 CyQUANT-NF cell proliferation assay:

For ex vivo assay of proliferation, splenocytes were isolated from vehicle or plumbagin treated mice (2 mg/kg body weight, 24 h). Two million splenocytes were stimulated with Con A (10 µg/ml) in 2ml RPMI with 10% FCS for 72 h at 37 °C. Proliferation was estimated from the change in the total DNA content in each well using CyQuant assay (CyQUANT NF Cell Proliferation Assay Kit) according to manufacturer's protocol. Fluorescence signals were read from a 96-well plate using a plate reader (Fluostar Optima, BMG Labtech) with excitation at 485 nm and emission at 530 nm. Results were expressed as mean fluorescence intensity±SEM (four replicates per experiment).

4.1.7 Induction of Graft Versus Host Disease (GVHD):

Swiss mice were exposed to 600 cGy whole body gamma-radiation (WBI) (Gamma Cell 220, AECL Canada). To induce GVHD in immunocompromised Swiss mice, 10×10^6 splenocytes from C57BL/6 donors were injected i.v. 48 h after irradiation. Each mice in control group received vehicle treated splenocytes whereas each mice in the plumbagin group received splenocytes treated with 1 µM plumbagin for 4 h. The recipient mice were monitored daily to assess the signs of GVHD. In total, 10 recipient mice in control group and 10 recipient mice in the plumbagin treated group were evaluated. GVHD became evident from rapid and sustained weight loss as well as from features such as hunch back, diarrhoea, hair loss and death.

4.1.8 Skin Transplantation:

Allo-skin grafts were obtained from tail skin of C57BL/6 mice. The tissue was rinsed with PBS before transplantation. A graft bed of approximately $0.6cm^2$ was made by surgical incision on the dorso-lateral side of the recipient Swiss mice. Tail skin graft of the size approximately $0.6cm^2$ were grafted on to the excised upon wound to cover it and the survival/rejection of graft was monitored by daily observation. The day of complete necrosis and physical rejection of the grafted tissue was scored as the day of rejection.

4.1.8 Murine endotoxin-induced shock model:

Swiss mice were divided into four groups and plumbagin (2 mg/kgbw) or vehicle (PBS) was administrated intraperitoneally 2h prior to LPS administration. LPS (15×10^5 e.u/mice) was administrated intraperitoneally and mice from different treatment groups were sacrificed at 2h, 6h and 24h to examine various parameters associated with LPS

endotoxemia. Blood was collected through retro-orbital puncture. Serum samples were stored at –80°C until further use. For experiments in which mortality was the outcome parameter, separate sets of animals were used and mice were observed twice every day for 7 days after the injection of LPS and the mortality was recorded. Serum ALT, AST and blood urea nitrogen levels were analyzed using an automated serum analyzer.

4.1.10 Pro-oxidant measurements:

As described in chapter 3, section 3.1.5

4.1.11 Serum cytokines and NO assay:

Levels of serum TNF-α, IL-10, IL-6, IFN-γ, and IL-12 were measured by commercial ELISA kits (Opt EIA Elisa kits; BD Biosciences Pharmingen, San Diego, CA). Serum nitrite levels were estimated according to the method described earlier (Chapter 2, section 2.1.16).

4.1.12 Western blot analysis:

Cytosolic and nuclear proteins were extracted from snap-frozen liver sections using the NXTRACT CelLytic nuclear extraction kit according to manufacturer's protocol. The cytosolic fractions (30 μg) were resolved by SDS-PAGE (10%) and transferred to nitro-cellulose membrane. After the membrane was blocked in 5% nonfat powdered milk, the membrane was incubated overnight with the primary antibody specific to IκB-α or phospho-ERK, washed three times with Tris-buffer saline containing 0.05% Tween 20 (TBST) and further incubated with horseradish peroxidase-labeled secondary antibody for 1 h. The membranes were washed, and specific bands were visualized on X-ray films using Enhanced Chemiluminiscence Kit (Roche, Germany). The membrane was stripped and reprobed with actin β or ERK antibody.

4.1.13 Electrophoretic mobility shift assay

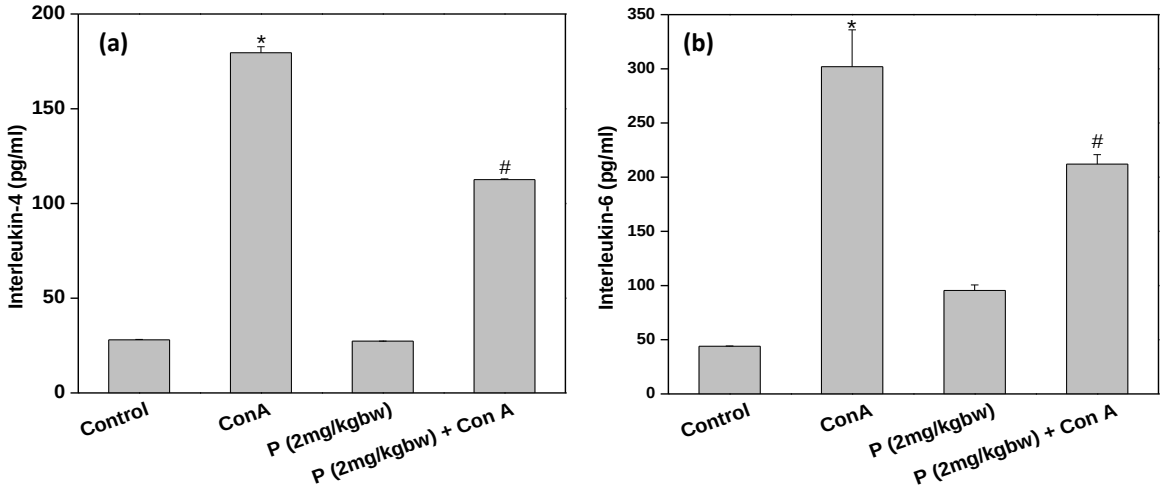
Nuclear proteins were extracted from snap-frozen liver sections using the NXTRACT CelLytic nuclear extraction kit according to manufacturer's protocol. EMSA was performed by incubating 15 µg of nuclear proteins with 16 fmol of 32 P-end-labeled, 45-mer double stranded NF-κB oligonucleotides from the human immunodeficiency virus long terminal repeat (5′-TTGTTACAA**GGGACTTTCCGCTGGGGACTTTCC**AGGGAGGCGTGG-3′; boldface indicates NF-κB binding sites) in the presence of 0.5 µg of poly (2′-deoxyinosinic-2′-deoxycytidylic acid) (poly (dI–dC)) in binding buffer (25mMHEPES, pH 7.9, 0.5mMEDTA, 0.5mMdithiothreitol,1% Nonidet P 40, 5% glycerol, and 50mMNaCl) for 30min at 37 °C. The DNA–protein complex was separated from free oligonucleotide on 6.6% native polyacrylamide gels using buffer containing 50 mM Tris, 200 mM glycine, and 1 mM EDTA, pH8.5. The dried gel was exposed on phosphorimage plate and the radioactive bands were visualized using a phosphorImage plate scanner (Amersham Biosciences,USA).

4.1.14 Statistical Analysis: The statistical significance of the differences in respect of all parameters studied between different treatment groups was assessed by student's t-test. Statistical significance of survival was assessed using Log-rank test.

4.2 RESULTS

4.2.1 Lymphocytes from plumbagin treated mice showed decreased responsiveness to Con A stimulation:

Fig4.1. shows the cytokine production and proliferation response of lymphocytes isolated from plumbagin (2 mg/kg body weight, 24 h) treated mice as compared to that from vehicle treated control mice. The lymphocytes from plumbagin treated mice showed a significantly lower production of IL-4, IL-6 and IFN-γ cytokines (Fig. 4.1a–c) as compared to that in lymphocytes taken from vehicle treated control mice when stimulated with Con A. Interestingly, the Con A induced proliferation response of T cells was also significantly diminished by plumbagin administration as compared to that in cells from control mice (Fig. 4.1d).



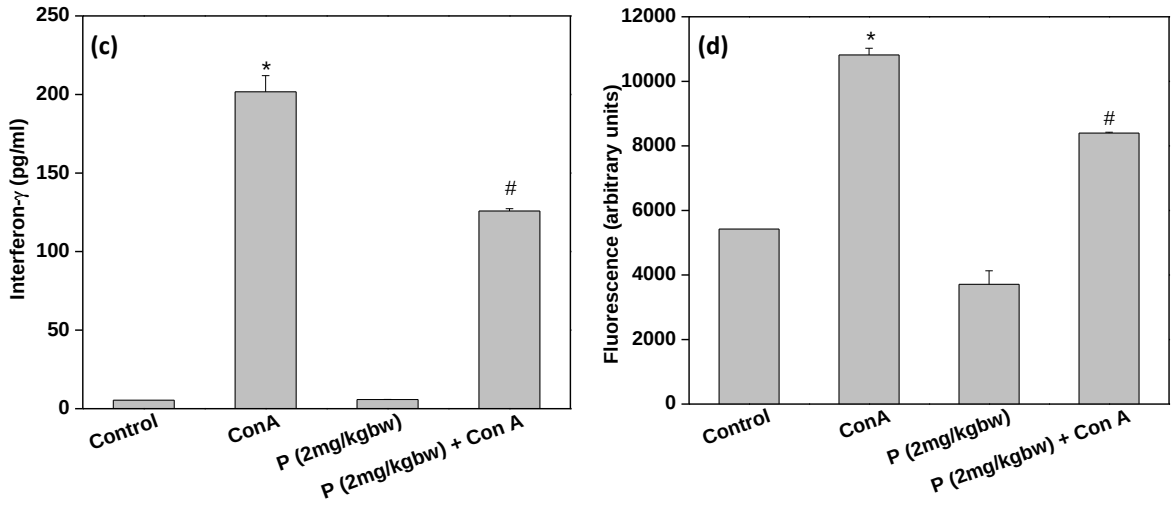


Fig. 4.1 Effect of plumbagin treatment on Con A induced lymphocyte proliferation and cytokine secretion in vivo: Mice were injected i.p with plumbagin in 25% PEG (2 mg/kg body weight). The mice in control group were treated with an equal volume of vehicle (25% PEG in saline). Splenic lymphocytes were isolated 24 h after injection and were stimulated with the Con A at 37 °C in RPMI for 24h for cytokine measurement and 72 h for estimating mitogen induced proliferation. The concentration of cytokines in the culture supernatant of cells isolated from control and plumbagin treated mice was estimated using ELISA. Graphs show concentration of (a) IL-4 (b) IL-6 and (c) IFN-γ cytokines. Each bar represents mean±S.E.M. from three replicates and two such independent experiments were carried out. After 72 h of stimulation with Con A, the cell proliferation was assessed by CyQUANT assay (d) and graphs show the fluorescence intensity corresponding to proliferation of cells in each treatment group. 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 and #$p<0.01$, as compared to Con A stimulated cells.

4.2.2. Treatment of lymphocytes with plumbagin prior to allogenic transplantation delayed the induction of GVHD in mice:

To test immunosuppressive activity of plumbagin in GVHD model, splenocytes from C57BL/6J mice were incubated with plumbagin (1 µM, 4h) and transferred to WBI treated immunocompromised Swiss mice. The mice of vehicle treated group developed GVHD that led to 70% death within 45 days (Fig. 4.2a), demonstrating typical symptoms of GVHD, including alopecia, scleroderma, hunched posture, diarrhea, and progressive weight loss (Fig 4.2c). However, in the plumbagin treated group 90% of the mice survived in better health for more than 45 days (Fig. 4.2a). Furthermore, the mice treated with plumbagin experienced inconspicuous weight loss as compared to control group (Fig.

4.2b). Therefore, plumbagin significantly prevented death and weight loss induced by allo-lymphocyte transfer.

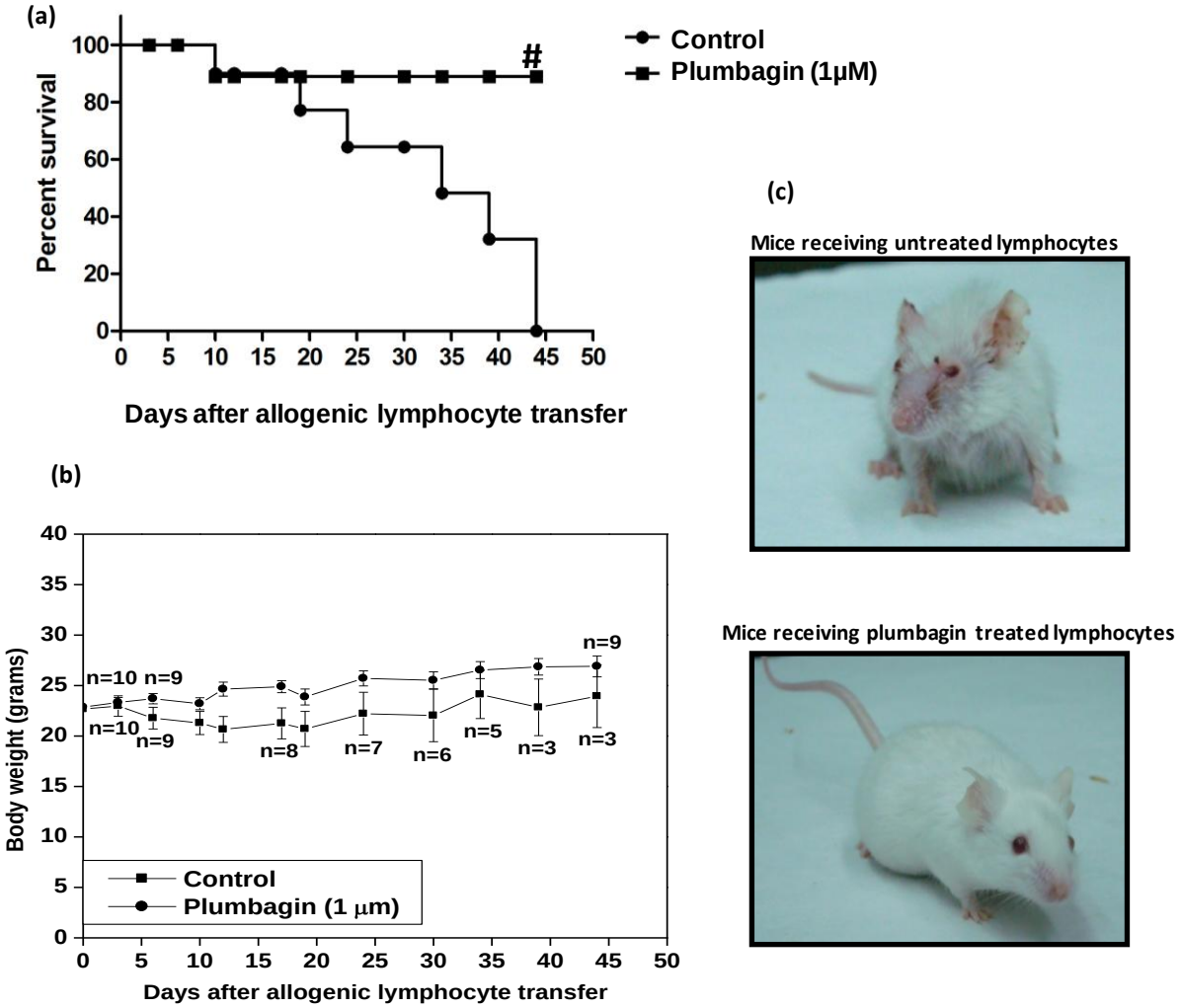


Fig. 4.2. Plumbagin prevented mortality and weight loss in GVHD mice: Ten million lymphocytes from C57BL/6 donor mice were injected i.v. into immunocompromised Swiss mice 48 h after WBI (600 cGy). Ten mice were included in each group. Control group mice received vehicle treated cells whereas the plumbagin group received cells treated with 1 μM plumbagin for 4 h. (a) Kaplan-Meier survival analysis of the immunocompromised mice reconstituted with allogenic lymphocytes treated with plumbagin or vehicle. Statistical significance of survival was assessed using Log rank test. #$p< 0.05$ as compared to control group (b) Changes in the bodyweight of the mice after allotransplantation. Data points represent mean±SEM from 10 mice. (c) A representative image of mice receiving untreated and plumbagin treated allogenic lymphocytes is shown.

4.2.3 Plumbagin administration significantly delayed rejection of allograft in mice:

The in vivo immunosuppressive efficacy of plumbagin was studied in a mouse model of allograft transplant. In this model allo-skin grafts were obtained from tail skin of C57BL/6 mice. A graft bed of approximately 0.6cm^2 was made by surgical incision on the dorso-lateral side of the recipient Swiss mice. Tail skin graft of the size approximately 0.6cm^2 were grafted on to the excised upon wound to cover it and the survival/rejection of graft was monitored by daily observation. The day of complete necrosis and physical rejection of the grafted tissue was scored as the day of rejection. It was observed that administration of plumbagin (2 mg/kgbw) significantly delayed rejection of allograft in mice as compared to untreated mice.

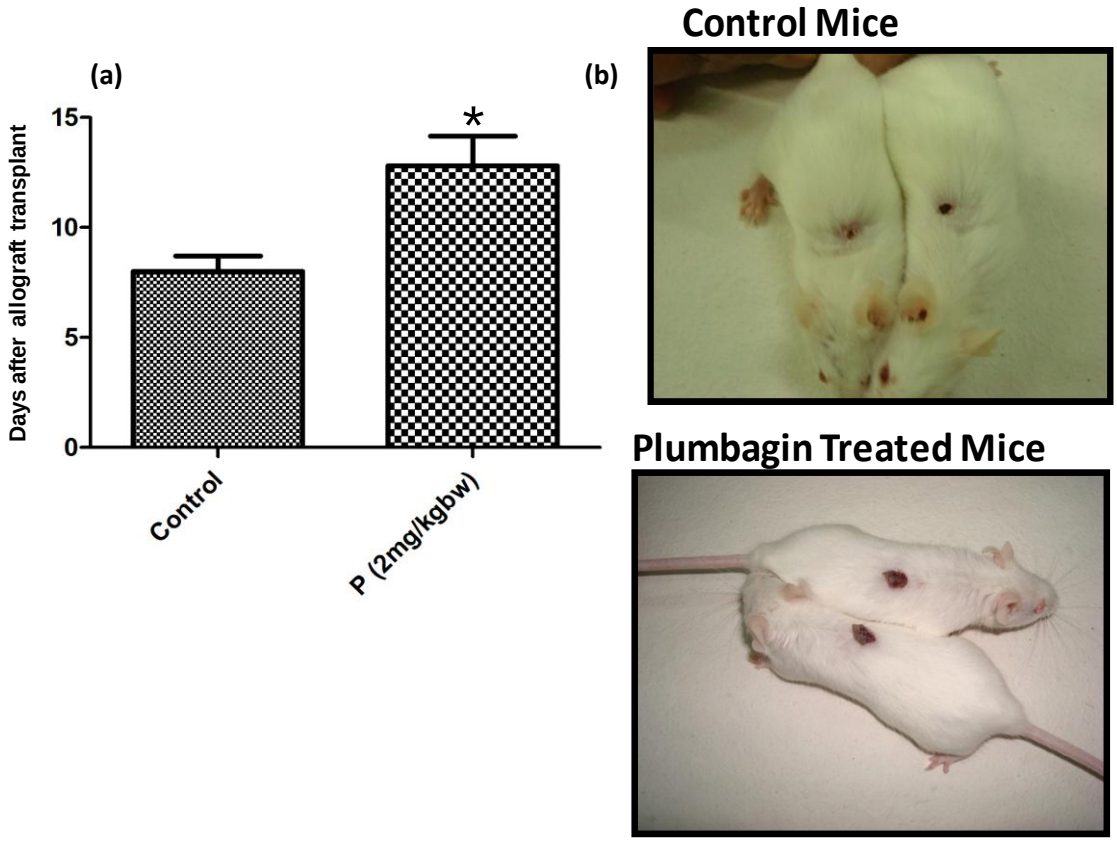


Fig. 4.3. Plumbagin delayed allograft rejection in mice: allo-skin grafts obtained from tail skin of C57BL/6 mice were grafted on to the dorso-ventral side of Swiss mice and survival/rejection of graft was monitored. *p<0.05,as compared to control mice. (c) A representative image of mice from control and plumbagin treated group is shown.

4.2.4 Plumbagin alleviated LPS induced lethal endotoxin shock in mice:

In the present experiments we have used a well-established model of LPS induced endotoxic shock to determine whether plumbagin, by virtue of its potent anti-inflammatory properties, is capable of exerting immunomodulatory effects on LPS-induced lethal endotoxemia. Mice were challenged i.p. with 15 x 10^5 endotoxin units/mice, a dose predetermined to be lethal in 80 to 90% of the animals, in combination with either PBS or 2mg/kgbw of plumbagin given 2h prior to LPS injection. It was observed that plumbagin administration was able to rescue mice from lethal endotoxic shock.

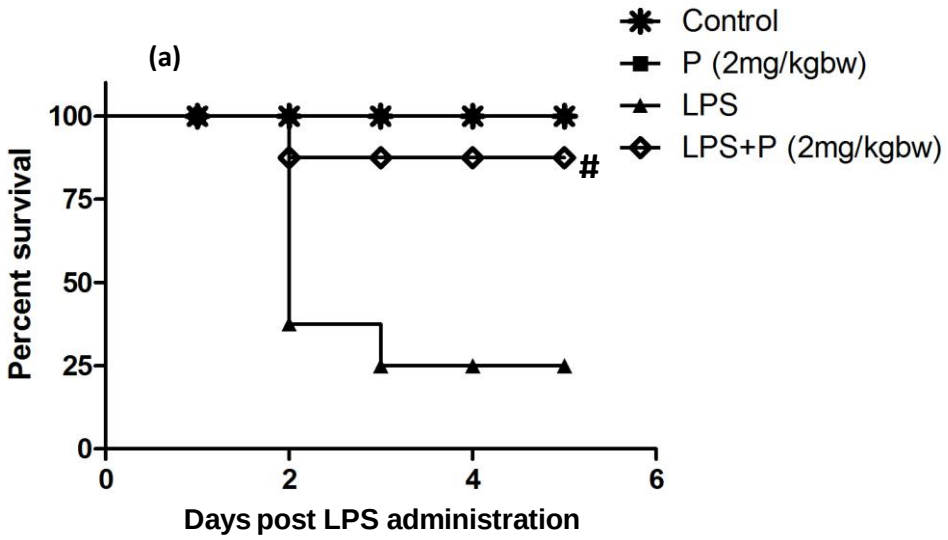


Fig. 4.4. Protective effects of plumbagin on LPS-induced lethality in mice. (a) Mice were pretreated intraperitoneally with plumbagin (2mg/kgbw) 2h before treatment with LPS (15 x 10^5 endotoxin units/mice, intraperitoneally). Survival was observed for 12, 24, 36 and 48 h after injection of LPS and Kaplan-Meier survival analysis was performed. Statistical significance of survival was assessed using Log rank test. #$p < 0.05$ as compared to LPS group.

4.2.5 Effect of plumbagin administration on LPS induced increase in the serum nitrite levels:

To determine the molecular mechanism of the observed therapeutic benefit of plumbagin in LPS endotoxemia model, the levels of serum nitrite was estimated 6h after LPS administration. There was a significant increase in the levels of nitrite in mice from the LPS group as compared to the control group (Fig. 4.5a). The serum levels of nitrite in mice from the plumbagin group were significantly lower as compared to the LPS 6 h group (Fig. 4.5a).

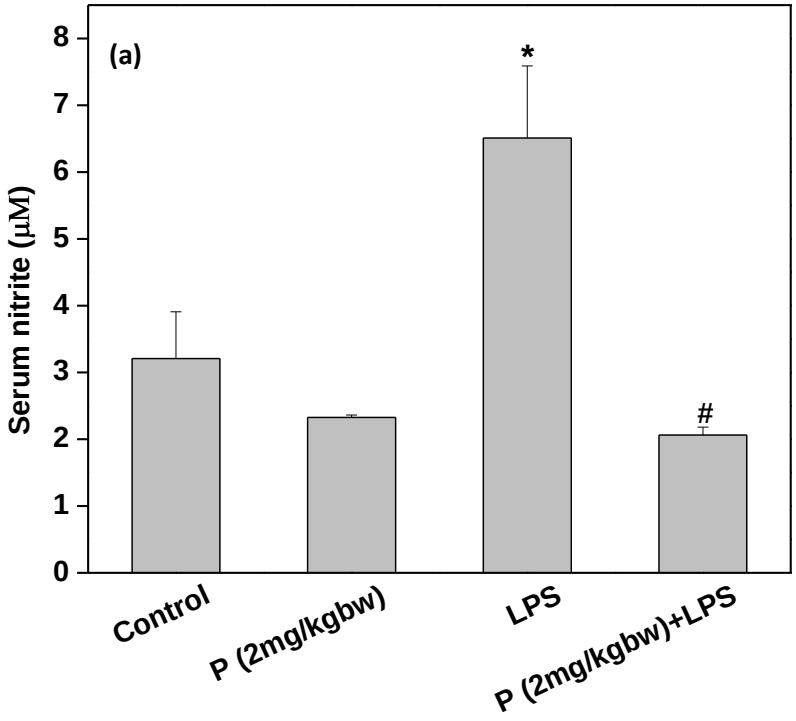


Fig 4.5 Serum nitrite levels: Serum nitrite levels from different treatment groups were monitored at 6 h after LPS administration. *p<0.05 as compared to control group and #p<0.05 as compared to LPS group.

4.2.6. Effect of plumbagin administration on LPS induced increase in the serum cytokine levels:

There was a significant increase in the levels of TNF-α , IL-6, IL-10, IL-12 and IFN-γ in mice from the LPS group as compared to the control group at the time points studied (Fig. 6a – e). The serum levels of all pro-inflammatory cytokines in mice from plumbagin treated group were significantly lower as compared to LPS treated group.

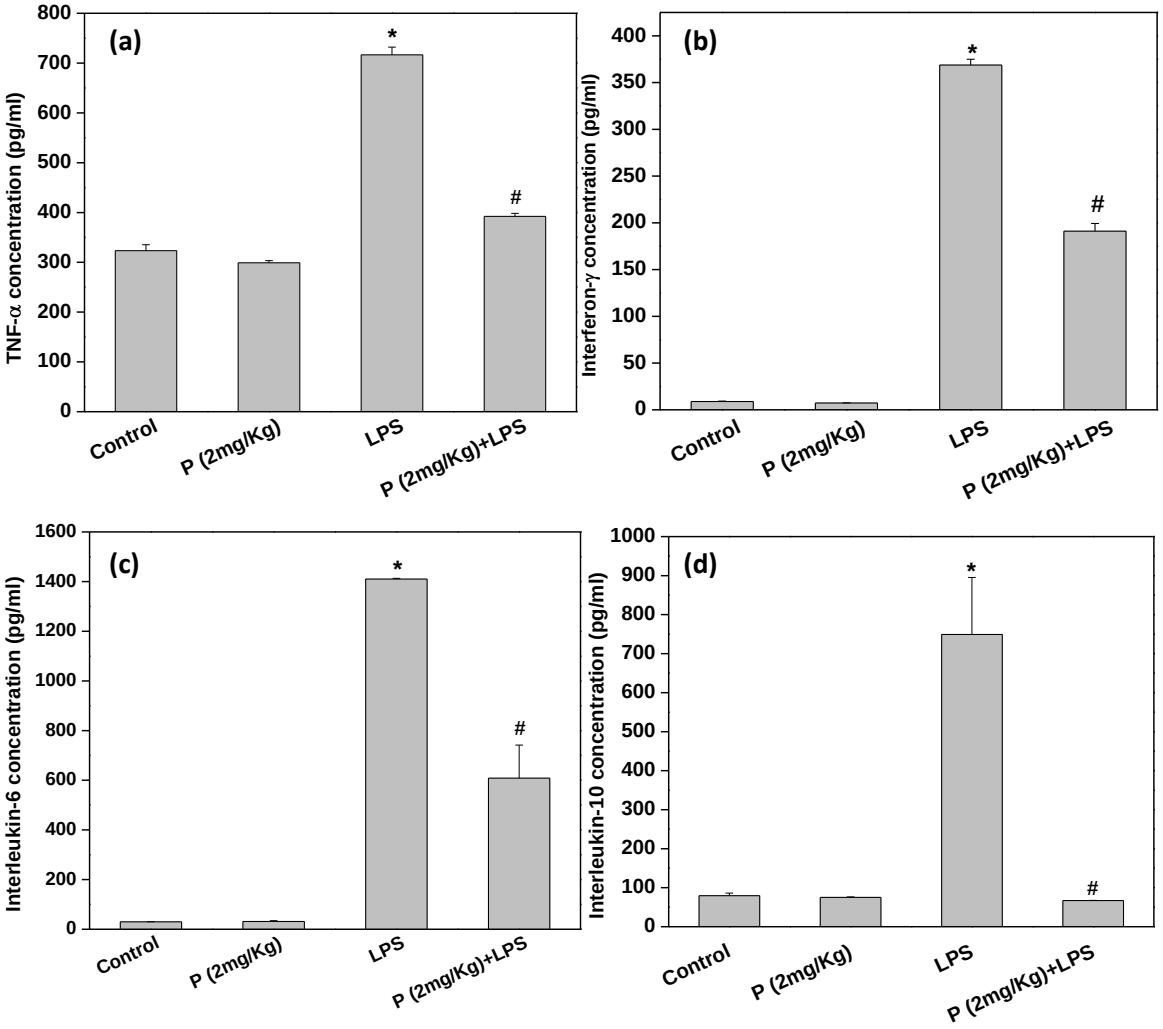


Fig. 4..6 (a – d) Effect of plumbagin on serum cytokines in LPS-treated mice: Cytokine levels from various groups were monitored at 2h (TNF-α and IL-10) and 6h (IL-6, IL-12 and IFN-γ) of LPS administration. *p< 0.05 as compared to control. #p < 0.05 as compared to LPS group.

4.2.7 Effect of plumbagin administration on LPS induced increase in the ROS levels in peritoneal macrophages and splenocytes:

There was a significant increase in the levels of ROS in peritoneal macrophages and splenic lymphocytes in mice from the LPS group as compared to the control group (Fig. 4.7a and b) as measured using DCF fluorescence. The ROS levels of peritoneal macrophages and splenic lymphocytes in mice from plumbagin treated group were significantly lower as compared to LPS treated group (Fig. 4.7a and b).

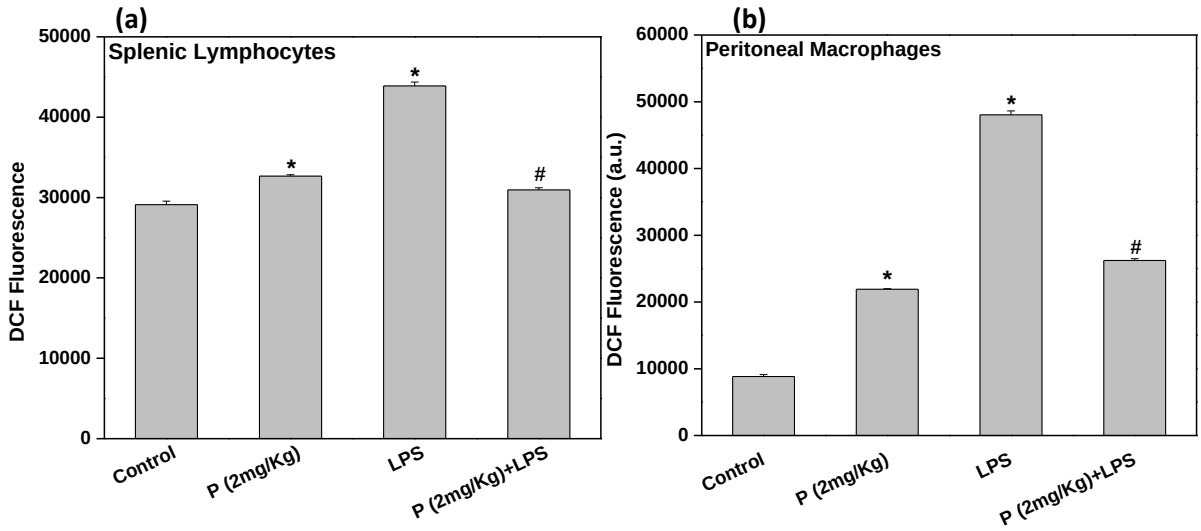


Fig. 4.7 (a and b) Effect of plumbagin on levels of ROS in peritoneal macrophages and splenic lymphocytes in LPS treated mice: ROS levels cells in from various groups were monitored at 2 h and 6 h of LPS administration. *p< 0.05 as compared to control. #p < 0.05 as compared to LPS group.

4.2.8 Effect of plumbagin on LPS induced increase in the levels of serum AST, ALT and blood urea nitrogen: Serum isolated from mice 6 h after LPS injection had significantly elevated levels of aspartate aminotransferase (AST) (Fig 4.8a), alanine aminotransferase (ALT) (Fig 4.8b) and blood urea (Fig 4.8c) nitrogen as compared to the mice from control group. The increased levels of both these enzymes as well as blood urea nitrogen were reduced in mice from the plumbagin treated group, when compared to the LPS group (Fig 4.8 a - c).

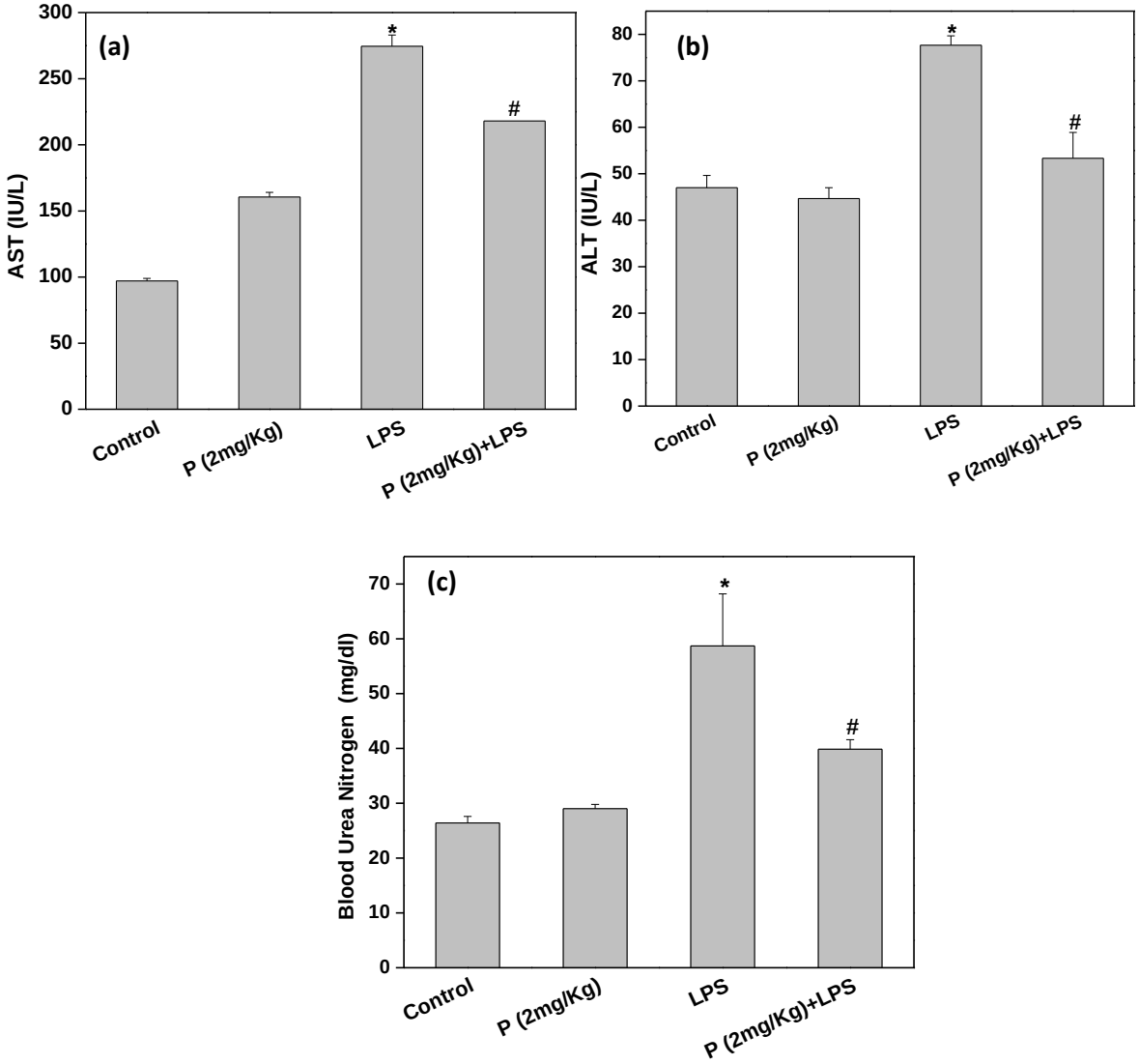


Fig 4.8 Effect of plumbagin on serum AST, ALT and blood urea nitrogen levels in LPS-treated mice: (a) ASAT, (b) ALAT and (c) Blood urea nitrogen levels from various groups were monitored at 24h after LPS administration. *$p < 0.05$ as compared to control mice. #$p < 0.05$ as compared to LPS group.

4.2.9 Effect of plumbagin on LPS induced MAPKinase and NF-κB activation in mice:

We have earlier observed that plumbagin inhibits MAPKinase and NF-κB activation in RAW cells in vitro and also rescued mice from lethal endotoxic shock. To elucidate the in vivo mechanism of action of plumbagin, its ability to modulated MAPKinase and NF-κB activation in mice was studied. It was observed that plumbagin was able to suppress LPS induced activation of NF-κB (Fig. 4.9a) and MAPKinases activation (Fig. 4.9b) in liver.

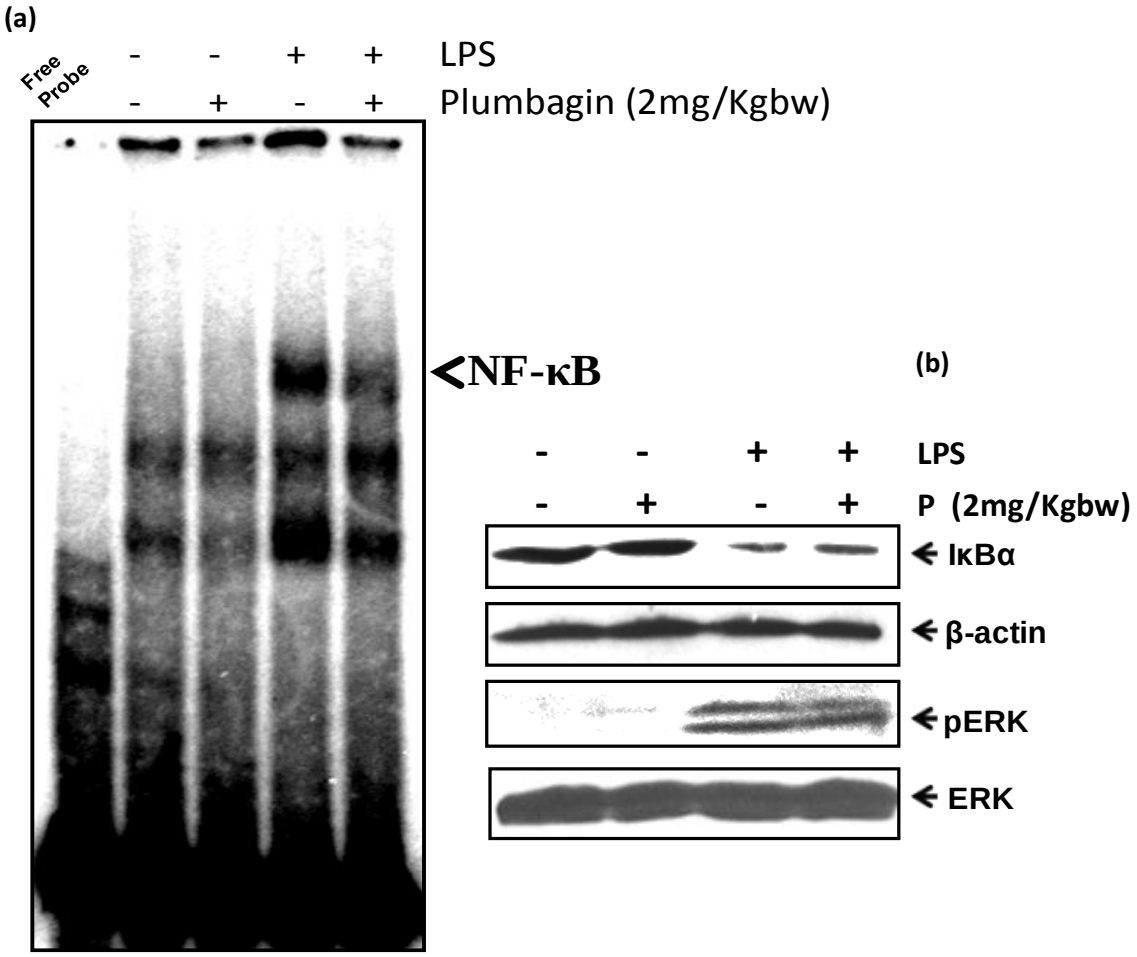


Fig 4.9 Plumbagin inhibited LPS-induced NF-κB and MAPKinase activation in LPS treated mice: Liver tissue was removed 24h after LPS injection and cytosolic and nuclear extracts were prepared. (a) The nuclear extracts were analysed for NF-κB activation by EMSA. (b) Cytoplasmic extracts were fractionated on 10% SDS-PAGE and electro-transferred onto nitrocellulose membrane. Western blot analysis was performed using IκBα, pERK and total-ERK antibody. β-actin expression in each group was used as loading control for IκBα.

CHAPTER - V

Discussion and Conclusions

5.1 DISCUSSION

The immune system is a complex network of molecules, cells and organs which provide defense against pathogenic microorganisms and non-infectious foreign substances. The immune cells are derived from hematopoietic stem cells in the bone marrow and circulate in the blood and lymph, form complex microstructures in specialized lymphoid organs and infiltrate virtually every tissue. Their anatomic organization in lymphoid organs and their ability to circulate throughout the body and ability to migrate between blood and lymphoid tissues are crucial components of host defense. There are two fundamentally different types of responses to invading microbes known as innate and adaptive immune responses. Innate (natural) responses occur to the same extent every time the infectious agent is encountered, whereas acquired (adaptive) responses improve on repeated exposure to a given infection. The innate response uses phagocytic cells (neutrophils, monocytes, and macrophages), cells that release inflammatory mediators (basophils, mast cells, and eosinophils), and natural killer cells. The molecular components of innate responses include complement, acute-phase proteins, and cytokines such as the interferons. Acquired immune responses involve the proliferation of antigen-specific B and T cells, which occurs when the surface receptors of these cells bind to antigen. T cells help B cells to make antibody and can also eradicate intracellular pathogens by activating macrophages and by killing virally infected cells. Innate and acquired responses usually work in close co-ordination to eliminate pathogens.

Inflammation is a localized protective reaction of cells/tissues of the body to allergic or chemical irritation, injury and/or infections. The symptoms of inflammation are characterized by pain, heat, redness, swelling and loss of function that result from dilation of the blood vessels leading to an increased blood supply and from increased

intercellular spaces resulting in the movement of leukocytes, protein and fluids into the inflamed regions [242]. The inflammatory cascade consists of a intricate network of complex processes and is divided into two parts i.e. acute and chronic which could either be beneficial or detrimental to the organism.

1. Acute inflammation is characterized by rapid onset and is believed to be a defense mechanism aimed at killing of bacteria, virus and parasites while still facilitating wound repairs. It is characterized by the exudation of fluids and plasma proteins and the migration of leukocytes, most notably neutrophils into the injured area.

2. Chronic inflammation is of a more prolonged duration and manifests histologically by the presence of lymphocytes and macrophages, resulting in fibrosis and tissue necrosis. The persistent chronic inflammation increases the development of the degenerative diseases including rheumatoid arthritis, atherosclerosis, heart disease, Alzheimer, asthma, acquired immunodeficiency syndrome (AIDS), cancer, congestive heart failure (CHF), multiple sclerosis (MS), diabetes, gout and inflammatory bowel disease.

Common to these forms of inflammation is that they have an afferent phase, in which the presence of a 'foreign material' is sensed by some types of cell, and an efferent phase, in which an inflammatory response is generated to eliminate the perceived hostile intruder. The purpose of the inflammatory response to microorganisms is obvious, and the response is beneficial and necessary to protect the integrity of the body as long as it does not become unnecessarily destructive or long-lasting. Irrespective of the cause of the inflammation, the response involves four major events.

1. **An increased blood supply to the site of inflammation.**

2. **Increased capillary permeability** which permits larger molecules, not normally capable of traversing the endothelium, to do so and thus delivers some soluble mediators to the site of inflammation.

3. **Leukocyte migration from the capillaries into the surrounding tissue**. This is promoted by release of chemoattractants from the site of inflammation and by the upregulation of adhesion molecules on the endothelium.

4. **Release of mediators from leucocytes at the site of inflammation**. These may include lipid mediators (e.g. prostaglandins, leukotrienes), peptide mediators (e.g. cytokines), reactive oxygen species (e.g. superoxide), amino acid derivatives (e.g. histamine) and enzymes (e.g. matrix proteases) depending upon the cell type involved, the nature of the inflammatory stimulus, the anatomical site involved and the stage during the inflammatory response. These mediators normally would play a role in host defence, but when produced inappropriately or in an unregulated fashion, they can cause damage to host tissues, leading to disease. Several of these mediators may act to amplify the inflammatory process acting, for example, as chemoattractants. Some of the inflammatory mediators may escape the inflammatory site into the circulation and from there they can exert systemic effects. For example, the cytokine IL-6 induces hepatic synthesis of the acute phase protein C-reactive protein (CRP), while the cytokine TNF-α elicits metabolic effects within skeletal muscle, adipose tissue and bone.

Several classes of drugs, such as corticosteroids, NSAIDs, and biologics, are used to treat the inflammatory disorders. The main problem is that these drugs possess several adverse effects or are too expensive to be used. Corticosteroids have long been used for the management of rheumatoid arthritis and IBD diseases, but they suffer from some serious adverse effects, such as hypertension, hyperglycemia, muscular weakness,

increased susceptibility to infection etc. The development of effective and safe immunosuppressive regimens for the treatment of inflammatory disorders, organ transplant rejection and autoimmune and allergic reactions represents a major goal of current clinical research.

Plants have been the basis of many traditional medicine systems throughout the world for thousands of years and they represent an exhaustive source of "raw materials" in order to find and synthesize new molecules with pharmacological activity [10]. Natural Products have played a pivotal role in immunosuppressive drug discovery as evident by the launch of the cyclosporin (1983), tacrolimus (1993), sirolimus (1999) and mycophenolate sodium (2003), and the semi-synthetic mycophenolate mofetil (1995), everolimus (2004) and fingolimod (2010). In addition, the natural product derived aspirin (acetylsalicylic acid) discovered in the late 1890s is still used widely as an analgesic and anti-inflammatory, while corticosteroids and β2 agonists modeled on adrenaline (e.g., salbutamol and salmeterol) are used to help control asthma [167]. Natural products offer consummate chemical diversity with structural complexity and biological effectiveness.

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is found in the plants of *Plumbaginaceae, Droseraceae, Ancestrocladaceae, and Dioncophyllaceae* families. Plumbagin is also present along with a series of other structurally related naphthoquinones in the roots, leaves, bark, and wood of *Juglans regia* (English walnut, Persian walnut, and California walnut), *Juglans cinerea* (butternut and white walnut), and *Juglans nigra* (black walnut). Preparations derived from black walnut have been used as hair dyes and skin colorants in addition to being applied topically for the treatment of acne, inflammatory diseases, ringworm, and fungal, bacterial, and viral infections. The root of *Plumbago zeylanica* (also called Chitrak), a major source of plumbagin, has been used in traditional

Indian medicine since 750 BC as an antiatherogenic, cardiotonic, hepatoprotective, and neuroprotective agent [243, 244]. The active principle, plumbagin, was first isolated in 1829 [245]. Plumbagin has been shown to exert several therapeutic biological effects including anticancer, antiproliferative, chemopreventive, chemotherapeutic, and radiosensitizing properties in experimental animals as well as in tumor cells in vitro [246-248].

A recent report suggested that the anti-carcinogenic, apoptotic and radiosensitizing effects of plumbagin could be attributed to its ability to suppress NF-κB activation in tumor cells [14]. One of the primary physiological roles of nuclear factor-kappa B (NF-κB) is in the immune system. 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, thereby regulating various aspects of innate and adaptive immune responses. In addition, NF-κB also contributes to the development and survival of the cells and tissues that carry out immune responses in mammals. In most cells, NF-κB complexes are inactive, residing primarily in the cytoplasm with any of the family of inhibitory IκB proteins. When the pathway is activated, the IκB protein is degraded and the NF-κB complex enters the nucleus to modulate target gene expression. In almost all cases, the common step in this activating process is mediated by an IκB kinase (IKK) complex, which phosphorylates IκB and targets it for proteasomal degradation [249]. There are several reports in literature that correlate NF-κB activation with inflammation in a wide array of diseases and animal models. Further, numerous studies using gene targeting and inhibitors of NF-κB that have established that NF-κB plays a central role in inflammatory processes. Hence, agents that target NF-κB can be used to manipulate the immune system to prevent and treat inflammation associated disease.

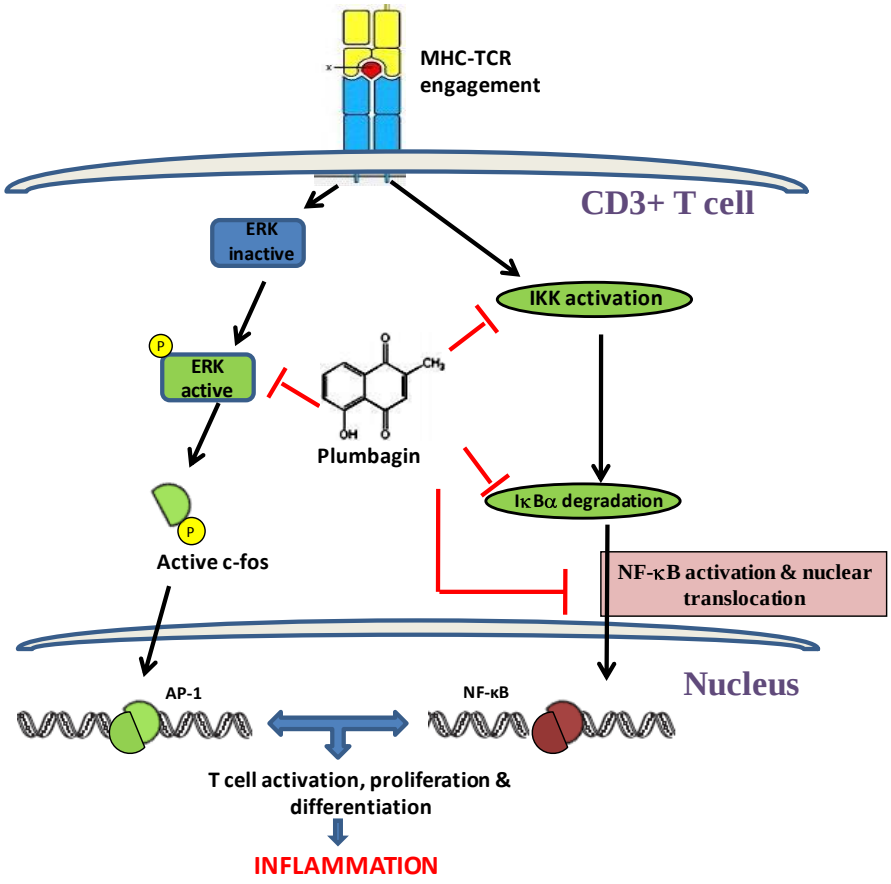
T cell activation plays a central role in the regulation of immune responses, and hence its pharmacologic inhibition has provided a powerful approach towards this objective. It is wellestablished that T cell activation proceeds in a sequence of complex events that comprise two major phases. The first one, which ensues triggering of the T cell receptor (TCR)/CD3 complex by antigen and of co-stimulatory molecules (e.g. CD28) by counter receptors on antigen-presenting cells (e.g. B7.1 and B7.2) results in the transcriptional activation of a group of immediate/early genes including lymphokine genes and leads from a quiescent (G0) to a competent (G1) state of T cells. The second phase involves the response of such competent T cells to secreted growth-promoting lymphokines (e.g. IL-2 or IL-4) in an autocrine or paracrine fashion, and drives their entry into the proliferation cycle through G1/S phase progression, with subsequent clonal expansion and acquisition of T cell effector functions. Both of these phases have proven amenable to pharmacologic intervention for immunosuppressive purpose.

In the present report the immunomodulatory effects of plumbagin were investigated in terms of lymphocyte activation, cytokine production and proliferation. The biochemical mechanisms that may contribute to its immunomodulatory activity were also elucidated. It was observed that plumbagin entered cells (Fig 2.1) and inhibited Con A induced proliferation of T cells, with significant inhibition at 50 nM (Fig. 2.3). These anti-proliferative effects of plumbagin were not due to increased apoptosis in T cells (Fig 2.2) but due to its ability to induce cell cycle arrest in G1 phase (Fig. 2.4b). The immunosuppressive effects of plumbagin were observed to be not just limited to CD4+ and CD8+ T cells (Fig. 2.6b and 2.7b), but it also suppressed LPS induced proliferation of B cells (Fig 2.12) suggesting a common mechanism its of action in these lymphocyte

subsets. Cytokines secreted by different cells participating in the immune response are known to play a critical role in the manifestation of successful pathogen clearance. Any alteration in this highly regulated network of cytokines by external or internal factors may result in undesirable consequences. IL-2, TNF-α, and IFN-γ are secreted by Th1 type cells and can activate macrophages and promotes cell-mediated immune responses against invasive intracellular pathogens. Th2 (IL-4, IL-5, IL-6, IL-10, and IL-13) cytokines promote humoral immune responses against extracellular pathogens [250]. IL-2 is the major growth factor for the clonal expansion of activated T cells and plays a crucial role in the progression of antigen activated T cells from G1 to S/G2/M phase of the cell cycle [251]. IFN-γ is an effector cytokine produced by activated Th1 cells and plays an important role in clearance of intracellular pathogens [252] by up-regulating the genes involved in pathogen recognition, antigen processing and presentation [253]. IL-4 is also an effector cytokine needed for the differentiation of naïve T helper cells into Th2 effector cells and it promotes humoral immunity [254]. It also plays a central role in the pathogenesis of allergic inflammation [255]. Plumbagin suppressed both Th1 and Th2 cytokines secreted by activated lymphocytes in response to both polyclonal and antigen specific stimuli in vitro by splenic lymphocytes (Fig 2.5 a – d) as well as by purified CD4+ T cells and CD8+ T cells (Fig 2.6c – e and 2.7c - e). Interestingly, plumbagin also mitigated LPS induced secretion of nitric oxide, TNF-α, IL-6 and PGE-2 by RAW cells (Fig 2.17b – e) and IL-1β, TNF-α and IL-6 in splenic adherent macrophages (Fig 2.18a - c). These results suggested that plumbagin acts on several cell types in exhibiting anti-inflammatory activity. Detailed studies on the mechanism of anti-inflammatory effects of plumbagin revealed that it acts both by inhibiting early and late events in T cell activation as well as by suppressing the upregulation of co-stimulatory molecules. The co-stimulatory molecules on APC (CD80 and CD86) bind to cognate receptors (CD28) on T cells and provide necessary signals for

complete activation and survival of T cells [256]. T cells that recognize antigen in the absence of costimulation, either fail to respond and die or enter a state of unresponsiveness known as T cell anergy [257, 258]. Plumbagin significantly inhibited mitogen induced upregulation of CD86 and CD80 on splenic APC (Fig. 2.13 and 2.14). These results indicate that suppression of co-stimulatory signal and possible induction of anergy may also contribute to the observed anti-inflammatory effects of plumbagin. Further it is well known that interaction of T cells with APC via TCR and MHC in the presence of appropriate co-stimulatory stimuli enhanced surface expression of several early and late activation markers. T cell activation involves the expression of several surface molecules such as CD25 and CD69 whose transcriptional regulation is dependent on coordinated activation of NFATc and NFκB [259]. Plumbagin completely inhibited the expression of CD69 and CD25 in Con A activated cells (Fig. 2.8 and 2.9). Similar inhibitory effects have been reported in rapamycin treated lymphocytes stimulated with Con A in vitro [260]. The inhibition of mitogen induced early as well as late activation markers like CD69 (early activation marker) (Fig. 2.8), CD25 (IL-2 receptor-α) (Fig. 2.9), CD54 (ICAM-1) (Fig. 2.10) and CD71 (transferrin receptor) (Fig. 2.11) clearly indicates that plumbagin suppressed early as well as late events during T cell activation. Experiments showing the inhibitory effects of plumbagin on activation and co-stimulatory markers on B cells (CD80 and CD86) also emphasize its ability to block the co-stimulatory pathway (Fig 2.13 and 2.14). Thus, our results indicated that plumbagin might be acting via inhibiting T cell activation and co-stimulatory pathways leading to induction of anergy. Interestingly, plumbagin was able to inhibit Con A induced cytokine secretion in murine lymphocytes even when added up to 6 h post-mitogenic stimulation (Fig. 2.15) which demonstrated its potential as a therapeutic agent. Further, lymphocytes treated with plumbagin for 4 h and washed prior to stimulation and rested for upto 72h also showed decreased secretion of IL-

2 in response to Con A stimulation indicating that a transient exposure of cells to plumbagin is sufficient to induce immunosuppression (Fig. 2.16). Since many of these lymphocyte activation markers, costimulatory molecules and cytokine genes are under NF-κB and AP-1 regulation, experiments were performed to examine the effect of plumbagin on this transcription factors and MAPkinases. Both ERK and JNK signaling pathways are vital mediators of a number of cellular processes including growth, proliferation, and survival of T cells [261, 262]. We observed that plumbagin was able to inhibit mitogen induced phosphorylation of ERK and IKK and degradation of IκBα indicating its potential as an immunosuppressant (Fig 3.8a). Further, we also observed that plumbagin was able to inhibit mitogen induced nuclear levels of NF-κB in splenic lymphocytes (Fig 3.7c) and purified CD4+ T cells (Fig 3.7d).



Scheme 5.1: Molecular targets of plumbagin. The signaling molecules inhibited by plumbagin are highlighted by red arrows.

Plumbagin was earlier shown to exhibit antibacterial action through generation of pro-oxidants [263]. It generated reactive oxygen species (ROS) in tumor cells leading to DNA damage and cytotoxicity [194, 207, 209]. It was shown that plumbagin directly inhibited the binding of NF-κB to its consensus target sequence by modifying a critical cystine-38 residue on p65 in tumor cells. This suppressive effect of plumbagin was shown to be sensitive to thiol-containing antioxidant, dithiothreitol [14]. Further, several investigators have shown that cellular redox status plays an important role in the biological effector functions of lymphocytes and leukocytes [264 265, 266]. Since oxidative stress has been shown to modulate signaling pathways through modulation of thiol groups present on proteins and glutathionylation of many proteins [109, 111, 267, 268], we hypothesized that the anti-inflammatory effects of plumbagin may be due to its ability to perturb the redox balance in cells leading to modification of critical signaling molecules required for activation of lymphocytes. Further, the modulation of intracellular redox by plumbagin and its mechanism is not fully understood. To test this hypothesis, we investigated the effect of plumbagin on cellular redox status. We also examined the effects of different antioxidants (thiol/non-thiol) on immunosuppressive and anti-inflammatory effects of plumbagin.

Cellular redox status is determined by balance between levels of pro-oxidants (ROS and RNS) and antioxidants (glutathione peroxidase, superoxide dismutase, and catalase) and rate of cellular respiration/metabolism. The ratio of GSH to GSSG is also considered to be intrinsic determinant of the cellular redox status. Cellular redox has been shown to play a critical role in lymphocyte activation, survival, and proliferation [269, 270 265]. Exposure of T cells to ROS scavenging agents like MnTBAP and chlorophyllin has been shown to increase cellular survival via up-regulation of anti-apoptotic gene expression [260, 271, 272]. On the contrary, exposure of T cells to oxidizing agents like H_2O_2,

xanthine/xanthine oxidase has been shown to suppress T cell activation, proliferation, and cytokine production via suppression of NF-κB [273]. In the present report we found that plumbagin disrupted cellular redox homeostasis (Fig. 3.1a and 3.2). We observed that plumbagin treatment increased DCF fluorescence (relatively specific for H_2O_2) as well as DHE fluorescence (specific for superoxide radical) but no change in the fluorescence of HPF fluorescence (relatively specific for hydroxyl radical) (Fig 3.1a,c and d). To rule out the possibility of direct oxidation of H_2DCF to DCF by plumbagin, we employed an oxidation insensitive analogue of DCFDA known as fluorescein diacetate (FDA) and observed that plumbagin treatment did lead any significant change in the fluorescence levels (Fig 3.1b) thus confirming the ability of plumbagin to modulated basal ROS levels. The increased levels of ROS in plumbagin-treated cells could be due to (i) an increase in the activities of NADPH oxidase or (ii) xanthine oxidase or (iii) influx from mitochondrial electron transport chain or (iv) modulation of free thiol levels. Since plumbagin-induced increase in intracellular ROS was not affected by specific inhibitors of NADPH oxidase, xanthine oxidase, and mitochondrial complex I (Fig. 3.2b), the role of first three of the above possibilities toward plumbagin-induced disruption of cellular redox was not apparent. Therefore, the possibility of plumbagin depleting GSH in lymphocytes was investigated. It was observed that plumbagin decreased MCB–GSH adduct fluorescence in lymphocytes (Fig. 3.2a). Hence, it may be concluded that plumbagin exerted its effects by depleting GSH in lymphocytes. DEM, a well-known depletor of GSH [274], accentuated ROS levels in plumbagin-treated lymphocytes compared to that by plumbagin alone (Fig. 3.1a). Further, cell permeable thiol antioxidant NAC significantly lowered ROS levels in plumbagin-treated cells (Fig. 3.2b). These data clearly suggested that depletion of intracellular thiols by plumbagin could be responsible for increased ROS levels in lymphocytes [275]. Although plumbagin was previously reported to induce ROS generation

in tumor cells, the mechanism of this phenomenon was not known. In this study, for the first time we show GSH depletion as a source of ROS generation in normal lymphocytes following plumbagin treatment. In normal cells, ROS homeostasis is maintained by antioxidant enzymes like superoxide dismutase, catalase, and glutathione peroxidase. The changes in ROS levels by exogenous agents have also been shown to affect the expression/activity of these enzymes. It was observed that the activity of catalase was significantly higher in plumbagin-treated cells (Fig. 3.3a). This may be due to cellular response to increased levels of H_2O_2 following treatment with plumbagin. However, the activity of SOD enzyme was not altered (Fig. 3.3b).

Earlier reports by several investigators have shown that agents that increase oxidative stress are capable of activating redox-dependent transcription factor NF-E2-related factor-2 (Nrf-2) [276]. In agreement with this, a recent report showed that plumbagin is able to increase nuclear localization and transcriptional activity of Nrf2 in human neuroblastoma cells [277]. In order to determine whether increased ROS levels or decreased thiol content or both were responsible for immunosuppressive action of plumbagin, effect of thiol and non-thiol antioxidants was investigated on suppression of mitogen induced T cell activation by plumbagin. Addition of DTT or NAC would increase the intracellular thiols whereas GSH being cell impermeable may inhibit plumbagin's action by extracellular interaction with it. It was observed that suppression of mitogen induced cytokines secretion and T cell proliferation by plumbagin were abrogated by thiol-containing antioxidants GSH, DTT and NAC, but not by non-thiol antioxidants suggesting that modulation of cellular thiol levels was very critical for immunosuppression by plumbagin (Fig. 3.4). Further, we observed that the anti-inflammatory action of well-known immunosuppressants rapamycin and Ly294002 could not be abrogated by thiol or non-thiol anti-oxidants (Figure 3.5). These results clearly demonstrate a novel mechanism

of action of plumbagin as compared to the existing immunomodulatory agents (Scheme 5.1). Interestingly, the immunosuppressive effects of plumbagin could be reversed by addition of reducing agent DTT to lymphocytes after plumbagin treatment (Fig. 3.6) indicating that the perturbation of cellular redox status by plumbagin is primarily responsible for its biological activity.

Changes in cellular thiol levels have been shown to affect multiple signaling pathways in different cell lines. Many of the proteins and transcription factors like P65, IKK, thioredoxin reductase, PI3kinase/AKT, and Keap1 have been shown to be highly sensitive to changes in cellular redox status [14, 278-281 282]. Our present results show that disruption of cellular redox by plumbagin affected specific signaling events following T cell activation with mitogen (including NF-κB, ERK, Bcl-2, Bcl-xL, and cyclin A) which are involved in cellular survival and proliferation (Fig. 3.8a and b). However, plumbagin did not inhibit signaling events required for activation of transcription factor AP-1 (P38MAPkinase and JNK) and signals emanating from costimulatory molecule (AKT) in activated T cells (Fig. 3.8c). These results were in agreement with our earlier report showing that plumbagin did not suppress AP-1 in tumor cells [14]. However, the mechanism of suppression of certain signaling events by plumbagin without affecting other activation signals in T cells is not completely understood. These results also indicate that plumbagin has specific targets in cells which differ from the targets of pharmacological drugs like rapamycin, FK506, and cyclosporine which inhibit mTOR and calcineurin, respectively. Most quinones are known to mediate their cellular effects through redox cycling and modulation of cellular thiols [275]. The modulation of thiol groups on proteins could be in terms of direct reaction with plumbagin or glutathionylation of proteins or formation of disulfide bridge between proteins (Scheme 5.2). Our results for the first time demonstrate that plumbagin indeed interacts with and forms an adduct with GSH (Fig. 3.9). Absorption

spectroscopy and HPLC clearly indicated that plumbagin interacts with GSH and forms a stable adduct having a different molecular weight (Fig 3.9a and b). This was further confirmed using LC-MS analysis, which revealed that the product of interaction between plumbagin (m/z=188) with GSH (m/z=306) was plumbagin–GSH adduct (m/z=494) (Fig 3.9c).

Cell free system

Plumbagin + G-SH → Plumbagin-S-G

Plumbagin-SG + Plumbagin-SG → G-S-S-G

In live cells

Direct interactions

Plumbagin + G-SH → Plumbagin-SG

Plumbagin-S-G + Plumbagin-S-G → G-S-S-G

Plumbagin + Protein-SH → Plumbagin-S-Protein

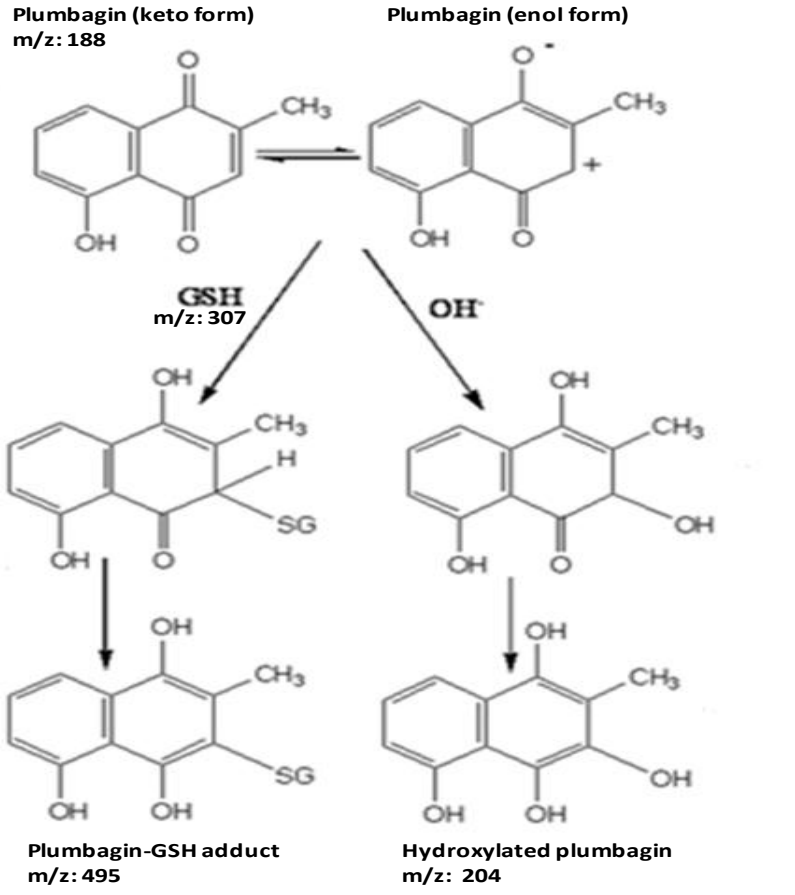
Plumbagin-S-Protein + G-SH → Protein-S-S-G

ROS mediated interactions

Protein-SH+ROS → Protein-S^{-}

G-SH + ROS → G-S-S-G / G-$S^{\bullet}$ / G-S-OH

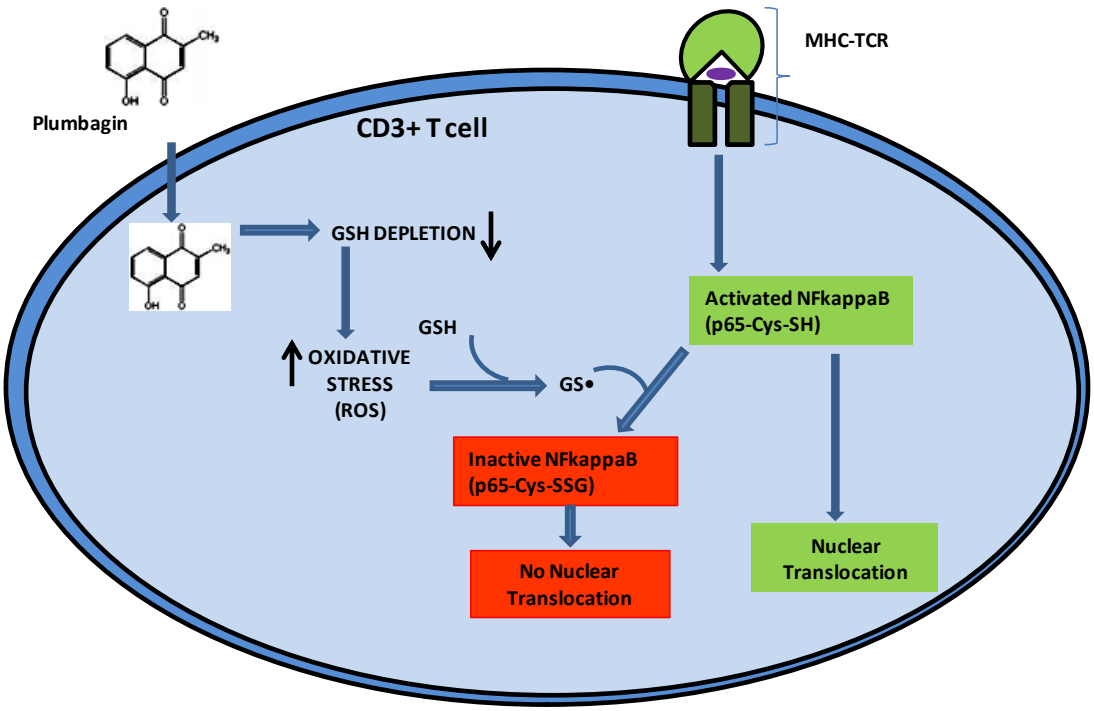
Protein-S^{-} + G-S-S-G / G-$S^{\bullet}$ / G-S-OH → P-S-S-G



Scheme 5.2: Possible chemical reactions and products of reaction of plumbagin with GSH and protein thiols.

Further, we also showed that plumbagin modulates thiol groups present on the proteins as evinced from decrease in iodoacetamide reactive thiol groups of the proteins (Fig. 3.10a). The results using biotin-glutathione ethyl ester (Bio-GEE) for the first time demonstrated formation of PSSG (protein–glutathione adduct) in plumbagin-treated cells (Fig. 3.10b). Glutathionylation is often considered to be a process that protects sensitive cysteinyl residues from irreversible oxidation and may be preceded by spontaneous reaction of glutathione (GSH) with a corresponding oxidized derivative, for example, S-nitrosyl (–SNO), sulfenic acid (–SOH), or a thiyl radical (GS•) [283]. Reversible posttranslational modifications of proteins by glutathionylation are thought to protect against irreversible oxidation [121]. In addition to this protective effect, glutathionylation also results in protein-specific functional changes during the regulation of signaling mediators. Moreover, transcription factors that function in cell growth, differentiation, and apoptosis appear to be regulated by glutathionylation [284]. Through the introduction of GSH within their DNA-binding sites, glutathionylation also inhibits the DNA-binding activity of c-Jun and NF-κB [118, 119]. Collectively, these data suggest that glutathionylation is a physiologically relevant mechanism for controlling the activation of key signaling pathways. Recent studies have now found that electrophiles can give rise to thiyl radicals and, consequently, propagate protein glutathionylation [285, 286]. These electrophiles cause GSH depletion and then interact with the sulfhydryl residues of proteins to modulate signal transduction [287, 288]. On the other hand, electrophiles, including plumbagin have also been reported to activate NF-E2–related factor-2 (Nrf2) a transcription factor which been shown to control the expression of many thiol-regulating enzymes, including glutathione S-transferase, glutamylcysteine synthetase, and thioredoxin reductase [277, 289, 290, 291]. Earlier reports have shown that IKK and IκBα, whose activation is suppressed by plumbagin in lymphocytes, are regulated by glutathionylation suggesting that NF-κB activation is regulated by redox modulation [292,

[293]. Such reactions have been shown to occur in cells exposed to oxidative stress [240]. NF-κB serves as a prototype for transcription factors whose activity is dependent upon the redox status of protein thiols. A previous study has shown that the NO-mediated S-nitrosylation of a cysteine within the RHD of p65 can function to inhibit the DNA-binding activity of this protein, thereby affecting the transcription of a broad range of inflammatory mediators [294]. The in vitro glutathionylation of p50 has also been shown to inhibit its DNA-binding activity [118]. However a previous study has shown that that the inhibitory effects of plumbagin upon NF-κB were exerted through its ability to directly inhibit the binding of NF-κB to DNA. In this study we demonstrate that apart from directly inhibiting the binding of NF-κB with DNA, plumbagin can also induce protein glutathionylation and the inhibition of nuclear translocation of NF-κB may also be mediated by induction of p65 glutathionylation by plumbagin (Fig 3.11) which may be the potential mechanism underlying the inhibition of NF-κB (Scheme 5.3). Further studies were carried out to demonstrate the immunosuppressant activity of plumbagin in vivo. Lymphocytes isolated from mice injected with plumbagin were observed to be hyporesponsive to mitogenic stimulation as studied in terms of their ability to proliferate and secrete cytokines (Fig 4.1).



Scheme 5.3: Proposed model of the inhibitory effects of plumbagin upon NF-κB activation. Increase in the intracellular ROS levels via GSH depletion promotes p65 glutathionylation, which then inhibits p65 nuclear translocation.

Further, we studied the in vivo anti-inflammatory potential of plumbagin using mouse models of graft-versus-host-disease, allograft transplant and endotoxin induced septic shock. GVHD is a frequent complication of allogenic bone marrow transplant in which the engrafted donor T cells attack the recepients' organs and tissues. Clinically, cyclosporine A and tacrolimus have been used in organ transplantation to prevent graft-versus-host disease [295]. However, these drugs are reported to show undesirable side effects that needs to be overcome before they can be considered completely safe and can be used in other inflammatory disorders and autoimmune disease [296, 297]. Modulation of NF-κB activation pathway leading to suppression of the alloreactive T cells has been shown to have therapeutic potential in the prevention of graft rejection [298]. It was shown that cardiac graft rejection was slower when the transplantation was performed in p50 and p52

deficient mice [299]. Further, the mice expressing transdominant IκBα did not reject transplanted hearts from allogenic donors [300]. The lifelong immunosuppression profoundly reduces the quality of life of BMT recipients as it renders them susceptible to opportunistic infections. A range of stimuli produced during the course of GVHD, like TNF-α and Interleukin-1, lead to phosphorylation of IKK resulting in the degradation of IκBα, allowing the translocation of NF-κB into the nucleus which leads to the activation of a plethora of target genes involved in multiple inflammatory responses. One of the strategies to counter the induction of GVHD is to identify agents that have the potential to specifically target alloreactive T cells whilst being harmless to unstimulated lymphocytes. One of the major highlight of the present study was that plumbagin treatment was not only able to abrogate the symptoms associated with GVHD but also significantly increased the survival of mice receiving allogenic lymphocytes (Fig. 4.2a and b). It is well known that one of the initial events in GVHD is T cell activation followed by cytokine secretion and hence the in vitro and in vivo immunosuppressive and anti-inflammatory properties of plumbagin substantiate its importance in the prevention of acute GVHD. These effects of plumbagin may possibly be attributed to its ability to suppress NF-κB activation in alloreactive T cells.

Due to the strong immunogenicity, skin grafts require potent immunosuppressant drugs to prevent rejection. T-cell play a central role in allograft rejection and infiltration of these cells into grafted organs is believed to be an important component of graft rejection. In addition to infiltration, the cytotoxic differentiation of intragraft CD8+ T cells contributes significantly to rejection of the allograft. Since plumbagin was able to inhibit T cell responses both in vitro and in vivo studies were carried out to elucidate the efficacy of plumbagin to prolong allograft survival in mouse model of skin transplant (Fig.4.3).

Plumbagin was able to significantly prolong the survival of allograft indicating its potent ability in inhibiting immune responses in vivo.

Septic shock is the systemic inflammatory response to infection frequently associated with hypotension, hypoperfusion, tissue injury, and multiple organ failure. Severe sepsis is characterized by an intravascular activation of the host's inflammatory pathways releasing potent inflammatory mediators into the circulation. The LPS (endotoxin) component of the bacterial cell wall is the main causative agent of this toxicity [301, 302]. Experimental models of endotoxic shock have demonstrated that a single injection of LPS into animals can produce changes that are characteristic of the septic shock syndrome in humans. Endotoxin exerts its effect by inducing potent macrophage activation, with the sequential release of pro-inflammatory cytokines such as TNF-α, IL-12, IL-Iβ , IL-6, and IL-8 [303]. The central importance of these cytokines in the pathogenesis of endotoxic shock is suggested by the fact that high circulating levels of these cytokines can be found in the serum of both humans and animals during endotoxemia, Macrophage can be activated by LPS to overproduce inflammation mediators including nitric oxide (NO), TNF-a, ET-1, thromboxaneA2 and ROS [304,305]. The mitogen activated protein kinase (MAPK) pathway in macrophages is one of the most extensively investigated intracellular signaling cascades involved in LPS-induced pro-inflammatory responses. This pathway includes extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and c-Jun N-terminal kinase (JNK) [306, 307]. MAPK activation leads to the activation of transcription factors such as NF-κB or AP-1 in macrophages. NF-κB, which is a major transcription factor, is largely involved in the expression of pro-inflammatory genes. We had observed that plumbagin inhibited LPS induced activation and release of inflammatory mediators nitric oxide, Cox-2, TNF-α and IL-6 by RAW cells (Fig 2.17b – e) and IL-1β, TNF-α and IL-6 in splenic adherent macrophages (Fig 2.18a - c). Further we

observed that similar to that observed in lymphocytes plumbagin modulated the redox status of RAW cells. It increased the basal ROS levels and depleted GSH levels in these cells indicating a similar mechanism of action of plumbagin in macrophages as compared to lymphocytes (Fig 3.12a and b). Plumbagin also inhibited the activation of MAPKinase and NF-κB pathway in RAW cells (Fig 3.12 c and d). Based on these observations we hypothesized that plumbagin may be able to abrogate LPS induced endotoxic shock and rescue mice from lethal septic shock. Indeed, we observed that plumbagin was able to recue mice from lethal LPS-induced septic shock (Fig 4.4). Detailed mechanistic studies revealed that plumbagin administration to mice abrogated LPS induced increase in the serum levels of nitric oxide, tumor necrosis factor-α and IL-12, IL-6, IL-10 and IFN-γ which are central mediators of endotoxin induced mortality (Fig 4.5 and 4.6). In agreement with these results, LPS induced increase in the serum levels of serum aspartate aminotransferase, alanine aminotransferase and blood urea nitrogen were also inhibited by plumbagin (Fig 4.8). Plumbagin administration to mice also inhibited LPS induced activation of NF-κB and ERK in liver (Fig 4.9). These results clearly demonstrate the in vivo anti-inflammatory efficacy of plumbagin in ameliorating inflammatory disorders.

In conclusion, in the present study, we have shown the novel anti-inflammatory and immunosuppressive effects of plumbagin in vitro and in vivo. These results highlight a potential application of plumbagin as an immunosuppressive agent which may be used in the treatment of inflammatory disorders. This study also showed a ROS-independent mechanism of anti-inflammatory action of plumbagin. Modulation of cellular thiols played a more significant role than increased ROS levels in biological actions of plumbagin. Any possible therapeutic use of plumbagin for amelioration of inflammation or immunosuppression would require inhibition of effector functions in activated lymphocytes. The use of a phytochemicals with a favorable toxicity profile is particularly

helpful in chronic inflammatory conditions such as autoimmune diseases where protracted use of traditional immunosuppressants such as corticosteroids and cytotoxic agents (methotrexate, azathioprine, cyclosporine, etc.) is associated with cumulative long-term toxicities. Thus, plumbagin may find potential clinical application as an anti-inflammatory or immunosuppressive agent for the treatment of disease conditions mediated by activated and memory lymphocytes, even when used after the triggering event has occurred. For the first time, evidence for a role for glutathionylation of cellular proteins as a mechanism of anti-proliferative action of plumbagin is provided. Further, mechanistic basis for potential therapeutic application of plumbagin as an immunosuppressive or anti-inflammatory drug is highlighted. In this regard, plumbagin-regulated glutathionylation is likely to be an important mechanism by which inflammatory pathways and signaling mediators are modulated and, thus, may also provide new insights into novel anti-inflammatory strategies.

5.2 CONCLUSIONS

The major conclusions drawn from this study are:

1. Plumbagin inhibited mitogen induced lymphocyte proliferation and cytokine secretion by splenic lymphocytes and B cells.
2. Plumbagin did not induce cell death in splenic lymphocytes and induces cell cycle arrest in G1 phase of the cell cycle.
3. Plumbagin inhibited anti-CD3/CD28 induced proliferation and cytokine secretion by CD4+ T cells and CD8+ T cells.
4. Plumbagin also inhibited upregulation of activation markers and co-stimulatory molecules involved in T cell and B cell activation.
5. Plumbagin inhibited LPS induced nitric oxide production and cytokine secretion by RAW cells and splenic adherent macrophages.
6. Plumbagin treatment increased basal ROS levels and depleted GSH levels in lymphocytes leading to an alteration in the redox status of the cells.
7. Immunosuppressive effects of plumbagin were mediated via a novel redox dependent mechanism which is independent of ROS but dependent on GSH.
8. Anti-inflammatory effects of plumbagin were mediated by inhibition of NF-κB and MAPKinase activation in lymphocytes.
9. Plumbagin interacted with GSH and formed an adduct.
10. Suppressive effects of plumbagin on mitogen-induced signaling events were sensitive to GSH.
11. Plumbagin interacted with free thiol groups present on proteins and also induced protein S-glutathionylation in lymphocytes.
12. Plumbagin induced glutathionylation of P65 subunit of NF-κB in lymphocytes.

13. Plumbagin modulated cellular redox status in RAW cells and inhibited LPS induced MAPKinase and NF-κB activation in these cells.
14. Lymphocytes from plumbagin treated mice showed decreased responsiveness to Con A stimulation.
15. Treatment of lymphocytes with plumbagin prior to allogenic transplantation delayed the induction of GVHD in mice.
16. Plumbagin administration significantly delayed rejection of allograft in mice.
17. Plumbagin rescued mice from endotoxin shock induced mortality and morbidity.
18. Plumbagin administration abrogated LPS induced increase in the serum nitric oxide, cytokine, blood urea nitrogen, AST and ALT levels. It also inhibited LPS induced increase in the ROS levels in peritoneal macrophages and splenocytes.
19. Plumbagin was able to suppress LPS induced activation of NF-κB and MAPKinases activation in liver tissue of mice.

5.3 FUTURE PROSPECTS

1. Further mechanistic studies are required to identify other putative molecular targets of plumbagin like Nrf-2 and PPARγ which are known to play a crucial role in regulating immune responses.
2. Role of interactions between NF-κB and Nrf-2 during inflammatory responses needs to be studied
3. As an extension of these studies carried out using mouse models, the effect of plumbagin on dendritic cells needs to be studied and the mechanism of action needs to be elucidated.
4. Further studies are required to identify the detailed mechanism of plumbagin induced glutathionylation of cellular proteins and to identify other proteins that may be getting glutathionylated following plumbagin treatment.
5. Since plumbagin induces cell cycle arrest and is anti-inflammatory its ability to protect lymphocytes against radiation induced cell death needs to be explored.

CHAPTER - VI

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macrophages stimulated with lipopolysaccharide and interferon-gamma. J Infect Dis 1999;179:939-944.

ANNEXURE

1. Trypan blue dye solution:

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

Trypan Blue 0.5g+ NaCl 0.9g+ 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 0.9g+ Na_2HPO_4 0.126 g+ NaH_2PO_4 0.0451g+ Distilled Water 100ml

5. Tris–Borate EDTA (5X):

TRIZMA base 54g+ Boric acid 27.5g+0.5M EDTA 20ml (pH: 8.0)+ Distilled Water 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 + H3PO4 1.25ml+ Distilled Water 50ml

8. Neutral Buffered Formalin For Tissue Fixation:

NaH2PO4 4g+ Na2HPO4 6.5g+ 40% Formaldehyde 100ml+ Distilled Water 900ml

9. Coating Buffer for ELISA:

(0.1 M Sodium Carbonate): NaHCO3 8.4g+ Na2Co3 3.56g+ 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. Store at 4^0 C.

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 4^0 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 at the time of use. Store at – 20^0C.

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 4^0C.

1X transfer buffer for Western Blot-2000ml

20% v/v methanol in DW:400 ml

DW:1200 ml

Use immediately.

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 4^0C.

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 4^0C.

26. Protease Inhibitors

1M NaF	-10 µl
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 -20^0C.

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

Publications related to Ph.D. Work

1: **Checker Rahul**, Sharma D, Sandur SK, Subrahmanyam G, Krishnan S, Poduval TB, Sainis KB. Plumbagin inhibits proliferative and inflammatory responses of T cells independent of ROS generation but by modulating intracellular thiols. J Cell Biochem. 2010 Aug 1;110(5):1082-93.

2: **Checker Rahul**, Sharma D, Sandur SK, Khanam S, Poduval TB. Anti-inflammatory effects of plumbagin are mediated by inhibition of NF-kappaB activation in lymphocytes. Int Immunopharmacol. 2009 Jul;9(7-8):949-58.

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Anti-inflammatory effects of plumbagin are mediated by inhibition of NF-kappaB activation in lymphocytes

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ABSTRACT

Plumbagin (5-hydroxy-2-methyl-1, 4-naphthoquinone), a quinone isolated from the roots of *Plumbago zeylanica* was recently reported to suppress the activation of NF-κB in tumor cells. NF-κB, a ubiquitous transcription factor, plays a central role in regulating diverse processes in leukocytes like cellular proliferation, expression of immunoregulatory genes and apoptosis during innate and adaptive immune responses. Consequently, plumbagin might affect the biological functions of leukocytes participating in various immune responses. The present report describes novel immunomodulatory effects of plumbagin. Plumbagin inhibited T cell proliferation in response to polyclonal mitogen Concanavalin A (Con A) by blocking cell cycle progression. It also suppressed expression of early and late activation markers CD69 and CD25 respectively, in activated T cells. At these immunosuppressive doses (up to 5 μM), plumbagin did not reduce the viability of lymphocytes. Further, the inhibition of T cell proliferation by plumbagin was accompanied by a decrease in the levels of Con A induced IL-2, IL-4, IL-6 and IFN-γ cytokines. Similar immunosuppressive effects of plumbagin on cytokine levels were seen in vivo. To characterize the mechanism of inhibitory action of plumbagin, the mitogen induced IκB-α degradation and nuclear translocation of NF-κB was studied in lymphocytes. Plumbagin completely inhibited Con A induced IκB-α degradation and NF-κB activation. Further, plumbagin prevented Graft Versus Host Disease-induced mortality in mice. To our knowledge this is the first report showing the immunomodulatory effects of plumbagin in lymphocytes via modulation of NF-κB activation.

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1. Introduction

For management of inflammation and survival of allograft, a broad range of immunosuppressive drugs like calcineurin inhibitors (cyclosporine A), adjuvants (azathioprine, mycophenolate mofetil, sirolimus) and steroids have been used [1]. These drugs, however, have undesirable side effects like metabolic derangements, toxicities, development of infections and cancers. One of the major complications in patients receiving repeated blood transplantation is Graft-versus-host disease (GVHD) which is characterized by undesired immune activation and proinflammatory cytokine production leading to tissue destruction. The objective remains to identify novel agents that can be used in combination with the current drugs to optimize treatment for acute inflammation and prolong graft survival while limiting the side effects.

Phytochemicals derived from traditional medicine have shown promising results as immunosupressors and immunomodulators [2]. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), a quinone found in the plants of Droseraceae, Plumbaginaceae, Ancestrocladaceae and Dioncophyllaceae families, has been shown to possess potent anti-tumor activity [3]. It has been shown to inhibit the growth of Raji, Calu-1, HeLa and Wish cell lines in vitro [4]. A recent report showed that plumbagin induced cell cycle arrest and apoptosis in human melanoma A375.S2 cells through ROS/JNK pathway [5]. Further, plumbagin exhibited chemotherapeutic potential in BRCA1 mutated/defective ER-positive cancers [6]. Apart from its anti-cancer properties plumbagin has also been shown to act as a radio sensitizer in mouse Ehrlich melanoma cells [7].

Plumbagin has been shown to augment the bactericidal activity of macrophages at low concentrations whereas it had inhibitory effects at higher concentrations [8]. A recent randomized double blind study using plant based formulation containing plumbagin as one of the active components showed positive results in the management of chronic obstructive pulmonary disease in humans [9]. However, there are no reports on the immunomodulatory effects of plumbagin in lymphocytes. A thorough examination of the immunomodulatory properties of plumbagin will facilitate its use for application in human clinical trials.

It is well known that transcription factor NF-κB is central to a series of cellular processes like inflammation, cell proliferation and apoptosis and

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is of particular importance in modulating the expression of immunoregulatory genes [10]. Lately, plumbagin was shown to inhibit constitutive as well as inducible NF-κB activation and NF-κB regulated genes in tumor cells. It also inhibited DNA binding ability of NF-κB [11]. Several studies have shown that inhibition of NF-κB activation is a relevant strategy for alleviation of GVHD induced tissue damage [12,13]. It was shown that cardiac graft rejection was slower when the transplantation was performed in p50 and p52 deficient mice [14]. Further, the mice expressing transdominant IκB-α did not reject transplanted hearts from allogenic donors [15]. Based on these observations, we speculated that plumbagin may show immunomodulatory effects and might have significant clinical application in prevention of acute GVHD. To test this hypothesis, the immunomodulatory effects of plumbagin were studied in murine lymphocytes in vitro and in vivo and its ability to prevent induction of acute GVHD was tested in a mouse model.

2. Materials and methods

2.1. Chemicals

Plumbagin, RPMI 1640 medium, HEPES, ethylenediaminetetraacetic (EDTA), ethylene glycol tetraacetic acid (EGTA), phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, benzamidine, dithiothreitol (DTT), Nonidet P-40, polyethylene glycol (PEG) and propidium Iodide (PI) were purchased from Sigma Chemical Co. (USA). Carboxy fluorescein diacetate succinimidyl ester (CFSE) was procured from Molecular Probes, The Netherlands. Fetal calf serum (FCS) was obtained from GIBCO BRL. Concanavalin A was purchased from Calbiochem, USA. ELISA sets for detection of cytokines (IL-2, IL-4, IL-6 and IFN-γ) and fluorescently labeled antibodies and respective isotype controls were procured from BD Pharmingen (USA). Antibodies against IκB-α and β-actin were obtained from Cell Signaling Technologies (USA). CyQUANT cell proliferation assay kit was purchased from Molecular Probes, Invitrogen. All other chemicals were purchased from reputed local manufacturers.

2.2. Animal maintenance

Six to eight week old inbred Swiss male mice, weighing approximately 20–25 g, reared in the animal house of Bhabha Atomic Research Centre were used. They were housed at 23 ± 3 °C with a 12/12 hour light/dark cycle and were given mouse chow and water ad libitum. The guidelines issued by the Institutional Animal Ethics Committee of Bhabha Atomic Research Centre, Government of India, regarding the maintenance and dissections of small animals were strictly followed.

2.3. Treatment with plumbagin

A 100 mM solution of plumbagin was prepared in dimethyl sulfoxide (DMSO), stored as small aliquots at −20 °C, and then diluted as needed in cell culture medium. In all in vitro experiments, cells were treated with plumbagin for 4 h in 2-mercapto ethanol (ME) free RPMI medium and were further stimulated with Con A (10 μg/ml) without washing the cells. DMSO was used as vehicle control in vitro. In all in vivo experiments, mice were injected i.p with plumbagin in 25% PEG (2 mg/kg body weight) in a 0.2 ml volume. The mice in control group were treated with an equal volume of vehicle (25% PEG in saline).

2.4. Proliferation assay

Splenocytes were obtained by squeezing the spleen through a nylon mesh in a petri plate containing RPMI medium. The RBC were lysed by brief hypotonic shock. Splenocytes were stained with CFSE (20 μM, 5 min, 37 °C) and washed three times using ice-cold RPMI medium containing 10% FCS, 100 IU/ml penicillin and 100 mg/ml streptomycin (complete medium, CM). Two million splenocytes were treated with plumbagin (50 nM to 5 μM, 4 h) and were stimulated with Con A (10 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 flowcytometer (BD FACSAria). Percent daughter cells that showed a decrease in CFSE fluorescence intensity were calculated using BD FACSDiva software and were expressed as daughter cells.

For ex vivo assay of proliferation, splenocytes were isolated from vehicle or plumbagin treated mice (2 mg/kg body weight, 24 h). Two million splenocytes were stimulated with Con A (10 μg/ml) in 2 ml RPMI WITH 10% FCS for 72 h at 37 °C. Proliferation was estimated from the change in the total DNA content in each well using CyQuant assay (CyQUANT NF Cell Proliferation Assay Kit) according to manufacturer's protocol. Fluorescence signals were read from a 96-well plate using a plate reader (Fluostar Optima, BMG Labtech) with excitation at 485 nm and emission at 530 nm. Results were expressed as mean fluorescence intensity ± SEM (four replicates per experiment).

2.5. Estimation of cell cycle and apoptosis

The percentage of cells in different phases of cell cycle (G1, S + G2/M) and percentage of apoptotic cells was estimated by flowcytometry. For cell cycle analysis splenocytes were treated with plumbagin (50 nM to 5 μM, 4 h) and stimulated with Con A (10 μg/ml) for 72 h at 37 °C in RPMI medium supplemented with 10% FCS. Vehicle treated cells served as a control. To study the effect of plumbagin on cell viability, two million splenocytes were treated with plumbagin (5 μM) for 24 h and 72 h at 37 °C in 2 ml RPMI medium supplemented with 10% FCS. Vehicle treated cells served as a control. At the end of incubation period cells were washed with PBS and incubated with 1 ml of staining solution (0.5 μg/ml propidium iodide, 10 μg/ml ribonuclease A, 0.1% sodium citrate and 0.1% Triton X-100) overnight [2]. A total of 20,000 cells were acquired in Partec PAS III flow cytometer and analyzed using FloMax® software. The pre G_1 population represented the apoptotic cells. Undivided cells were in G_1 phase of cell cycle (2n DNA content). The population showing more than 2n DNA represented cells in S + G2/M phase of cell cycle. RN1, RN2 and RN3 in flowcytometric histograms stand for Region 1 (hypodiploid/apoptotic cells), Region 2 (cells in G0/G1 phases of cell cycle) and Region 3 (Cells in S/G2/M phases of cell cycle) respectively.

2.6. Measurement of cytokine secretion

The concentration of IL-2, IL-4 and IFN-γ and IL-6 in the supernatant of control vehicle treated cells and cells stimulated with Con A (10 μg/ml) for 24 h after plumbagin (50 nM to 5 μM, 4 h) treatment was estimated using cytokine ELISA sets (BD Pharmingen, USA).

2.7. Antibody staining

Splenocytes were treated with plumbagin (5 μM and 2.5 μM, 4 h) and were further stimulated with Con A (10 μg/ml). After 24 h, cells (0.5×10^6) were stained with PE conjugated CD25 antibody or CD69 antibody and 20,000 cells in each group were acquired in a flowcytometer (Partec PAS III) [16]. The frequency of CD25+ and CD69+ cells in each treatment group was determined using FLOMAX ® software.

2.8. Western blot analysis

Splenocytes were treated with plumbagin (5 μM, 4 h) and were stimulated with Con A (10 μg/ml) for 1 h at 37 °C and cytosolic extract prepared as explained in Sandur et al. [11]. Vehicle treated cells served as a control. Briefly, the cells were washed with ice-cold phosphate-buffered saline and suspended in 0.1 ml lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.5 mg/ml

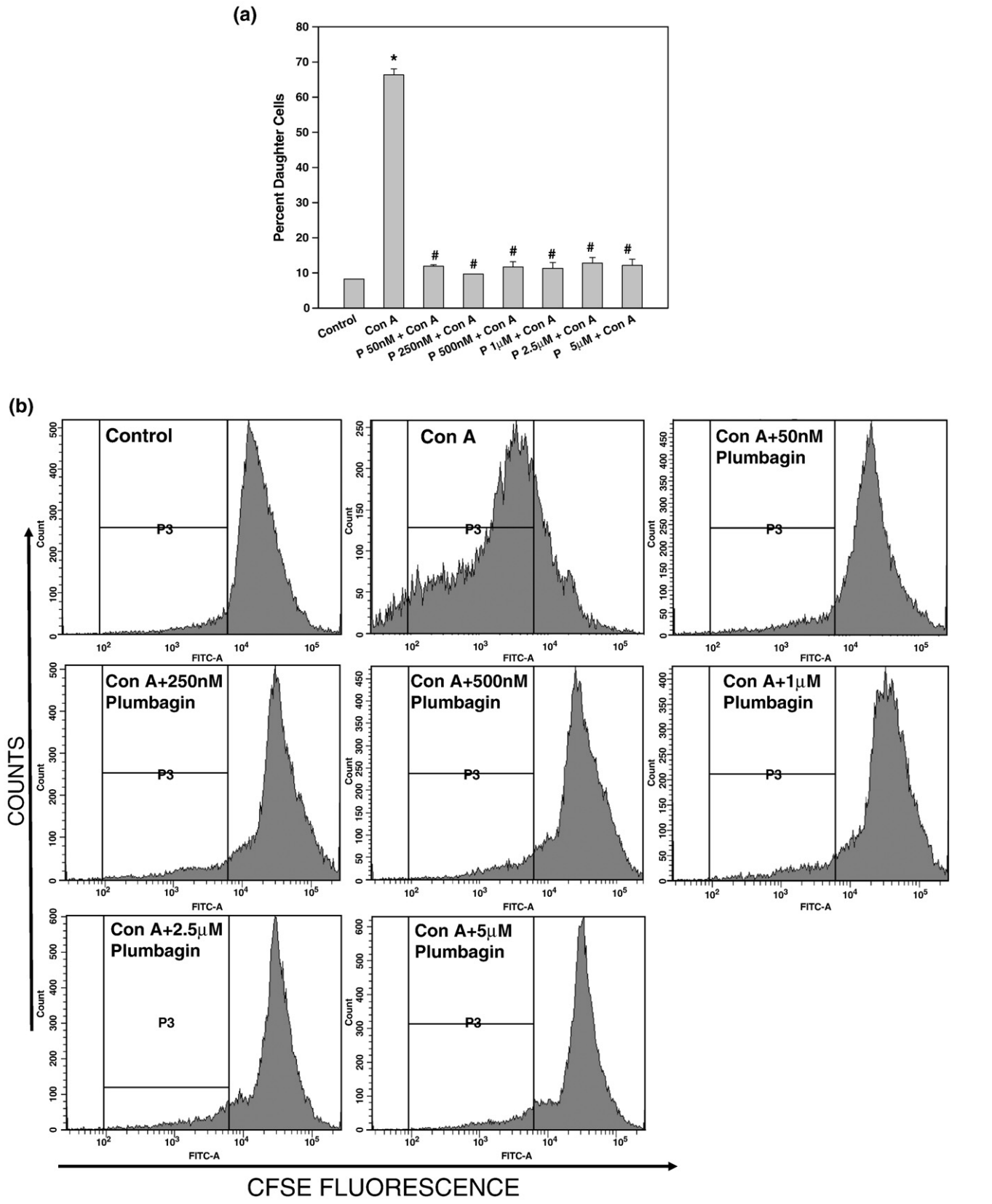

Fig. 1. Effect of plumbagin on Con A induced T cell proliferation in vitro: CFSE labeled lymphocytes were treated with plumbagin (50 nM to 5 μM, 4 h) and stimulated with the Con A (10 μg/ml) at 37 °C in complete medium for 72 h. Twenty thousand cells were acquired in a flowcytometer. Vehicle treated cells served as control. Percent daughter cells were calculated from decrease in mean fluorescence intensity. (a) Each bar represents percentage of daughter cells in each treatment group. Each bar represents mean ± S.E.M. from three replicates and two such experiments were carried out. *$p<0.01$, as compared to vehicle treated cells and #$p<0.01$, as compared to Con A stimulated cells. (b) Representative flowcytometric histograms of CFSE labeled cells representing plumbagin induced suppression of Con A induced proliferation.

benzamidine). The cells were allowed to swell on ice for 15 min, after which 25 µl of 10% nonidet P-40 was added and tubes were vortexed. The supernatants containing proteins from cytosolic fraction were collected by centrifuging the cells at 8000 rpm for 6 min at 4 °C. The pellet was suspended in nuclear extraction buffer for performing EMSA as described below. Protein estimation was carried out by Bradford method using Bio-Rad Protein Assay Kit (Cat No 500-0006). Equal amounts of protein (30 µg) were resolved by SDS-PAGE (10%) and transferred to nitro cellulose membrane. After the membrane was blocked in 5% nonfat powdered milk, the membrane was incubated overnight with the primary antibody specific to IκB-α (1:1000 dilution), washed three times with Tris-buffer saline containing 0.05% Tween 20 (TBST) and further incubated with horseradish peroxidase-labeled secondary antibody for 1 h. The membranes were washed, and specific bands were visualized on X-ray films using Enhanced Chemiluminiscence Kit (Roche, Germany). The membrane was stripped and reprobed with actin β antibody.

2.9. Electrophoretic mobility shift assay

The splenocytes were treated with plumbagin (5 µM, 4 h) and were stimulated with Con A (10 µg/ml) for 1 h at 37 °C and nuclear extracts were prepared [11]. The nuclear pellets were resuspended in 25 µl of ice-cold nuclear extraction buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2.0 pg/ml leupeptin, 2.0 µg/ml aprotinin, and 0.5 mg/ml benzamidine), and the tubes were incubated on ice for 15 min with intermittent agitation. This nuclear extract was microcentrifuged for 5 min at 12,000 rpm, and the supernatant was collected in fresh tubes and frozen at −70 °C. EMSA was performed by incubating 15 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′-TTGTTA-CAA**GGGACTTTCC**GCTG**GGGACTTTCC**AGGGAGGCGTGG-3′; boldface indicates NF-κB binding sites) in the presence of 0.5 µg of poly (2′-deoxyinosinic-2′-deoxycytidylic acid) (poly (dI–dC)) in binding buffer (25 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.5 mM dithiothreitol, 1% Nonidet P 40, 5% glycerol, and 50 mM NaCl) for 30 min at 37 °C. Poly (dI–dC) is a synthetic polynucleotide used as a nonspecific competitor DNA to reduce the background. The DNA–protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels using buffer containing 50 mM Tris, 200 mM glycine, and 1 mM EDTA, pH 8.5. The dried gel was exposed to phosphorimage plate and the radioactive bands were visualized using a PhosphorImage plate scanner (Amersham Biosciences, USA).

2.10. Induction of Graft Versus Host Disease (GVHD)

Swiss mice were exposed to 600 cGy whole body gamma-radiation (WBI) (Gamma Cell 220, AECL Canada). To induce GVHD in immuno-compromised Swiss mice, 10×10^6 splenocytes from C57BL/6 donors were injected i.v. 48 h after irradiation. Each mice in control group received vehicle treated splenocytes whereas each mice in the plumbagin group received splenocytes treated with 1 µM plumbagin for 4 h. The recipient mice were monitored daily to assess the signs of GVHD. In total, 10 recipient mice in control group and 10 recipient mice in the plumbagin treated group were evaluated. GVHD became evident from rapid and sustained weight loss as well as from features such as hunchback, diarrhoea, hair loss and death.

2.11. Statistical analysis

The statistical analysis was done using ANOVA with Microcal Origin 6.0 software followed by post-hoc analysis using Schiffe's test. * refers to $p<0.01$, as compared to vehicle treated control and # refers to $p<0.01$, as compared to Con A stimulated cells.

3. Results

3.1. Plumbagin inhibited Con A induced T cell proliferation

The effect of plumbagin on Con A induced lymphocyte proliferation as assessed by CFSE dye dilution is shown in Fig. 1a. The bars represent percentage of daughter cells obtained after 72 h. Plumbagin completely inhibited the Con A (10 µg/ml)-induced proliferation of

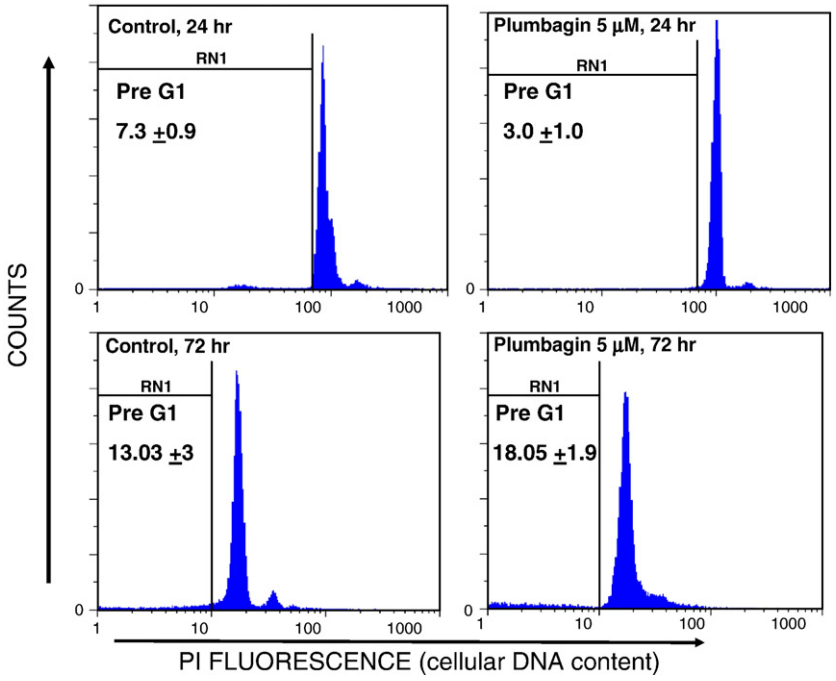

Fig. 2. Effect of plumbagin on unstimulated lymphocytes: Lymphocytes were treated with plumbagin (5 µM) at 37 °C in complete medium for 24 and 72 h after which the cells were stained with propidium iodide solution and twenty thousand cells were acquired in a flowcytometer. Vehicle treated cells served as control. Percentage apoptosis (pre-G1 peak) in lymphocytes was estimated and is shown in the histograms. Two such independent experiments were carried out and results from one such experiment are shown. Data points represent mean ± S.E.M. from three replicates and two such experiments were carried out. RN1 in flowcytometric histograms stands for Region 1 (hypodiploid/apoptotic cells).

lymphocytes. Majority of the plumbagin treated cells failed to enter cell division cycle as shown by lack of decrease in the CFSE fluorescence intensity in flowcytometric histograms (Fig. 1b). The inhibition of T cell proliferation by plumbagin was not due to cytotoxicity because lymphocytes incubated with plumbagin (5 μM) for 24 h and 72 h did not show any loss of viability as measured by trypan blue dye exclusion (data not shown) and also did not show any increase in apoptosis (preG1 peak) as compared to that in control cells as estimated by PI staining (Fig. 2).

3.2. Plumbagin inhibited Con A induced cell cycle progression in lymphocytes

Fig. 3a shows the effect of plumbagin on cell cycle progression in Con A stimulated lymphocytes as assessed by PI staining. The bars represent the percentage of cells in different phases of cell cycle 72 h after stimulation with Con A (10 μg/ml). The fraction of cells in S + G2/M phase of cell cycle in plumbagin treated lymphocytes stimulated with Con A was significantly lower than that in lymphocytes stimulated with Con A alone (Fig. 3b). Also percentage of cells in G1 phase of cell cycle in plumbagin treated lymphocytes stimulated with Con A was significantly higher than that in lymphocytes stimulated with Con A alone indicating that plumbagin induced cell cycle arrest at G1 stage in activated T cells (Fig. 3b).

3.3. Plumbagin inhibited Con A induced cytokine production

Fig. 4 shows the secretion of IL-2 (Fig. 4a), IL-4 (Fig. 4b), IL-6 (Fig. 4c) and IFN-γ (Fig. 4d) cytokines in plumbagin (50 nM, 1 μM and

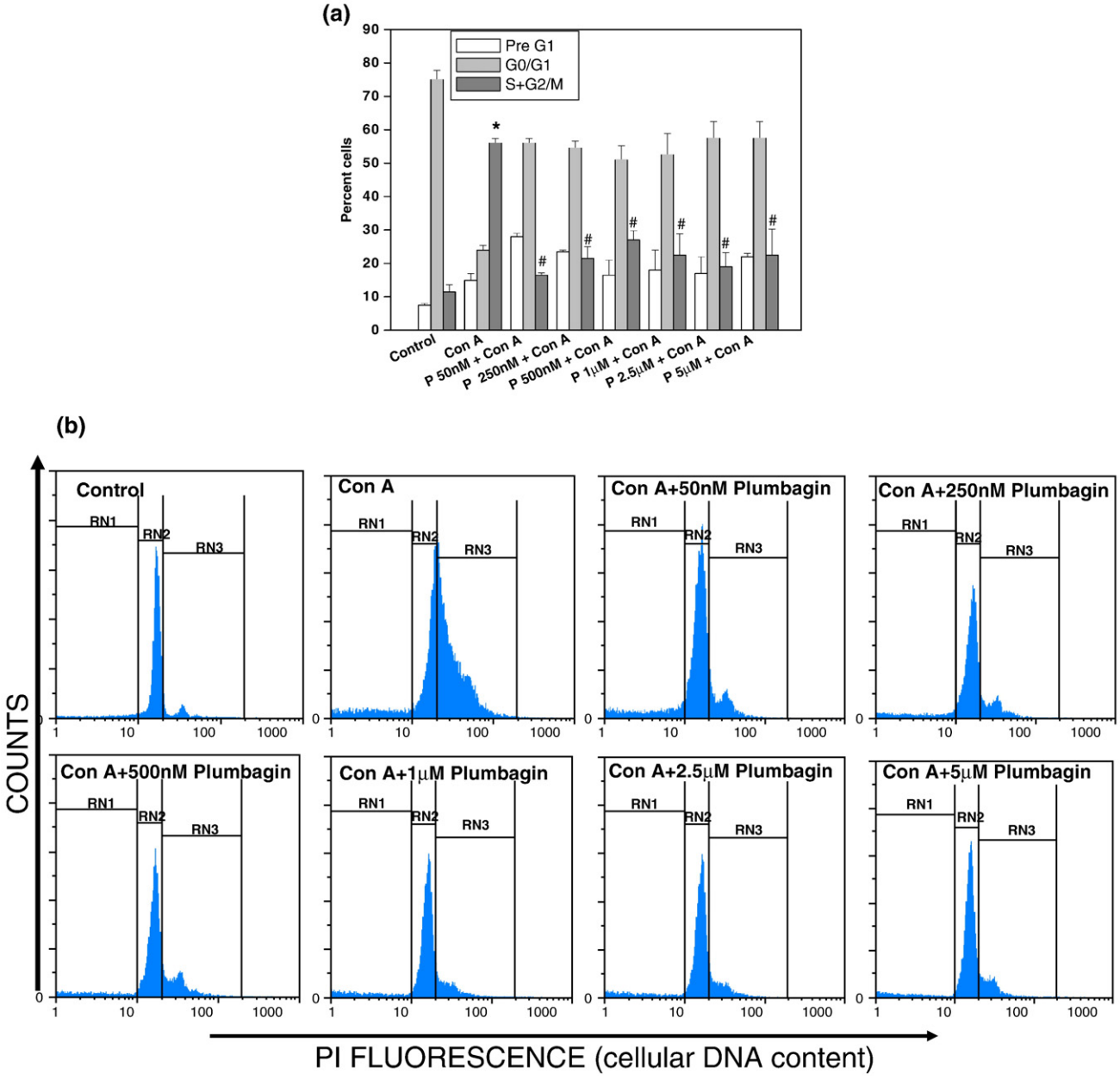

Fig. 3. Effect of plumbagin on Con A induced cell cycle progression: Lymphocytes were treated with plumbagin (50 nM to 5 μM, 4 h) and stimulated with the Con A (10 μg/ml) at 37 °C in complete medium for 72 h. The cells were stained with propidium iodide and twenty thousand cells were acquired in a flowcytometer. Vehicle treated cells served as control. (a) The hollow bars represent percentage of cells containing less than 2n DNA (sub-G1/apoptotic cells), light gray bars show cells containing 2n DNA (in G1 phase) and the dark gray bars indicate the cells containing more than 2n DNA (in S + G2/M phase). Each bar represents mean ± S.E.M. from three replicates and two such experiments were carried out. *$p<0.01$, as compared to vehicle treated cells and #$p<0.01$, as compared to Con A stimulated cells. (b) Representative flowcytometric histograms of PI labeled cells representing plumbagin induced inhibition of cell cycle progression. RN1, RN2 and RN3 in flowcytometric histograms stand for Region 1 (hypodiploid/apoptotic cells), Region 2 (cells in G0/G1 phases of cell cycle) and Region 3 (Cells in S/G2/M phases of cell cycle) respectively.

5 μM) treated cells stimulated with Con A (10 μg/ml) as compared to that in the cells stimulated with Con A alone. Con A activated cells showed significantly higher secretion of IL-2, IL-4, IL-6 as well as IFN-γ as compared to that in control vehicle treated cells. Plumbagin treated cells showed significant inhibition of IL-2, IL-6 and IFN-γ production at 50 nM plumbagin. Complete inhibition of IL-2, IL-4, IL-6 and IFN-α production was seen at 1 μM plumbagin (Fig. 4a–d).

3.4. Plumbagin inhibited expression of Con A induced T cell activation markers CD69 and CD25

Fig. 5 shows the expression of early and late T cell activation markers CD69 and CD25 (IL-2R-α) respectively in lymphocytes treated with plumbagin (2.5 and 5 μM, 4 h) and stimulated with Con A (10 μg/ml) as compared to that in the cells stimulated with Con A alone. Con A activated cells showed significantly higher expression of CD69 and CD25 as compared to that in control vehicle treated cells. Plumbagin treatment prior to Con A stimulation leads to a complete inhibition of both CD69 and CD25 expression (Fig. 5a and b).

3.5. Plumbagin suppressed Con A induced NF-κB activation

Fig. 6 shows the effect of plumbagin on Con A induced NF-κB activation in lymphocytes. The Con A (10 μg/ml) stimulated cells showed degradation of IκB-α in the cytosolic fraction and NF-κB activation in the nuclear fraction as compared to that in vehicle treated control cells. However, cells treated with plumbagin and then stimulated with Con A did not show IκB-α degradation (Fig. 6a and b) and NF-κB activation (Fig. 6c).

3.6. Lymphocytes from plumbagin treated mice showed decreased responsiveness to Con A stimulation

Fig. 7 shows the cytokine production and proliferation response of lymphocytes isolated from plumbagin (2 mg/kg body weight, 24 h) treated mice as compared to that from vehicle treated control mice. The lymphocytes from plumbagin treated mice showed a significantly lower production of IL-4, IL-6 and IFN-γ cytokines (Fig. 7a–c) as compared to that in lymphocytes taken from vehicle treated control mice when stimulated with Con A. Interestingly, the Con A induced proliferation response of T cells was also significantly diminished by plumbagin administration as compared to that in cells from control mice (Fig. 7d).

3.7. Treatment of lymphocytes with plumbagin prior to allogenic transplantation delayed the induction of GVHD in mice

To test immunosuppressive activity of plumbagin in GVHD model, splenocytes from C57BL/6J mice were incubated with plumbagin (1 μM, 4 h) and transferred to WBI treated immunocompromised Swiss mice. The mice of vehicle treated group developed GVHD that led to 70% death within 45 days (Fig. 8a), demonstrating typical symptoms of GVHD, including alopecia, scleroderma, hunched posture, diarrhea, and progressive weight loss. However, in the plumbagin treated group 90% of the mice survived in better health

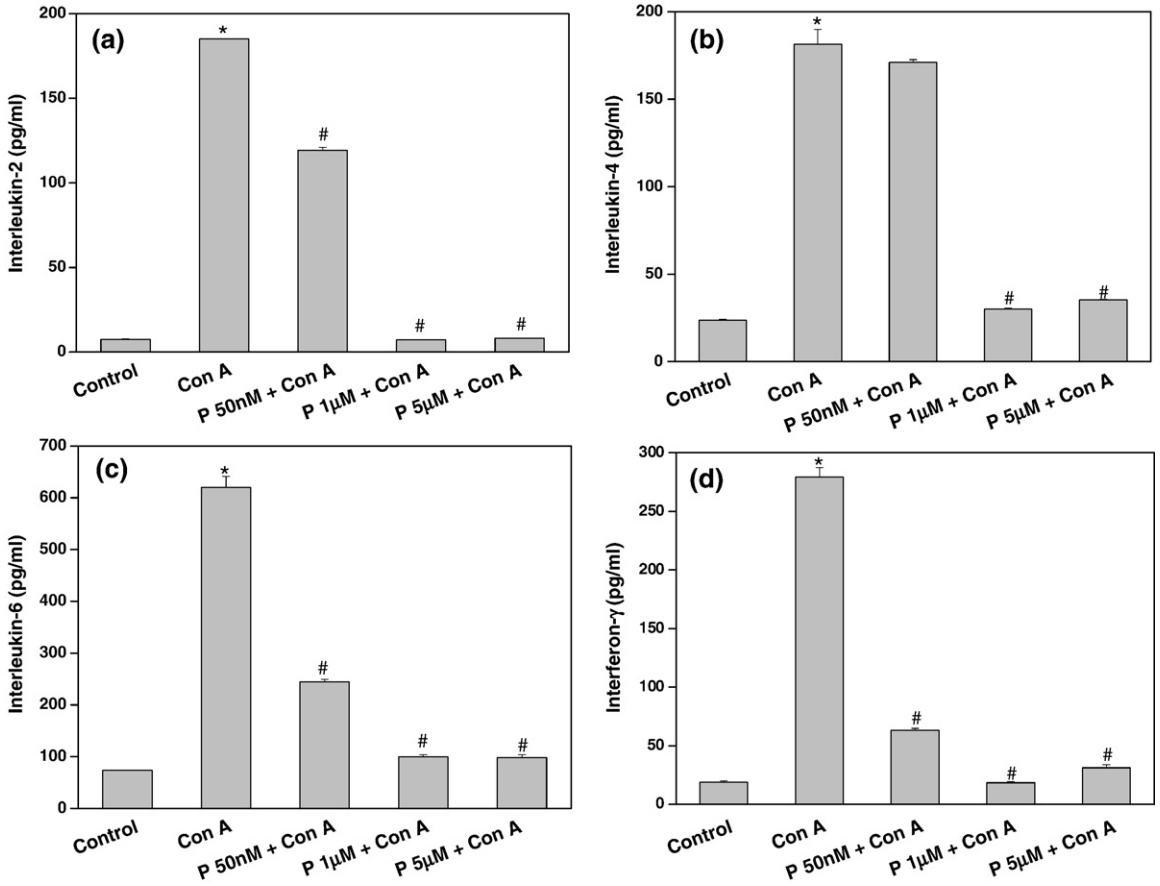

Fig. 4. Effect of plumbagin on Con A induced cytokine production in vitro: Lymphocytes were pre-treated with different concentrations of plumbagin for 4 h before stimulation with Con A (10 μg/ml) for 24 h at 37 °C in complete medium. Vehicle treated cells served as control. The concentration of cytokines in the supernatant was estimated using ELISA. Each bar represents concentration of (a) IL-2 (b) IL-4, (c) IL-6 and (d) IFN-γ. Each bar represents mean ± S.E.M. from three replicates and three 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.

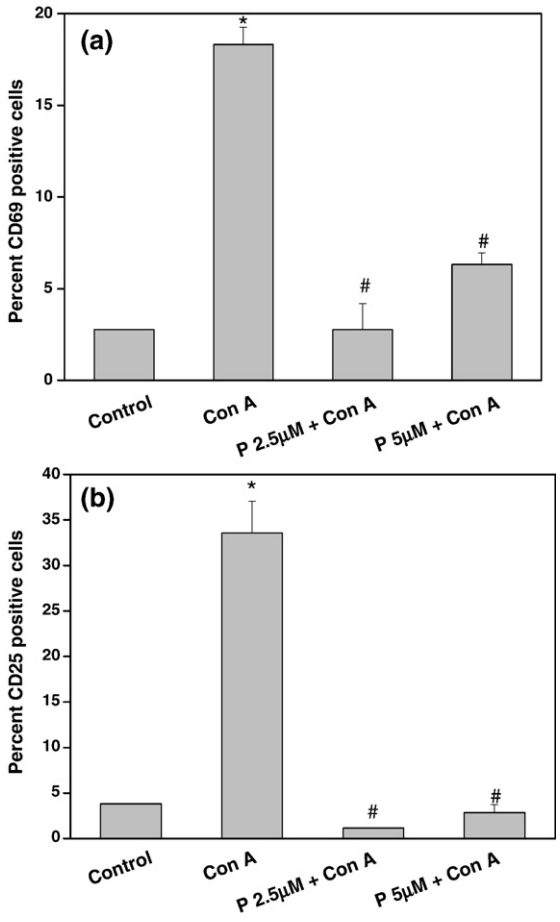

Fig. 5. Effect of plumbagin on CD69 and CD25 expression in Con A stimulated lymphocytes in vitro. Lymphocytes were treated with plumbagin (2.5 and 5 μM, 4 h) and then stimulated with Con A (10 μg/ml) for 24 h at 37 °C in complete medium. Vehicle treated cells served as control. In each group, 0.5×10^6 cells were stained with PE conjugated CD25 antibody or CD69 antibody. Each bar represents frequency of (a) CD69+ cells (b) CD25+ cells. Data points represent mean ± S.E.M. from three replicates and two such experiments were carried out. *$p<0.01$, as compared to vehicle treated cells and #$p<0.01$, as compared to Con A stimulated cells.

for more than 60 days (Fig. 8a). Furthermore, the mice treated with plumbagin experienced inconspicuous weight loss as compared to control group (Fig. 8b). Therefore, plumbagin significantly prevented death and weight loss induced by allo-lymphocyte transfer.

4. Discussion

A recent report suggested that the anti-carcinogenic, apoptotic and radiosensitizing effects of plumbagin could be attributed to its ability to suppress NF-κB activation in tumor cells [11]. Nuclear factor kappa-B plays a critical role in lymphocyte activation, proliferation and survival [17]. Thus, the inhibition of NF-κB activation by plumbagin may modulate functions of lymphocytes and immune responses.

In the present report the immunomodulatory effects of plumbagin were investigated in terms of lymphocyte activation, cytokine production and proliferation. The biochemical mechanisms that may contribute to its immunomodulatory activity were also elucidated. Plumbagin inhibited Con A induced proliferation of T cells, with significant inhibition at 50 nM (Fig. 1a). To our knowledge this is the first report showing the anti-proliferative effects of plumbagin in lymphocytes by inhibition of entry of cells into the S phase of the cell cycle (Fig. 3a). Similar results on inhibitory effects of plumbagin have been reported in tumor cells [4].

T cell activation involves the expression of several surface molecules such as CD25 and CD69 whose transcriptional regulation is dependent on coordinated activation of NFATc and NFκB [18]. Plumbagin completely inhibited the expression of CD25 and CD69 in Con A activated cells (Fig. 5). Similar inhibitory effects have been reported in rapamycin treated lymphocytes stimulated with Con A in vitro [16]. Thus, plumbagin appeared to inhibit an early activation event in T cell stimulation, similar to rapamycin.

IL-2 is the major growth factor for the clonal expansion of activated T cells and plays a crucial role in the progression of antigen activated T cells from G1 to S/G2/M phase of the cell cycle [19]. IFN-γ is an effector cytokine produced by activated TH1 cells and plays an important role in clearance of intracellular pathogens [20] by up-regulating the genes involved in pathogen recognition, antigen

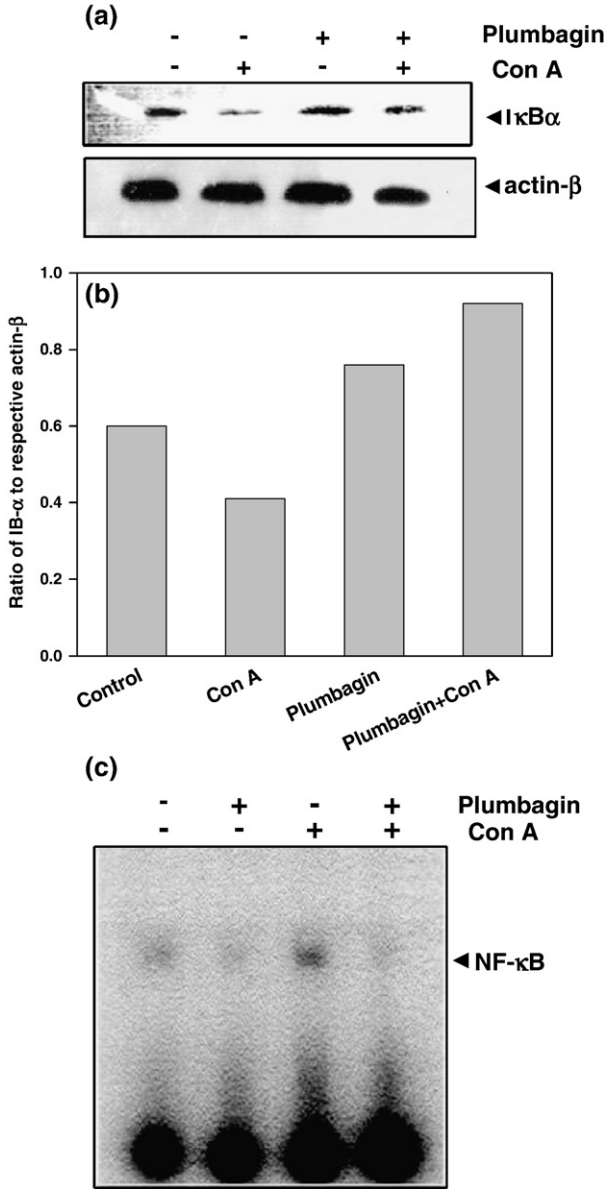

Fig. 6. Effect of plumbagin on Con A induced IκBα degradation and NF-κB activation. Lymphocytes were incubated with 5 μM plumbagin for 4 h and further stimulated with Con A (10 μg/ml) for 1 h. Cytoplasmic extracts were prepared and equal amount of protein in each group was fractionated on 10% SDS-PAGE and electro-transferred onto nitrocellulose membrane. Western blot analysis was performed using IκBα antibody. β-actin expression in each group was used as loading control (a). The ratio of intensity of each band with that of respective β-actin band is shown in (b). Lymphocytes were incubated with 5 μM plumbagin for 4 h and stimulated with Con A (10 μg/ml) for 1 h. The total nuclear proteins were prepared and analysed for NF-κB activation by EMSA (c). Two independent experiments were carried out and representative figure from one such experiment is shown.

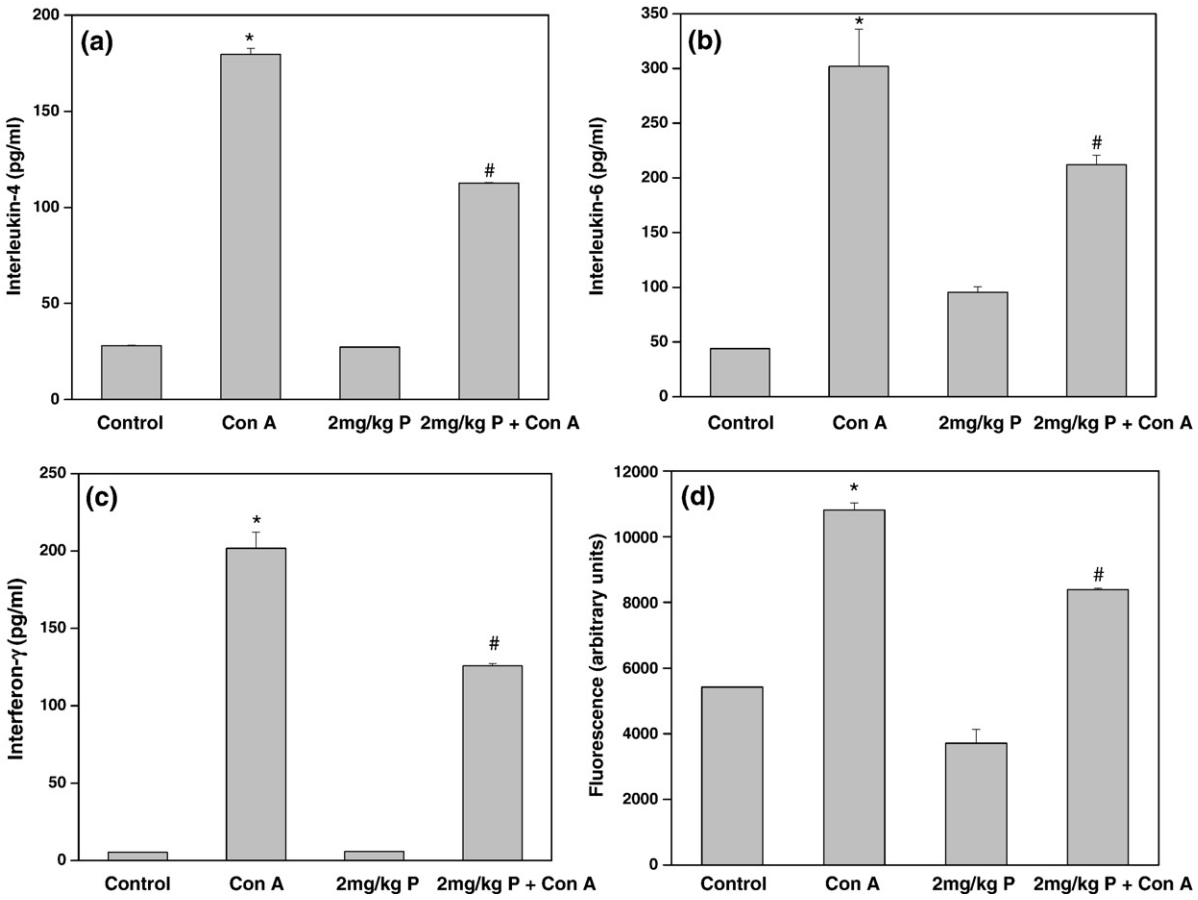

Fig. 7. Effect of plumbagin treatment on Con A induced lymphocyte proliferation and cytokine secretion in vivo. Mice were injected i.p with plumbagin in 25% PEG (2 mg/kg body weight). The mice in control group were treated with an equal volume of vehicle (25% PEG in saline). Splenic lymphocytes were isolated 24 h after injection and were stimulated with the Con A (10 μg/ml) at 37 °C in RPMI for 24 h for cytokine measurement and 72 h for estimating mitogen induced proliferation. The concentration of cytokines in the supernatant of cells isolated from control and plumbagin treated mice was estimated using ELISA. Each bar represents concentration of (a) IL-4 (b) IL-6 and (c) IFN-γ. Each bar represents mean ± S.E.M. from three replicates and two such independent experiments were carried out. After 72 h of stimulation with Con A, the cell proliferation was assessed by CyQUANT assay (d) and each bar represents the fluorescent intensity corresponding to proliferation of cells in each treatment group. 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 and #$p<0.01$, as compared to Con A stimulated cells.

processing and presentation [21]. IL-4 is also an effector cytokine needed for the differentiation of naïve T helper cells into TH2 effector cells and it promotes humoral immunity [22]. It also plays a central role in the pathogenesis of allergic inflammation [23]. Interestingly, plumbagin inhibited the production of IL-2 as well as effector cytokines (IFN-γ and IL-4) in Con A activated cells indicating that plumbagin may not only inhibit the activation and proliferation of naïve T cells but also inhibit the functions of differentiated effector and memory T cells (Fig. 4).

Interleukin-6 is a pleiotropic cytokine produced by TH17 cells and is involved in inflammation, TH17 differentiation and autoimmunity [24]. Deregulated overproduction of IL-6 has been shown to play a role in pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis, Castleman's disease, juvenile idiopathic arthritis and Crohn's disease [25]. Plumbagin significantly inhibited the secretion of IL-6, suggesting its role as a potential anti-inflammatory agent (Fig. 4c). Similarly, the Con A induced production of IL-4, IL-6 and IFN-γ and proliferation of lymphocytes isolated from plumbagin treated mice was severely impaired indicating its anti-inflammatory efficacy in vivo (Fig. 7).

The transcription factor NF-κB activation upon TCR cross linking is critical for T cell survival and activation [26,27]. In unstimulated T cells, NF-κB is sequestered in the cytoplasm by IκB-α. T cell activation results in phosphorylation and degradation of IκB-α, leading to translocation of NF-κB to the nucleus [28]. Phosphorylation of IκB-α is mediated by IκB kinase (IKK) complex, which contains two catalytic subunits, IKK-α and IKK-β, and one regulatory subunit, IKK-γ. NF-κB activation is required for complete T cell activation, survival and proliferation [29,30]. In addition to TCR mediated signals, NF-κB can be activated by several other agents like cigarette smoke, mitogens, hydrogen peroxide and TNF-α. Recently, TNF-induced NF-κB activation was shown to be inhibited by plumbagin [11]. Several reports suggest that production of IL-2, IL-4, IL-6 and IFN-γ cytokines and the expression of activation markers CD25 and CD69 is transcriptionally regulated by NF-κB [18,23,31–34]. Our results indicate that plumbagin inhibits Con A induced NF-κB activation in T cells by preventing IκB-α degradation (Fig. 6). We propose that suppression of T cell activation, proliferation and cytokine production by plumbagin treatment may be due to its ability to inhibit the NF-κB activation pathway.

Modulation of NF-κB activation pathway leading to suppression of the alloreactive T cells has been shown to have therapeutic potential in the prevention of graft rejection [12]. Also, acute GVHD mediated by undesired activation of donor immune cells is a major hurdle for successful bone marrow transplantation (BMT) and also in patients receiving multiple blood transfusions. Although many immunosuppressive drugs are available, none of them are able to completely abolish acute GVHD. The lifelong immunosuppression profoundly reduces the quality of life of BMT recipients as it renders them susceptible to opportunistic infections. A range of stimuli produced during the course of GVHD, like TNF-alpha and Interleukin-1, lead to the phosphorylation of IKK resulting in the degradation of IκB-α, allowing the translocation of NF-κB into the nucleus which leads to the activation of a plethora of

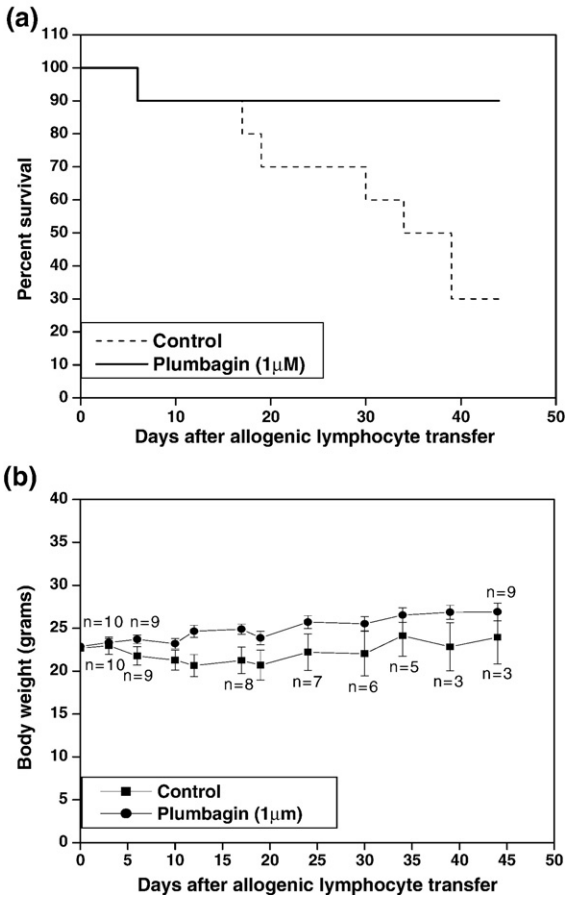

Fig. 8. Plumbagin prevented weight loss and mortality in GVHD mice: Ten million lymphocytes from C57BL/6 donor mice were injected i.v. into immunocompromised Swiss mice 48 h after WBI (600 cGy). Ten mice were included in each group. Control group mice received vehicle treated cells whereas the plumbagin group received cells treated with 1 μM plumbagin for 4 h. (a) Survival of the immunocompromised mice reconstituted with allogenic lymphocytes treated with plumbagin or vehicle. (b) Changes in the body weight of the mice after allotransplantation. Data points represent mean ± SEM from 10 mice.

target genes involved in multiple inflammatory responses [11,12]. One of the strategies to counter the induction of GVHD is to identify agents that have the potential to specifically target alloreactive T cells whilst being harmless to unstimulated lymphocytes [11,12]. Major highlight of the present study was that plumbagin treatment was not only able to abrogate the symptoms associated with GVHD but also significantly increased the survival of mice receiving allogenic lymphocytes (Fig. 8a and b). It is well known that one of the initial events in GVHD is T cell activation followed by cytokine secretion and hence the in vitro and in vivo immunosuppressive and anti-inflammatory properties of plumbagin substantiate its importance in the prevention of acute GVHD. These effects of plumbagin may possibly be attributed to its ability to suppress NF-κB activation in alloreactive T cells. Interestingly, plumbagin did not induce cytotoxicity in unstimulated lymphocytes (Fig. 2).

In summary, the present report describes the anti-inflammatory and immunosuppressive effects of plumbagin in mice. The findings also demonstrate the ability of plumbagin to protect mice from lethal GVHD. These results also highlight a potential application of plumbagin as an immunosuppressive agent which may be used in conjunction with the available drugs to establish regimens that prolong allograft survival.

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ARTICLE
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Plumbagin Inhibits Proliferative and Inflammatory Responses of T Cells Independent of ROS Generation But by Modulating Intracellular Thiols

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ABSTRACT

Plumbagin inhibited activation, proliferation, cytokine production, and graft-versus-host disease in lymphocytes and inhibited growth of tumor cells by suppressing nuclear factor-κB (NF-κB). Plumbagin was also shown to induce reactive oxygen species (ROS) generation in tumor cells via an unknown mechanism. Present report describes a novel role of cellular redox in modulation of immune responses in normal lymphocytes by plumbagin. Plumbagin depleted glutathione (GSH) levels that led to increase in ROS generation. The decrease in GSH levels was due to direct reaction of plumbagin with GSH as evinced by mass spectrometric and HPLC analysis. Further, addition of plumbagin to cells resulted in decrease in free thiol groups on proteins and increase in glutathionylation of proteins. The suppression of mitogen-induced T-cell proliferation and cytokine (IL-2/IL-4/IL-6/IFN-γ) production by plumbagin was abrogated by thiol antioxidants but not by non-thiol antioxidants confirming that thiols but not ROS play an important role in biological activity of plumbagin. Plumbagin also abrogated mitogen-induced phosphorylation of ERK, IKK, and degradation of IκB-α. However, it did not affect phosphorylation of P38, JNK, and AKT. Our results for the first time show that antiproliferative effects of plumbagin are mediated by modulation of cellular redox. These results provide a rationale for application of thiol-depleting agents as anti-inflammatory drugs. J. Cell. Biochem. 110: 1082–1093, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: ANTI-INFLAMMATORY; THIOL; REDOX; IMMUNE; PLUMBAGIN; GLUTATHIONYLATION

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is found in the plants of Plumbaginaceae, Droseraceae, Ancestrocladaceae, and Dioncophyllaceae families. Plumbagin is also present along with a series of other structurally related naphthoquinones in the roots, leaves, bark, and wood of *Juglans regia* (English walnut, Persian walnut, and California walnut), *Juglans cinerea* (butternut and white walnut), and *Juglans nigra* (black walnut). Preparations derived from black walnut have been used as hair dyes and skin colorants in addition to being applied topically for the treatment of acne, inflammatory diseases, ringworm, and fungal, bacterial, and viral infections. The root of *Plumbago zeylanica* (also called Chitrak), a major source of plumbagin, has been used in traditional Indian medicine since 750 BC as an antiatherogenic, cardiotonic, hepatoprotective, and neuroprotective agent [Padhye and Kulkarni, 1973; Tilak et al., 2004]. The active principle, plumbagin, was first isolated in 1829 [D'Astafort, 1829].

Plumbagin has been shown to exert several therapeutic biological effects including anticancer, antiproliferative, chemopreventive, chemotherapeutic, and radiosensitizing properties in experimental animals as well as in tumor cells in vitro [Naresh et al., 1996; Singh and Udupa, 1997; Hazra et al., 2002]. Earlier, we showed that plumbagin exerts its various activities through suppression of the transcription factor nuclear factor-κB (NF-κB). It suppressed

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constitutive as well as inducible NF-κB in various human tumor cell lines [Sandur et al., 2006]. Recently, we showed that plumbagin inhibited NF-κB activation in lymphocytes. The suppression of NF-κB by plumbagin resulted in inhibition of mitogen-induced activation and proliferation of lymphocytes [Checker et al., 2009]. It also inhibited lipopolysaccharide-induced activation and cytokine production in macrophages [Raghu et al., 2009]. We also showed that plumbagin suppressed homeostatic proliferation of autologous T cells in lymphopenic mice and graft-versus-host disease associated morbidity and mortality, which are known to require NF-κB activation [Checker et al., 2009; Sharma et al., 2009].

In addition to having anti-inflammatory and growth-modulatory effects, plumbagin exhibited antibacterial action through generation of pro-oxidants [Krishnaswamy and Purushothaman, 1980]. It generated reactive oxygen species (ROS) in tumor cells leading to DNA damage and cytotoxicity [Inbaraj and Chignell, 2004; Srinivas et al., 2004; Kawiak et al., 2007]. It was shown that plumbagin directly inhibited the binding of NF-κB to its consensus target sequence by modifying a critical cystine-38 residue on p65 in tumor cells. This suppressive effect of plumbagin was shown to be sensitive to thiol-containing antioxidant, dithiothreitol [Sandur et al., 2006]. Cellular redox status plays an important role in the biological effector functions of lymphocytes and leukocytes [Malmberg et al., 2001; Hildeman et al., 2003b; Klemke and Samstag, 2009]. Since oxidative stress has been shown to modulate signaling pathways through modulation of thiol groups present on proteins and glutathionylation of many proteins [Fratelli et al., 2002; Biswas et al., 2006; Winterbourn and Hampton, 2008; Dalle-Donne et al., 2009], we hypothesized that the anti-inflammatory effects of plumbagin may be due to its ability to perturb the redox balance in cells leading to modification of critical signaling molecules required for activation of lymphocytes. Further, the modulation of intracellular redox by plumbagin and its mechanism is not fully understood. To test this hypothesis, we investigated the effect of plumbagin on cellular redox status. We also examined the effects of different antioxidants (thiol/non-thiol) on immunosuppressive and anti-inflammatory effects of plumbagin. The biochemical and signaling mechanisms responsible for novel redox dependent anti-inflammatory action of plumbagin are described herein.

MATERIALS AND METHODS

REAGENTS

Plumbagin (practical grade), RPMI 1640 medium, HEPES, ethylenediaminetetraacetic (EDTA), ethylene glycol tetraacetic acid (EGTA), phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, benzamidine, dithiothreitol (DTT), glutathione (GSH), *N*-acetyl cysteine (NAC), nonidet P-40, propidium iodide (PI), Hoechst, monochlorobimane, maleic acid diethyl ester (DEM), dimethyl sulfoxide (DMSO), rotenone, allopurinol, diphenyleneiodonium, and antibodies against BCL-2, BCL-xL, Cyclin A, and β-actin were purchased from Sigma Chemical Co. (USA). A 100 mM solution of plumbagin was prepared in DMSO, stored as small aliquots at −20°C, and then diluted as needed in cell culture medium. Carboxy fluorescein diacetate succinimidyl ester (CFSE), 5-(and-6)-carboxy-2,7-dichlorofluorescein diacetate (DCF-DA), streptavidin agarose, biotin C2-iodoacetamide, glutathione ethyl ester biotin amide (Bio-GEE) were procured from Molecular Probes, Invitrogen. Fetal calf serum (FCS) was obtained from Gibco BRL. Concanavalin A (con A), Mn (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP), Trolox, Catalase Assay Kit, and Superoxide dismutase enzyme assay kit were purchased from Calbiochem (USA). ELISA sets for detection of cytokines (IL-2, IL-4, IL-6, and IFN-γ) were procured from BD Pharmingen (USA). Antibodies against p-ERK, ERK, p-IKKα/β, IKKα, IKKβ, IκB-α, p-P38, P38, p-JNK, JNK, p-AKT, and AKT were obtained from Cell Signaling Technologies (USA).

ANIMAL MAINTENANCE

Six- to eight-week-old inbred Swiss male mice, weighing approximately 20–25 g, reared in the animal house of Bhabha Atomic Research Centre were used. They were housed at 23 ± 3°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.

PROLIFERATION ASSAY

Lymphocytes were obtained by squeezing the spleen through a nylon mesh in a petri plate containing RPMI medium. The RBCs were lysed by brief hypotonic shock. Lymphocytes were stained with CFSE (20 μM, 5 min, 37°C) and washed three times using ice-cold RPMI medium containing 10% FCS, 100 IU/ml penicillin, and 100 mg/ml streptomycin. Two million lymphocytes were treated with different antioxidants for 2 h followed by plumbagin (1 μM, 2 h) and were stimulated with con A (5 μg/ml) for 72 h at 37°C in 2 ml RPMI with 10% FCS in a 95% air/5% CO_2 atmosphere. Vehicle (DMSO)-treated cells served as a control. Cell proliferation was measured by dye dilution in a flowcytometer (BD FACSAria). Percent daughter cells that showed a decrease in CFSE fluorescence intensity were calculated using FCS express (Version 3) software.

MEASUREMENT OF CYTOKINE SECRETION

The concentration of IL-2, IL-4, IFN-γ, and IL-6 in the supernatant of control vehicle-treated cells and cells stimulated with con A (5 μg/ml) for 24 h after plumbagin (1 μM) treatment was estimated using cytokine ELISA sets (BD Pharmingen).

WESTERN BLOT ANALYSIS

To determine the levels of protein expression, whole cell lysates were prepared [Takada and Aggarwal, 2003] and fractionated by SDS–PAGE. After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes, probed with the appropriate antibodies, and detected by enhanced chemiluminescence (Roche, Germany).

INTRACELLULAR GSH ASSAY

To measure intracellular GSH, lymphocytes were incubated with the indicated concentrations of DEM or treated with plumbagin for 4 h at 37°C. Monochlorobimane (final concentration, 40 μM, 30 min at 37°C) was loaded into cells [Hedley and Chow, 1994]. Fluorescence emission from cellular sulfhydryl-reacted monochlorobimane was measured using a spectrofluorimeter (BMG Labtech Optima).

Monochlorobimane is also known to react with small-molecular-weight thiols other than GSH but GSH forms the major monochlorobimane reactive thiol. Hence, MCB fluorescence is referred to as GSH levels in this manuscript. There are several reports in the literature measuring GSH levels using this dye [Bouzyk et al., 1997; Deas et al., 1997; Agrawal et al., 2007].

PRO-OXIDANT MEASUREMENTS

To detect intracellular ROS, lymphocytes were preincubated with 20 μM oxidation-sensitive DCF-DA for 25 min at 37°C before being treated with various concentrations of plumbagin. The oxidized form of the dye (DCF) acts as a control for changes in uptake, ester cleavage, and efflux [Checker et al., 2008]. After 1 h of incubation, the increase in fluorescence resulting from oxidation of H_2DCF to DCF was measured using a spectrofluorimeter.

HPLC SEPARATION OF PRODUCTS OF REACTION OF PLUMBAGIN WITH GSH

A Waters (Milford, MA) Millenium32 chromatographic system equipped with model 510 pumps, a U6K injector, a system interface module, and a diode array detector was used for sample analyses. Separations were achieved on Waters Spherisorb S3 ODS2 reverse phase column (4.6 mm × 150 mm). The column was equilibrated with HPLC grade water at a flow rate of 1 ml/min prior to injecting 25 μl of the reaction mixture. Elution was carried out using a gradient of acetonitrile from 0% to 100% over 20 min, beginning 2 min after injection of the sample. Absorbance of the effluent was measured using a diode array detector.

LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

LC–MS was performed using a Varian ProStar 410 AutoSampler in combination with Varian 1200 L LC–MS equipment triple quadrupole mass (QqQ) spectrometer with ESI source (Varian, Inc., USA). The column was 3 μm C18, 100 mm × 2.1 mm (Phenomex). A gradient mobile phase was used at 0.2 ml/min. The ESI source operated in positive ionization mode.

CONFOCAL MICROSCOPY

Confocal laser microscopy was used to study the entry of plumbagin into lymphocytes. Lymphocytes were incubated with or without plumbagin (10 μM) in medium containing 10% FBS. At the end of 24 h, the cells were centrifuged onto coverslips, fixed with paraformaldehyde, stained with Hoechst stain for nuclear staining, and mounted onto glass slides. Slides were examined using a LSM510 scanning module (Carl Zeiss Microscopy, Jena GmbH, Germany) with a krypton–argon laser, coupled to an Orthoplan Zeiss photomicroscope using a 488 nm laser line and a 510nm-band pass filter. Overlay images were recorded by superimposing simultaneous images from each channel.

LABELING, PULL DOWN, AND DETECTION OF PROTEIN THIOLATES

To detect protein thiolate anions, lymphocytes were preincubated with plumbagin for 4 h after which the cells were harvested and whole cell lysates were prepared. The lysates were incubated with BIAM (0.2 mM) for 30 min at 37°C and then incubated with streptavidin agarose beads (20 μl/mg of protein) for 1 h at 4°C. The agarose beads were separated by centrifugation, washed four times with RIPA buffer (1% NP-40, 0.1% SDS, 0.5 mg/ml sodium deoxycholate, 150 mM NaCl, and 50 mM Tris–HCl, pH 7.5) and boiled in SDS sample buffer. Proteins in the eluent were resolved by SDS–PAGE and detected by Coomassie blue staining [Ying et al., 2007].

PURIFICATION OF PUTATIVE *S*-GLUTATHIONYLATED PROTEINS USING BIO-GEE

Lymphocytes were incubated with Bio-GEE (250 μM, 1 h) prior to the addition of plumbagin at the concentrations indicated for 4 h with cycloheximide (1 μg/ml). Incubations were terminated, and a soluble protein extract was obtained as described above. The soluble proteins were then incubated with streptavidin agarose beads (20 μl/mg of protein) for 30 min at 4°C. The agarose beads were separated by centrifugation, washed four times with RIPA buffer, and boiled in SDS sample buffer. Proteins in the eluent were resolved by SDS–PAGE and detected by Coomassie blue staining [Sullivan et al., 2000].

STATISTICAL ANALYSIS

The statistical significance of the differences in respect of all parameters studied between untreated and con A stimulated cells in presence or absence of plumbagin or antioxidants was assessed by Student's "*t*"-test.

RESULTS

The aim of the present studies was to investigate the role of cellular redox status on immunomodulation by plumbagin. The antioxidants and pharmacological inhibitors used in these experiments did not affect viability of lymphocytes.

PLUMBAGIN MODULATES CELLULAR REDOX BY CHANGING ROS LEVELS, GSH CONTENT, AND ANTIOXIDANT ENZYME ACTIVITY

Figure 1A shows the levels of ROS in control and plumbagin-treated lymphocytes. Plumbagin (5 μM)-induced increase in ROS levels in lymphocytes was further augmented in presence of diethymaleate, a GSH depleting agent (Fig. 1A). To estimate the effect of plumbagin on intracellular GSH levels, lymphocytes were incubated with plumbagin or DEM for 4 h. Plumbagin-treated cells showed significantly lower levels of intracellular GSH as compared to untreated cells (Fig. 1B). The positive control, DEM showed depletion of GSH to a similar extent as plumbagin (Fig. 1B). Further, to identify the intracellular source of ROS in plumbagin-treated cells, lymphocytes were incubated with inhibitors of xanthine oxidase (allopurinol, AP, 1 mM), NADPH oxidase (DPI, 50 μM), or mitochondrial complex I inhibitor (rotenone, 10 μM). These inhibitors did not abrogate the plumbagin-induced increase in ROS levels in lymphocytes (Fig. 1C). Incubation of lymphocytes with NAC (10 mM) prior to addition of plumbagin, on the other hand, led to decrease in ROS production (Fig. 1C). All the inhibitors of ROS generation used in our experiment reduced the DCF fluorescence suggesting that basal ROS levels decreased upon addition of these

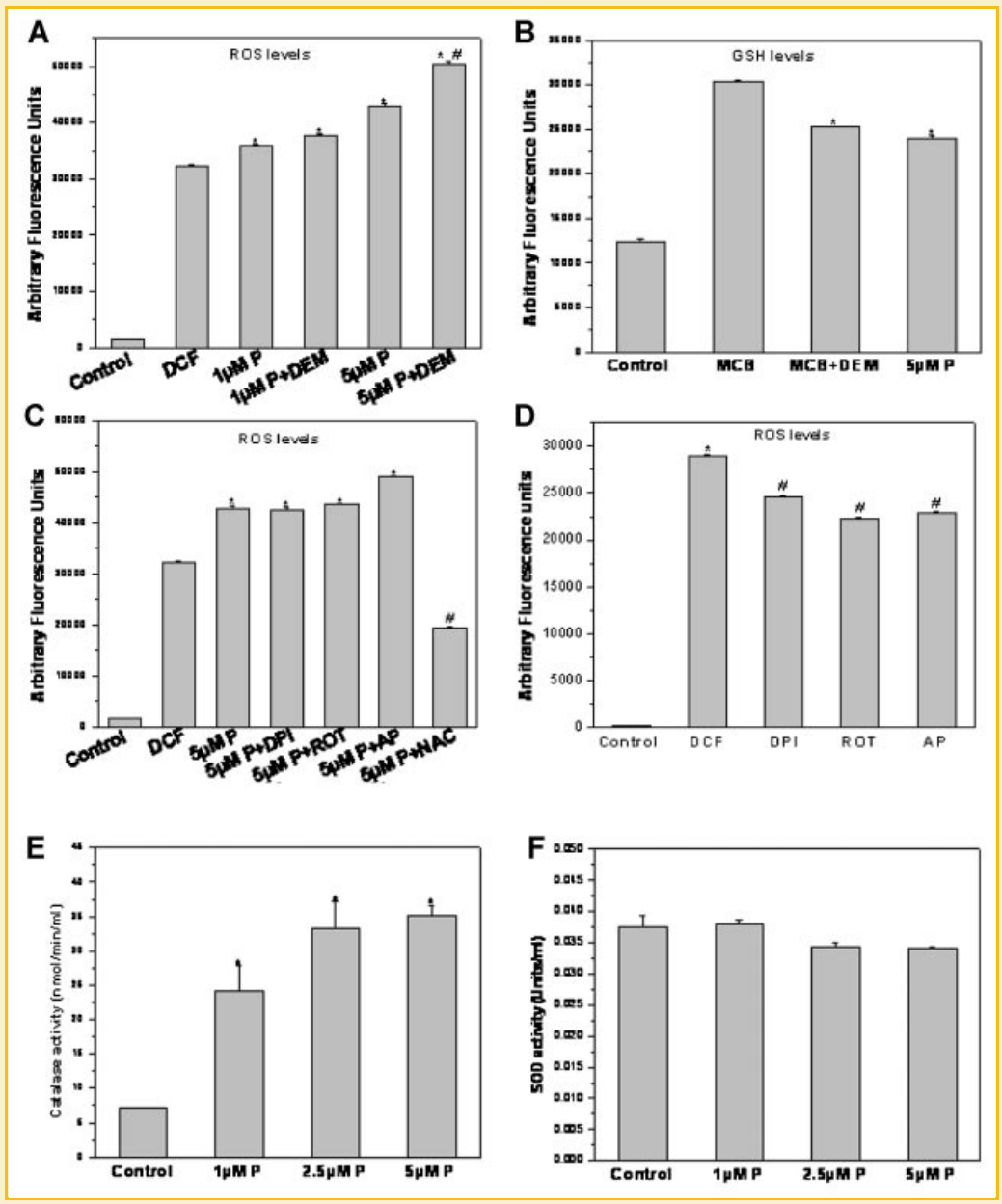


Fig. 1. A: Plumbagin increased ROS levels in lymphocytes. Lymphocytes were stained with DCF-DA (20 μM, 30 min, 37°C) and were treated with plumbagin (1 and 5 μM) in presence or absence of DEM. Each bar shows mean DCF-DA fluorescence ± SEM from four replicates and three such independent experiments were carried out. $^{*}P < 0.01$ as compared to untreated cells, $^{\#}P < 0.01$ as compared to plumbagin (5 μM)-treated cells. B: Plumbagin depleted intracellular thiols. Lymphocytes were treated with plumbagin or DEM (90 μM) for 4 h at 37°C and stained with MCB (final concentration 40 μM) for 30 min. Fluorescence emission was measured at 490 nm following excitation at 394 nm. Each bar shows mean MCB–GSH adduct fluorescence ± SEM from four replicates and three such independent experiments were carried out. $^{*}P < 0.01$ as compared to untreated cells stained with MCB. C: Effect of different inhibitors of ROS generation or *N*-acetylcysteine (NAC, 10 mM) on plumbagin-mediated increase in ROS. Lymphocytes were incubated with inhibitors of NADPH oxidase, xanthine oxidase, or mitochondrial complex I (diphenyl iodonium, DPI, 50 μM or allopurinol, AP, 1 mM or rotenone, ROT, 10 μM, respectively) or NAC (10 mM) for 2 h and stained with DCF-DA. The cells were treated with plumbagin for 1 h at 37°C and fluorescence emission was measured at 520 nm following excitation at 488 nm. Each bar shows mean DCF fluorescence ± SEM from four replicates and two such independent experiments were carried out. $^{*}P < 0.01$ as compared to untreated cells stained with DCF-DA. $^{\#}P < 0.01$ as compared to plumbagin-treated cells. D: Effect of different inhibitors of ROS generation on basal ROS levels. Lymphocytes were incubated with inhibitors of NADPH oxidase, xanthine oxidase, or mitochondrial complex I (diphenyl iodonium, DPI, 50 μM or allopurinol, AP, 1 mM or rotenone, ROT, 10 μM, respectively) for 2 h and stained with DCF-DA. Fluorescence emission was measured at 520 nm following excitation at 488 nm. Each bar shows mean DCF fluorescence ± SEM from four replicates and two such independent experiments were carried out. $^{*}P < 0.01$ as compared to untreated control cells. $^{\#}P < 0.01$ as compared untreated cells stained with DCF-DA. E: Plumbagin enhanced catalase activity but not SOD activity. Lymphocytes were incubated with plumbagin for 4 h and the activity of catalase (E) and SOD (F) enzymes was measured according to manufacturer's protocol. Each bar represents catalase activity (E) and SOD activity (F). $^{*}P < 0.01$ as compared to vehicle-treated cells. Each bar shows mean ± SEM from three replicates and two such independent experiments were carried out.

inhibitors to lymphocytes (Fig. 1D). The common groups in Figure 1A,C (Control, DCF, 5 μM P) are from the same experiment.

Since plumbagin induced increase in ROS levels, the activities of antioxidant enzymes like catalase and SOD in lymphocytes treated with plumbagin were measured. Plumbagin-treated cells showed significantly higher catalase activity as compared to untreated cells (Fig. 1E) without altering the SOD activity in lymphocytes (Fig. 1F). These results suggest that plumbagin regulates redox status of lymphocytes by modulation of thiols, mainly GSH levels.

IMMUNOSUPPRESSIVE EFFECTS OF PLUMBAGIN IN LYMPHOCYTES WERE ABROGATED BY THIOL ANTIOXIDANTS

Figure 2 shows the modulation of immunosuppressive effects of plumbagin by different antioxidants. Thiol-containing antioxidants (DTT, GSH, and NAC) abrogated the antiproliferative effect of plumbagin in mitogen-activated lymphocytes (Fig. 2A–C). However, antioxidants that do not contain a thiol group (MnTBAP and trolox) did not restrain the antiproliferative action of plumbagin (Fig. 2D). Similarly, anti-inflammatory action of plumbagin as seen by suppression of mitogen-induced cytokines was also curbed by thiol-containing antioxidants, GSH and NAC, (Fig. 3A–D) but not by non-thiol antioxidants (Fig. 3E). Both thiol and non-thiol antioxidants per se did not affect con A induced IL-2 secretion (Fig. 3F).

PLUMBAGIN INHIBITED CYTOKINE PRODUCTION IN ACTIVATED LYMPHOCYTES

Our present results pointed toward a novel redox-dependent immunosuppressive action of plumbagin. Our previous reports have shown effective immunosuppressive action of plumbagin in naïve T cells and B cells. Experiments were carried out to determine if plumbagin could inhibit effector functions in T cells when it was added after stimulation with mitogen. Figure 4 clearly shows that plumbagin effectively suppressed cytokine production in T cells even when it was added up to 60 min after their activation with a mitogen. To see the reversibility of plumbagin-mediated immunosuppression, splenic lymphocytes were treated with plumbagin (4 h) and cells were washed twice with medium. Cells were then rested for 24 h before stimulating with con A. Despite resting cells for 24 h, plumbagin suppressed con A induced IL-2 production. These results showed that immunosuppressive effects of plumbagin in lymphocytes are not reversible (Fig. S2).

SUPPRESSIVE EFFECTS OF PLUMBAGIN ON MITOGEN-INDUCED SIGNALING EVENTS WERE SENSITIVE TO GSH

Figure 5A shows the activation of ERK and IKK and degradation of IκB-α in mitogen-activated lymphocytes as compared to unstimulated cells. Plumbagin inhibited mitogen-induced activation of ERK and IKK and degradation of IκB-α in lymphocytes.

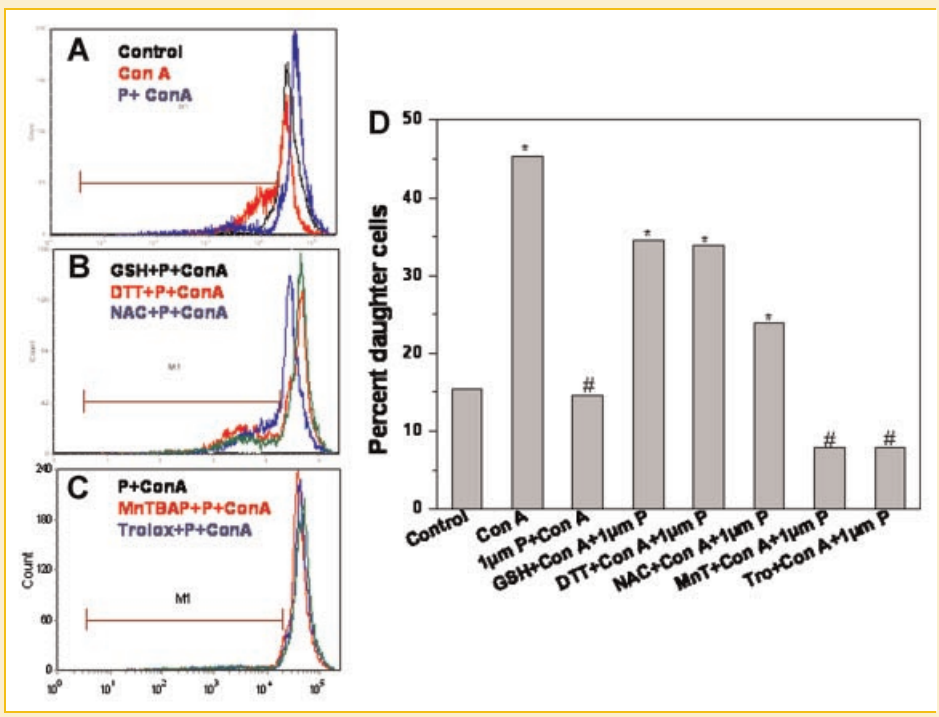


Fig. 2. Antiproliferative effects of plumbagin were abrogated by thiol-containing antioxidants. Lymphocytes were stained with CFSE and were incubated with different antioxidants (GSH 10 mM or NAC 10 mM or DTT 100 μM or MnTBAP 100 μM or trolox 100 μM) for 2 h. The cells were stimulated with con A in presence or absence of plumbagin for 92 h at 37°C in a 5%CO_2/99% air atmosphere. Cell proliferation was measured from CFSE dye dilution using a flowcytometer. Percent daughter cells were calculated using FCSexpress3 software. 95 Representative flowcytometric histograms showing effect of plumbagin on T-cell proliferation (A) and its modulation by thiol-containing antioxidants (B) and non-thiol antioxidants (C). The percentage of daughter cells in each group is shown in (D). Each bar shows mean ± SEM from three replicates and three such independent experiments were carried out. $^{*}P < 0.01$ as compared to untreated cells and $^{\#}P < 0.01$ as compared to untreated cells stimulated with con A.

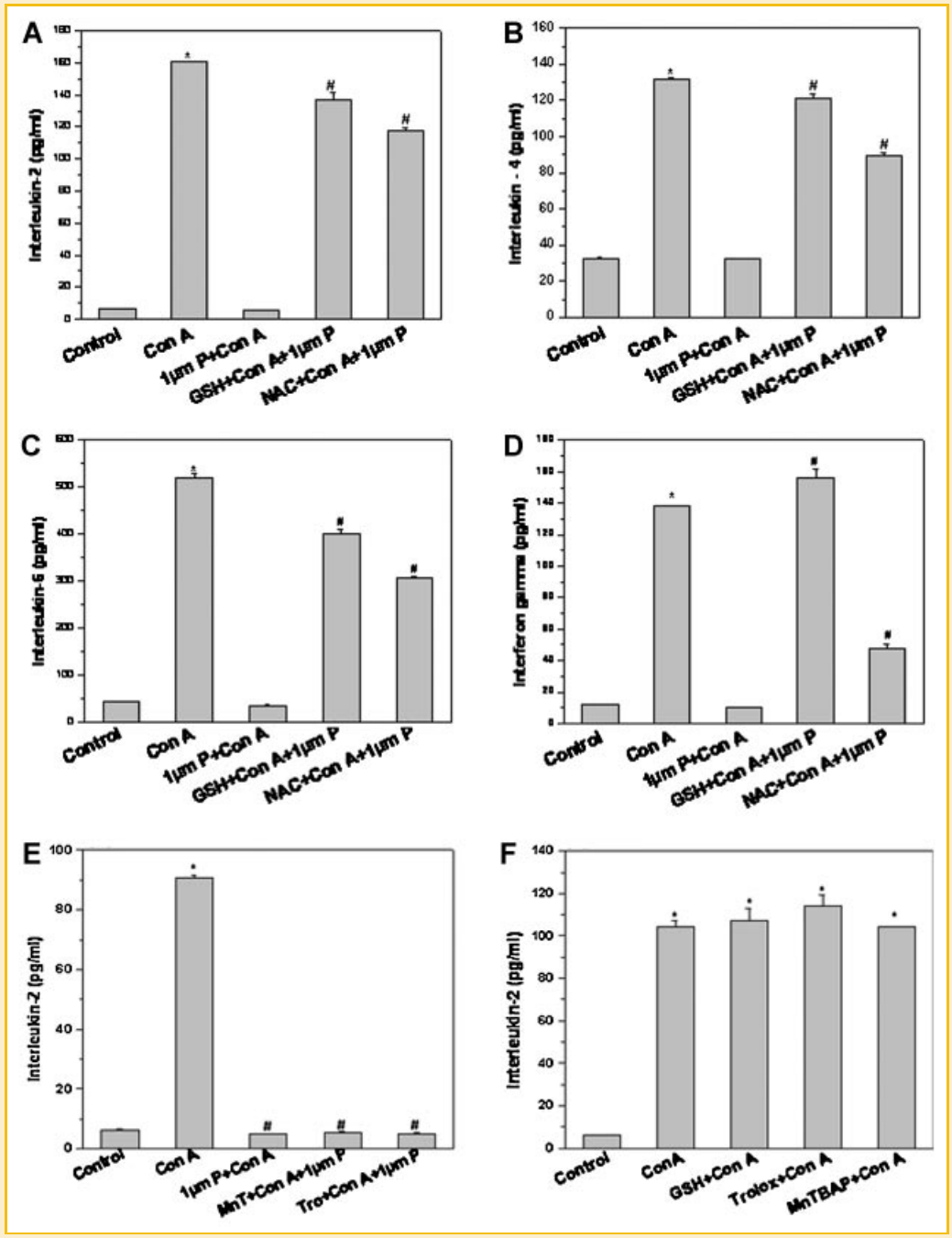


Fig. 3. Effect of thiol-containing antioxidants on cytokine production in plumbagin-treated lymphocytes stimulated with con A. Lymphocytes were incubated with different antioxidants (GSH or NAC) for 2 h. The cells were stimulated with con A in presence or absence of plumbagin for 24 h at 37°C in a 5%CO_2/95% air atmosphere. The concentration of IL-2 (A), IL-4 (B), IL-6 (C), and IFN-γ (D) in the culture supernatant was estimated by ELISA. Each bar shows mean ± SEM from three replicates and three such independent experiments were carried out. $^{*}P < 0.01$ as compared to control untreated cells. $^{\#}P < 0.01$ as compared to plumbagin-treated cells stimulated with con A. E: Effect of non-thiol antioxidants on IL-2 production in lymphocytes stimulated with con A in presence of plumbagin. Lymphocytes were incubated with different antioxidants (MnTBAP or trolox) for 2 h. The cells were stimulated with con A in presence or absence of plumbagin for 24 h at 37°C in a 5%CO_2/95% air atmosphere. The concentration of IL-2 (E) in the culture supernatant was estimated by ELISA. Each bar shows mean ± SEM from three replicates and three such independent experiments were carried out. $^{*}P < 0.01$ as compared to control untreated cells. $^{\#}P < 0.01$ as compared to cells stimulated with con A. F: Effect of thiol and non-thiol antioxidants on con A induced IL-2 production. Each bar represents mean ± SEM from three replicates and two such independent experiments were carried out. $^{*}P < 0.01$ as compared to control untreated cells.

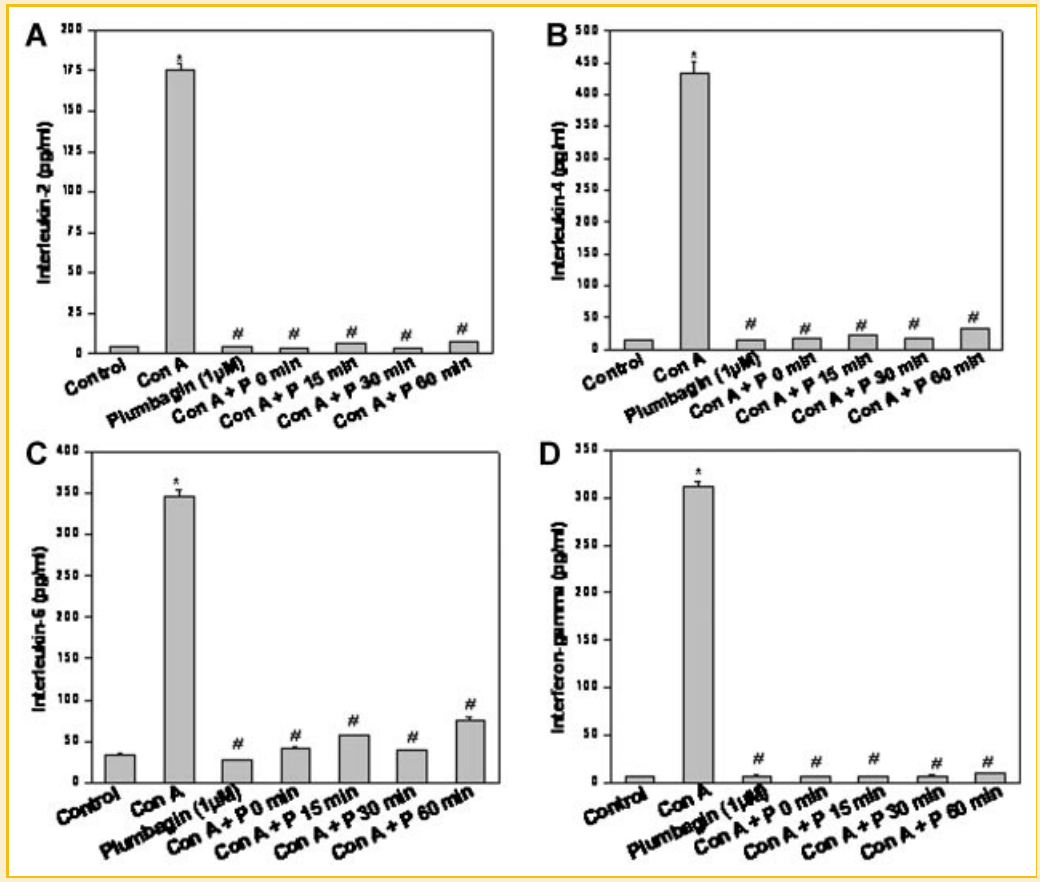


Fig. 4. Inhibition of cytokine production in activated lymphocytes by plumbagin. Lymphocytes were stimulated with con A (5 μg/ml) following which plumbagin was added at the indicated time points and the cells were cultured for 24 h at 37°C. Vehicle-treated cells served as control. The concentration of cytokines in the supernatant was estimated using ELISA. Each bar represents concentration of (A) IL-2, (B) IL-4, (C) IL-6, and (D) IFN-γ. Data points represent mean ± SEM 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.

Plumbagin down-modulated the expression of NF-κB-dependent genes like Bcl-2, Bcl-xL, and cyclin A (Fig. 5B). The suppressive action of plumbagin in activated lymphocytes was completely abrogated by GSH (Fig. 5A,B). At the same time, plumbagin did not suppress mitogen-induced activation of redox-sensitive molecule c-Jun N-terminal kinase (JNK) (Fig. 5C). The mitogen-induced activation of P38MAPkinase and atypical kinase (AKT) was also not suppressed by plumbagin as shown in Figure 5C suggesting that it has specific targets in cells.

PLUMBAGIN INTERACTED WITH GSH

Since antiproliferative and anti-inflammatory effects of plumbagin were sensitive to presence of thiol antioxidants, experiments were carried out to determine whether plumbagin physically interacted with thiol groups. Plumbagin was incubated with GSH and subjected to HPLC separation. Retention time of pure plumbagin on C18 column was 3.5 min (Fig. 6A, red line). This peak disappeared when plumbagin was preincubated with GSH and a new peak appeared at 17.5 min (Fig. 6A, blue dotted line). A minor peak also appeared at 13.5 min. These results indicated that the reaction of plumbagin with GSH formed a single major product which could be adduct of plumbagin with GSH. To characterize this adduct, LC–MS analysis was carried out. Molecular mass analysis revealed that the major peak corresponded to plumbagin–GSH adduct (M = 494) (Fig. 6B, upper panel). In addition to plumbagin–GSH complex, plumbagin also induced GSH to GSSG conversion. A peak corresponding to molecular mass 204 also appeared which may be hydroxylated plumbagin (Fig. 6B, lower panel). There were other minor peaks appearing at different molecular masses, which could not be characterized. However, these products did not appear in HPLC separation. This could be due to similar polarity of the products making them separate at the same retention time.

PLUMBAGIN ENTERED CELLS AND INDUCED GLUTATHIONYLATION OF PROTEINS IN LYMPHOCYTES

Figure 7A shows the confocal images of lymphocytes treated with plumbagin for 24 h and stained with Hoechst. Plumbagin entered lymphocytes and majority of it was localized in the nucleus (Fig. 7A). Since plumbagin reacted with GSH and also induced conversion of GSH to GSSG in cell-free systems and also depleted cellular GSH levels, experiments were carried out to determine whether plumbagin induces protein glutathionylation in cells. Lymphocytes were treated with plumbagin and cell lysates were incubated with BIAM. Using equal amount of protein in each group,

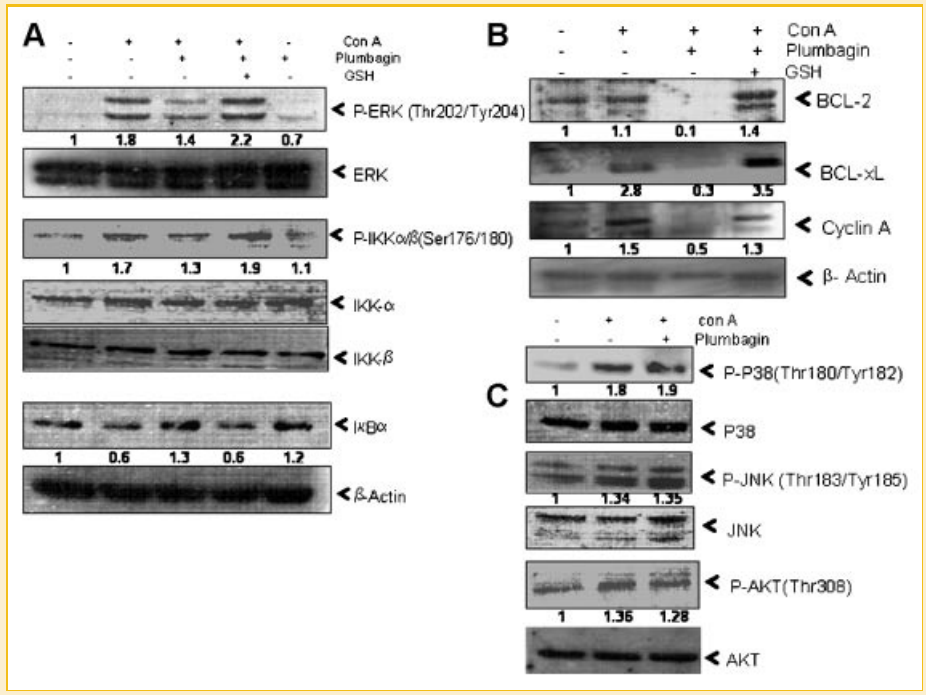


Fig. 5. Inhibition of proliferation/survival associated signaling molecules by plumbagin and their modulation by GSH in activated T cells. Lymphocytes were incubated with plumbagin (1 μM, 4 h) in presence or absence of GSH and were stimulated with con A (5 μg/ml) for 1 h (A,C) or 24 h (B). Whole cell lysates were prepared, fractionated on 10% SDS–PAGE, and electrotransferred to nitrocellulose membrane. Western blot analysis was performed using different antibodies specific for (A) P-ERK, ERK, P-IKK-α/β, IKK-α, IKK-β, IκB-α, (B) BCL-2, BCL-xL, Cyclin A, and (C) P-P38, P-38, P-JNK, JNK, P-AKT, AKT. β-Actin was used as loading control. Two such independent experiments were carried out.

non-glutathionylated proteins conjugated with BIAM were pulled down using streptavidin agarose beads and were resolved on SDS–PAGE. Lymphocytes treated with plumbagin showed marked decrease in free thiol groups on proteins (Fig. 7B). To assess the effect of plumbagin on protein glutathionylation, cells were incubated with cell permeable biotinylated-glutathione-ethyl ester (Bio-GEE). Bio-GEE loaded cells were treated with vehicle or plumbagin and cell lysates were prepared. Equal amount of protein in each group was used for precipitation of glutathionylated proteins using streptavidin agarose beads and resolved on SDS–PAGE. Coomassie staining showed a dose-dependent increase in specific glutathionylated proteins in plumbagin-treated samples as compared to that in untreated control cells (Fig. 7C).

DISCUSSION

Cellular redox status is determined by balance between levels of pro-oxidants (ROS and RNS) and antioxidants (glutathione peroxidase, superoxide dismutase, and catalase) and rate of cellular respiration/metabolism. The ratio of GSH to GSSG is also considered to be intrinsic determinant of the cellular redox status. Cellular redox has been shown to play a critical role in lymphocyte activation, survival, and proliferation [Flescher et al., 1994, 1998; Lahdenpohja et al., 1998; Hildeman et al., 2003b]. Exposure of T cells to ROS scavenging agents like MnTBAP and chlorophyllin has been shown to increase cellular survival via upregulation of antiapoptotic gene expression [Hildeman et al., 2003a; Sharma et al., 2007a,b]. On the contrary, exposure of T cells to oxidizing agents like H_2O_2, xanthine/xanthine oxidase has been shown to suppress T cell activation, proliferation, and cytokine production via suppression of NF-κB [Pahlavani and Harris, 1998]. The anti-inflammatory and immunosuppressive effects of plumbagin are known to be mediated via inhibition of NF-κB activation in lymphocytes [Checker et al., 2009; Sharma et al., 2009].

In the present report we found that plumbagin disrupted cellular redox homeostasis (Fig. 1A,B). The increased levels of ROS in plumbagin-treated cells could be due to (i) an increase in the activities of NADPH oxidase or (ii) xanthine oxidase or (iii) influx from mitochondrial electron transport chain or (iv) modulation of free thiol levels. Since plumbagin-induced increase in intracellular ROS was not affected by specific inhibitors of NADPH oxidase, xanthine oxidase, and mitochondrial complex I (Fig. 1C), the role of first three of the above possibilities toward plumbagin-induced disruption of cellular redox was not apparent. Therefore, it may be concluded that plumbagin exerted its effects by depleting GSH in lymphocytes. This was supported by decrease in MCB–GSH adduct fluorescence in plumbagin-treated cells (Fig. 1B). DEM, a well-known depletor of GSH [Plummer et al., 1981], accentuated ROS levels in plumbagin-treated lymphocytes compared to that by plumbagin alone (Fig. 1A). Further, cell permeable thiol antioxidant NAC significantly lowered ROS levels in plumbagin-treated cells (Fig. 1C). These data clearly suggested that depletion of intracellular thiols by plumbagin could be responsible for increased

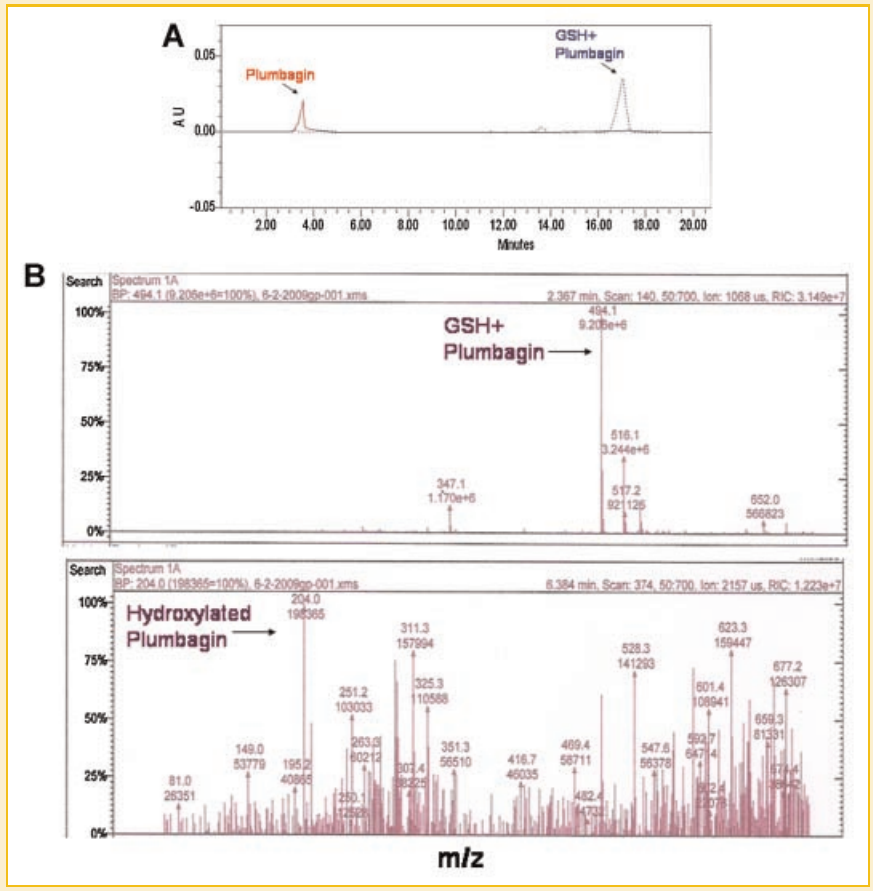


Fig. 6. A: HPLC separation of products of reaction of plumbagin with GSH. Plumbagin (100 μM) was mixed with GSH (10 mM) in 10 mM potassium phosphate buffer. After 1 h incubation at 37°C, 25 μl of each sample was subjected to HPLC. Effluent was monitored using a diode array detector. Red line (solid) indicates plumbagin alone and blue (dotted) line indicates reaction product(s) of plumbagin with GSH. B: Identification of products of reaction of plumbagin with GSH by mass spectrometry. Sample containing 100 μM plumbagin in 10 mM GSH was subjected to LC–MS analysis using ESI ion trap detector. Two major peaks corresponding to plumbagin–GSH adduct (mol. wt 494, upper panel) and hydroxylated product of plumbagin (mol. wt 204, lower panel) were observed following ESI–MS analysis. Two such independent experiments were carried out.

ROS levels in lymphocytes [Henry and Wallace, 1996]. Although plumbagin was previously reported to induce ROS generation in tumor cells, the mechanism of this phenomenon was not known. In this report, for the first time we show GSH depletion as a source of ROS generation in normal lymphocytes following plumbagin treatment.

In normal cells, ROS homeostasis is maintained by antioxidant enzymes like superoxide dismutase, catalase, and glutathione peroxidase. The changes in ROS levels by exogenous agents have also been shown to affect the expression/activity of these enzymes. It was observed that the activity of catalase was significantly higher in plumbagin-treated cells (Fig. 1E). This may be due to cellular response to increased levels of H_2O_2 following treatment with plumbagin. However, the activity of SOD enzyme was not altered (Fig. 1F) indicating that plumbagin may preferentially induce hydrogen peroxide and not superoxide anions in lymphocytes. The DCF-DA dye used in our experiments to detect intracellular ROS levels is known to be relatively specific for hydrogen peroxide which confirms the production of this pro-oxidant. Earlier reports by several investigators have shown that agents that increase oxidative stress are capable of activating redox-dependent transcription factor NF-E2-related factor-2 (Nrf-2) [Kang et al., 2005]. In agreement with this, a recent report showed that plumbagin is able to increase nuclear localization and transcriptional activity of Nrf2 in human neuroblastoma cells [Son et al., 2010].

In order to determine whether increased ROS levels or decreased thiol content or both were responsible for immunosuppressive action of plumbagin, effect of thiol and non-thiol antioxidants was investigated on suppression of mitogen-induced T-cell activation by plumbagin. Addition of DTT or NAC would increase the intracellular thiols whereas GSH being cell impermeable may inhibit plumbagin's action by extracellular interaction with it. It was observed that suppression of mitogen-induced cytokines and T-cell proliferation by plumbagin were ameliorated by thiol-containing antioxidants GSH, DTT, and NAC, but not by non-thiol antioxidants suggesting that modulation of cellular thiol levels was very critical for immunosuppression by plumbagin (Figs. 2 and 3).

Changes in cellular thiol levels have been shown to affect multiple signaling pathways in different cell lines. Many of the proteins and transcription factors like P65, IKK, thioredoxin

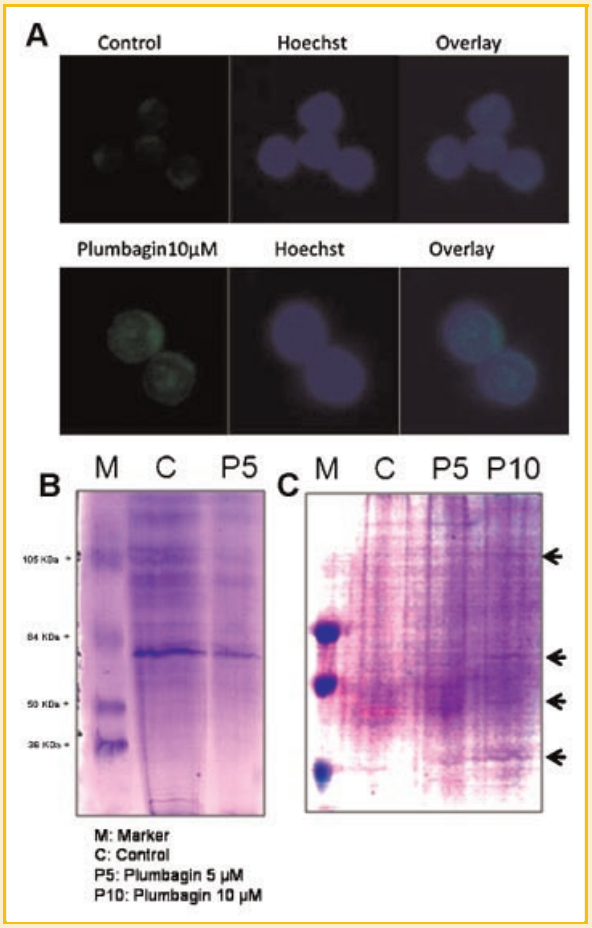


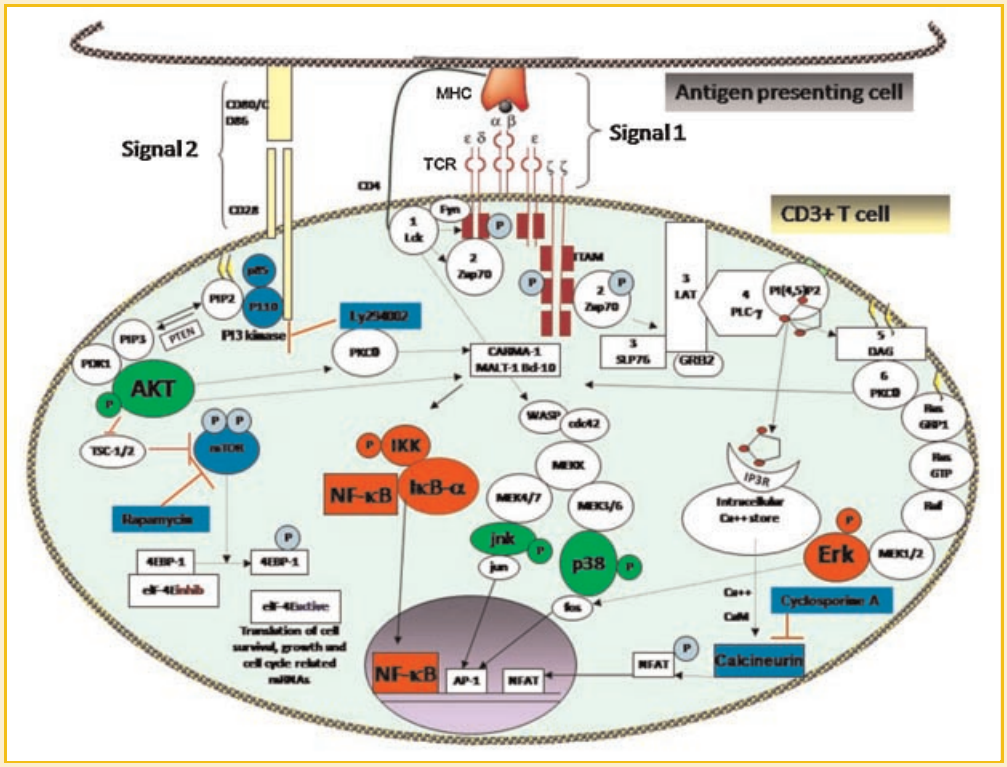
Fig. 7. A: Plumbagin enters lymphocytes. Lymphocytes were incubated with plumbagin for 24 h and fixed with paraformaldehyde. The cells were stained with Hoechst and observed under confocal microscope. B: Enrichment of iodoacetamide reactive sulfhydryl groups on proteins. Iodoacetamide (IAM) binds selectively to protein thiolate anions. The lysates of lymphocytes incubated with 5 μM plumbagin for 4 h were treated with BIAM (0.2 mM) for 30 min at 37°C. Streptavidin agarose beads (20 μl/mg of protein) were added to the lysates and incubated for 1 h at 4°C. The agarose beads were separated by centrifugation, washed four times with RIPA buffer, and boiled in SDS sample buffer. Proteins in the eluent were resolved by SDS–PAGE and detected by Coomassie blue staining. C: Purification of putative *S*-glutathionylated proteins. Protein extract was obtained as described under the Materials and Methods Section from Bio-GEE loaded lymphocytes incubated with plumbagin (0 or 5 or 10 μM, 4 h). Biotin-labeled proteins were extracted from total protein using streptavidin agarose beads. The beads were washed with RIPA buffer and boiled in SDS sample buffer. The eluted proteins were resolved by SDS–PAGE and were detected by Coomassie blue staining of the gel. Two such independent experiments were carried out.

reductase, PI3kinase/AKT, and Keap1 have been shown to be highly sensitive to changes in cellular redox status [Zhang and Hannink, 2003; Smart et al., 2004; Sandur et al., 2006; Gloire and Piette, 2009; Harikumar et al., 2009; Naughton et al., 2009]. Our present results show that disruption of cellular redox by plumbagin affected specific signaling events following TCR ligation with mitogen (including NF-κB, ERK, BCL-2, BCL-xL, and cyclin A) which are involved in cellular survival and proliferation (Fig. 5A,B). However, plumbagin did not inhibit signaling events required for activation of transcription factor AP-1 (P38MAPkinase and JNK) and signals emanating from costimulatory molecule (AKT) in activated T cells (Fig. 5C). These results were in agreement with our earlier report showing that plumbagin did not suppress AP-1 in tumor cells [Sandur et al., 2006]. However, the mechanism of suppression of certain signaling events by plumbagin without affecting other activation signals in T cells is not completely understood. These novel targets identified for immunosuppressive action of plumbagin differ from the targets of pharmacological drugs like rapamycin, FK506, and cyclosporine which inhibit mTOR and calcineurin, respectively. Scheme 1 highlights the signaling events that are unaffected by plumbagin (shown in green) and blocked by plumbagin (shown in red), or rapamycin, cyclosporine, and the PI3kinase inhibitor, Ly294002 (shown in blue).

Most quinones mediate their cellular effects through redox cycling [Henry and Wallace, 1996]. The modulation of thiol groups on proteins could be in terms of direct reaction with plumbagin or glutathionylation of proteins or formation of disulfide bridge between proteins. Our results for the first time demonstrate that plumbagin indeed formed an adduct with GSH and also converted GSH to GSSG in cell-free systems (Fig. 6B). Further, it was shown that plumbagin modulates thiol groups present on the proteins as evinced from decrease in iodoacetamide reactive thiol groups of the proteins (Fig. 7B). The results using Bio-GEE for the first time demonstrated formation of PSSG (protein–glutathione adduct) in plumbagin-treated cells (Fig. 7C). Earlier reports have shown that IKK and IκB-α, whose activation is suppressed by plumbagin in lymphocytes, is regulated by glutathionylation suggesting that NF-κB activation is regulated by redox modulation [Reynaert et al., 2006; Kil et al., 2008]. Such reactions have been shown to occur in cells exposed to oxidative stress [Sullivan et al., 2000].

Any possible therapeutic use of plumbagin for amelioration of inflammation or immunosuppression would require inhibition of effector functions in activated lymphocytes. Our earlier published results had shown that prior incubation of cells with plumbagin before stimulation with mitogen resulted in complete suppression of effector functions like cytokine production, proliferation, and GVHD [Checker et al., 2009; Raghu et al., 2009; Sharma et al., 2009]. Present results showed an efficient suppression of proinflammatory cytokine production in mitogen-activated lymphocytes by plumbagin even when it was added to the cells 1 h after stimulation (Fig. 4). The use of a phytochemicals with a favorable toxicity profile is particularly helpful in chronic inflammatory conditions such as autoimmune diseases where protracted use of traditional immunosuppressants such as corticosteroids and cytotoxic agents (methotrexate, azathioprine, cyclosporine, etc.) is associated with cumulative long-term toxicities. Thus, plumbagin may find potential clinical application as an anti-inflammatory or immunosuppressive agent for the treatment of disease conditions mediated by activated and memory lymphocytes, even when used after the triggering event has occurred.

Present report for the first time shows a ROS-independent mechanism of anti-inflammatory action of plumbagin. Modulation of cellular thiols played a more significant role than increased ROS levels in biological actions of plumbagin. For the first time, evidence for a role for glutathionylation of cellular proteins as a



Scheme. 1. T-cell receptor signaling and its inhibition by plumbagin and other pharmacological inhibitors. The signaling molecules highlighted in red were specifically inhibited by plumbagin and the molecules highlighted in green were not affected by plumbagin in activated T cells. Targets of pharmacological inhibitors (rapamycin, Ly294002, and cyclosporine) are highlighted in blue.

mechanism of anti-proliferative action of plumbagin is provided. Further, mechanistic basis for potential therapeutic application of plumbagin as an immunosuppressive or anti-inflammatory drug is highlighted.

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