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THE ROLE OF ARACHIDONIC ACID – CYCLOOXYGENASE PATHWAY IN THE ANTI-LEUKEMIC PROPERTIES OF SELENIUM

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ABSTRACT

The trace mineral selenium (Se) is an essential micronutrient of fundamental importance to human biology. Findings are unequivocal in linking the deficiency of selenium to conditions with increased oxidative stress and inflammation. Epidemiological studies suggest that supranutritional intake of selenium may be associated with a reduced risk of cancers. Many of these beneficial effects of selenium are thought to be due to the action of selenoproteins, which collectively form a part of the cellular antioxidant defense system. But the exact molecular mechanisms involved in these actions are still to be defined. The primary objective of this study is to understand the mechanisms associated with the anti-inflammatory and anti-carcinogenic effects of selenium, which would form the basis for exploring the role of supplemental selenium as a potential therapeutic agent.

Using a combination of *in vitro* and *ex vivo* models, the anti-inflammatory action of selenium was dissected with a focus on the regulation of the arachidonic acid (AA) - cyclooxygenase (COX) metabolism pathway. Supplementation of macrophages with supra-physiological levels of selenium showed that the AA was preferentially metabolized to electrophilic metabolites of prostaglandin D_2 (PGD_2). These include Δ^{12}-PGJ_2 and 15d-PGJ_2, which are structurally classified as cyclopentenone PGs (CyPGs). Interestingly, these lipid metabolites impacted the expression of downstream PG synthases via the differential modulation of two transcription factors, nuclear factor-kappa B (NF-κB) and peroxisome proliferator-activated receptor γ (PPARγ) to activate the expression of hematopoietic PGD_2 synthase (H-PGDS), and suppress that of microsomal PGE synthase-1 (mPGES-1). As a result, LPS-stimulation of Se-supplemented macrophages produced high levels of CyPGs when compared to the Se-deficient macrophages, while the levels of pro-inflammatory PGE_2 were suppressed.

To relate these effects *in vivo*, well-established models of murine leukemia were used, where the infection of progenitor cells with Friend erythroleukemia virus (FV), or expression of a fusion oncogene, BCR-ABL, in the hematopoietic stem cells (HSCs) led to the development of the disease through the formation of leukemic stem cells (LSCs). Se-supplemented mice were completely resistant to the disease, while Se-deficient and Se-adequate mice succumbed to leukemia. Treatment of Se-supplemented mice with a COX inhibitor, indomethacin, blunted the protective effect of selenium, while the administration of 15d-PGJ_2 to these mice rescued them. Further analysis showed that supplementation with selenium selectively targeted the LSCs for apoptosis via p53 pathway activation, without any effects on normal HSCs.

In a related study to examine the role of electrophilic compounds of natural origin, a botanical with anti-inflammatory properties, gambogic acid (GA), was demonstrated to inhibit NF-κB activation by forming covalent Michael adduct with ^{179}Cys in a key enzyme, inhibitor of kappa B kinase β (IKKβ). This is similar to that previously reported with the endogenously produced 15d-PGJ_2.

Taken together, the studies included in this thesis suggest that anti-oxidant and anti-inflammatory compounds can activate pathways of resolution by shunting AA-COX metabolism towards increased production of CyPGs, which can potentially affect the survival and/or proliferation of LSCs.

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LIST OF ABBREVIATIONS

Se – selenium
Sec – selenocysteine
Cys - cysteine
SECIS – selenocysteine insertion sequence
GPx – glutathione peroxidase
GSH – glutathione
GR – glutathione reductase
TR – thioredoxin reductase
DIO – deiodinase
SPS2 – selenophosphate synthetase 2
MSA – methylseleninic acid
SeMet – selenomethionine
p-XSC – 1, 4-phenylenebis(methylene) selenocyanate
BMDM – bone marrow -derived macrophages
AA – arachidonic acid
COX – cyclooxygenase
LOX - lipoxygenase
PG – prostaglandin
CyPG – cyclopentenone prostaglandin
H-PGDS – hematopoietic prostaglandin D_2 synthase
mPGES-1 – microsomal prostaglandin E synthase – 1
TXAS – thromboxane synthase
PGIS – prostacyclin synthase
GAPDH – glyceraldehyde 3-phosphate dehydrogenase
EMSA – electrophoretic mobility shift assay
ELISA – enzyme-linked immunosorbent assay
Δ^{12}-PGJ_2 – Δ^{12}-prostaglandin J_2
15d-PGJ_2 – 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2
iNOS – inducible nitric oxide synthase
TNF-α – tumor necrosis factor –α
LPS – lipopolysaccharide
NF-κB – nuclear factor - kappa B
IκB – inhibitor of kappa B
IKK – inhibitor of kappa B (IκB) kinase
PTN – parthenolide
PPARγ – peroxisome proliferator - activated receptor gamma
PPRE – PPAR response element
WBC – white blood cell

FV – Friend virus
FVP – Friend virus, polycythemia
AML – acute myeloid leukemia
CML – chronic myeloid leukemia
Se-def – selenium-deficient (0.01 ppm selenite) diet
Se-adeq/Se-adq – selenium-adequate (0.1 ppm selenite) diet
Se-suppl – selenium-supplemented (0.4 ppm selenite) diet
HSC – hematopoietic stem cell
CSC – cancer stem cell
LSC – leukemic stem cell
GA –gambogic acid
DGA – 9,10-dihydro gambogic acid
TfR1 – transferrin receptor 1
PKA – protein kinase A
PKC – protein kinase C
DP1 – prostanoid D receptor
CRTH2 (DP2) – Chemoattractant receptor-homologous molecule expressed on Th2 lymphocyte receptor
ATM – ataxia telangiectasia mutated

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I came to Penn State for my PhD in Molecular Toxicology with minimal exposure to fields such as molecular biology, genetics, or toxicology, and without any laboratorial experience. Equally excited and nervous, I decided to start my doctoral studies with a fresh mindset. Today, on the verge of completing my PhD degree requirements, I want to grab this opportunity to thank those who have made my life a lot easier. I sincerely apologize if I miss a few names here.

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Chapter 1

Literature Review

Selenium and Selenoproteins

Selenium (Se) is an essential trace element that belongs to the group of chalcogen elements, including oxygen, sulfur, and tellurium. A unique aspect about Se is that it is incorporated into proteins in form of the 21st amino acid selenocysteine (Sec). The structure of Sec is similar to that of cysteine (Cys), except that selenium replaces sulfur. In 1978, glutathione peroxidase (GPx) 1 was the first protein reported to contain Sec [1]. Cloning of GPx1 led to the surprising discovery that the codon for Sec was UGA [2], which serves as one of the three termination codons for non-selenoprotein genes. The Sec insertion sequence (SECIS), an element in the 3'-UTR of eukaryotic selenoprotein mRNAs, was discovered following the cloning of the selenoprotein DIO (deiodinase) 1 [3]. Using SECISearch, a bioinformatics-based online search engine, ~25 different selenoprotein genes were identified in different mammalian genomes [4, 5]. In general, selenoproteins exhibit diverse patterns of tissue distribution and subcellular localization, and these properties have provided important information regarding their physiological roles [6]. Subsequently, some of the selenoproteins have been functionally characterized.

Glutathione peroxidases, TRs (thioredoxin reductases), and DIOs were the first three classes of eukaryotic selenoproteins discovered [7]. The GPxs (five in human, four in mice) are chiefly selenoenzymes, and form an integral component of antioxidant glutathione pathways, providing protection from RONS (reactive oxygen & nitrogen species) [8]. GPxs possess peroxidase activity and use glutathione as a cofactor. GPx1 is one of the most abundant and ubiquitously expressed selenoproteins that is highly sensitive to changes in Se status. The TRs (TR1, 2, and 3) are oxidoreductases, which use NADPH for reduction of thioredoxin in cellular redox pathways, particularly serving to maintain reduced cysteine groups. The DIO1, 2, and 3 cleave iodine-

carbon bonds in the metabolism of thyroid hormones [9]. DIO1 or DIO2 catalyze the monodeiodination of prohormone T4 to the active form T3. DIO1 or DIO3 also irreversibly inactivate T4 and T3. Thus their relative tissue expression patterns are responsible for the amount of active thyroid hormone available to specific tissues at different stages of development [10]. A progressive reduction of the T3/T4 ratio (because of increased T4 levels) and of erythrocyte GPx activity has been observed with advancing age, which negatively correlated with increase in ROS [11, 12]. SelP and SelW were two additional selenoproteins that were identified early through biochemical studies in 1982 and 1993, respectively [13, 14]. SelP contains ten Sec residues in humans, and functions mainly to deliver selenium to other body organs after its synthesis in the liver. SelW derives its name from 'white muscle disease', a disorder commonly seen in livestock from regions with low selenium soil levels [14]. It is similar to the GPx family in terms of its interaction with glutathione, presence of a redox motif, and evidence suggesting its antioxidant properties in cells [15].

Sequencing of the human genome led to the identification and characterization of the following selenoproteins through sequence homology of the SECIS element. SelH expression is localized to the nucleus, and overexpression studies suggested that it was a redox-responsive DNA-binding protein and also functioned as a transcriptional regulator of genes involved in *de novo* glutathione synthesis and phase II detoxification [16]. Studies also showed that SelK was localized to the ER and plasma membrane, with a relatively high expression in human heart where it decreased levels of ROS through unknown mechanisms [17]. SelR functions as a methionine sulfoxide reductase [18], while SelS is a transmembrane protein important in the removal of misfolded proteins from the ER membrane, and protecting cells from oxidative damage and ER stress-induced apoptosis [19, 20]. SelN plays a potential role in early muscle development, and is involved in calcium mobilization from ER [21]. SelI is the mammalian form

of ethanolamine phosphotransferase I, involved in phospholipid synthesis [22]. SelV expression is specific to testes [23], but its potential role in male reproduction is still under investigation. Recently, the ER-stress regulated 15-kDa selenoprotein (Sep15) was shown to play a critical role in redox homeostasis and glycoprotein folding through its interaction with UDP glucose:glycoprotein glucosyltransferase, with evidence suggesting its function in the maintenance of lens transparency [24]. SelM is a thiol-disulphide oxidoreductase, localized to the ER, with an NMR structure very similar to that of Sep15, suggesting similar roles [25]. Selenophosphate synthetase (SPS) 2 is a selenoenzyme that catalyzes the conversion of selenide to selenophosphate, which is the selenium donor for Sec, and thus essential for the formation of all selenoproteins [26]. Other selenoproteins like SelT and SelO have largely unknown functions.

Selenoproteins are collectively essential for life, as demonstrated by early embryonic lethality in mice upon targeted disruption of ^{Sec}tRNA gene (*Trsp*) [27, 28]. The antioxidant activity of selenium, through GPx and other selenoproteins, may be particularly relevant in individuals with low dietary selenium intake and may explain the protective effects of selenium against cancer as well as cardiovascular disease, and viral and parasitic infections [29]. In addition, enhancement of immune function and inhibition of tumor cell proliferation, which are mostly dependent upon specific metabolic pools of selenium like selenodiglutathione, hydrogen selenide and methylselenol, have been observed at supplemental doses, but the exact mechanisms are poorly understood [29-31]. However, to truly understand the role of selenoproteins in physiological and pathological processes, it is important to review the unique machinery and steps involved in selenoprotein biosynthesis.

Selenoprotein Biosynthesis

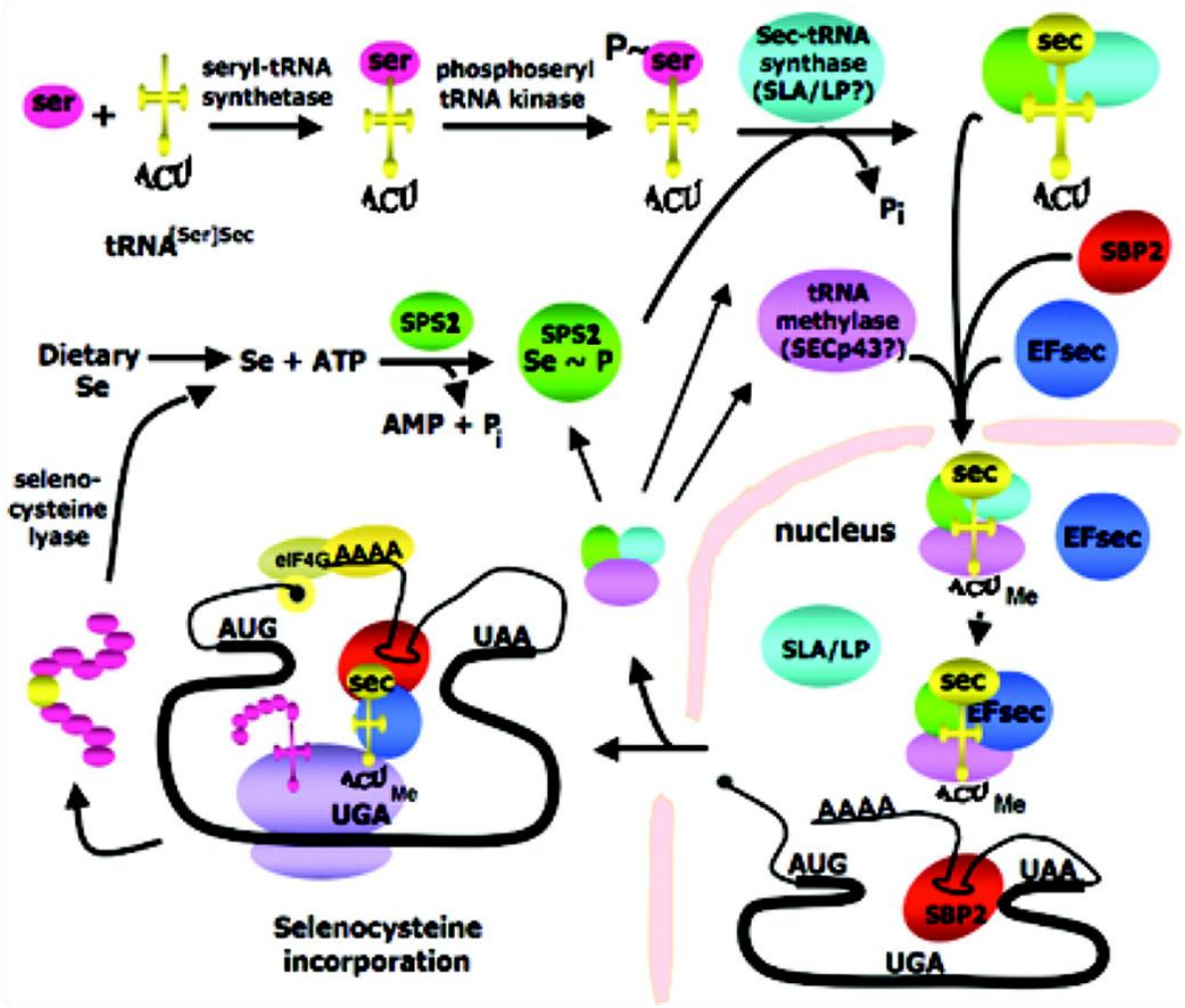

Fig. 1 Selenocysteine biosynthesis and incorporation into selenoproteins. Aminoacylation of Ser tRNA$^{[Ser]Sec}$ and its conversion to Sec tRNA$^{[Ser]Sec}$, via the actions of SPS2 (green) and the putative Sec tRNA$^{[Ser]Sec}$ synthase (aqua) are depicted along the top. Recruitment of SECp43 (purple) to the tRNA is depicted at the top right. Shuttling of the complex of Sec-tRNA$^{[Ser]Sec}$ and enzymes into the nucleus and association with EF_{sec} (blue), SBP2 (red), and the SECIS element are depicted along the right side, and cytoplasmic export and translation are shown along the bottom. Me, the methyl group added at the wobble base of Sec tRNA$^{[Ser]Sec}$. (Adapted and modified from Small-Howard A *et al.* Mol. Cell. Biol., 2006)

Selenium is co-translationally inserted into proteins as Sec. Strong evidence in support was provided by studies that showed that Sec is attached to its own tRNA and also biosynthesized on its own tRNA, which decodes the nonsense codon UGA [32, 33]. The central component in selenoprotein biosynthesis is Sec $tRNA^{[Ser]Sec}$, which is the only known tRNA that governs the expression of the entire class of selenoproteins [34]. Sec $tRNA^{[Ser]Sec}$ is initially aminoacylated with serine, which serves as a backbone for synthesis of Sec on the tRNA [35]. Although this reaction is catalyzed by seryl tRNA synthetase and the identity elements in Sec $tRNA^{[Ser]Sec}$ are for serine and not Sec, the amino acid inserted into the protein is Sec; hence this tRNA is termed Sec $tRNA^{[Ser]Sec}$ [34]. The next step involves the formation of an aminoacrylyl intermediate upon the removal of hydroxyl group from serine by a pyridoxal phosphate-dependent Sec synthase, as characterized in *E.coli* [36]. Monoselenophosphate, the active form of selenium is then donated to this intermediate in Sec biosynthesis. The enzyme selenophosphate synthetase 2 (SPS2), a selenoprotein, catalyzes the synthesis of monoselenophosphate from selenide (H_2Se) and ATP [37]. The biosynthesis of Sec $tRNA^{[Ser]Sec}$ is thus completed. The next step is incorporation of Sec into the nascent polypeptide during translation. In addition to Sec $tRNA^{[Ser]Sec}$ and the in-frame UGA codon in the selenoprotein mRNA transcript, several other factors are required for donation of Sec to protein, including (i) the *cis*-acting Sec insertion sequence (SECIS) element; (ii) the SECIS-binding protein 2 (SBP2); (iii) the Sec-specific elongation factor (EF_{sec}); and (iv) the ribosomal protein L30 [6, 38-42]. The highly conserved stem-loop structured SECIS elements, present in the 3' untranslated regions (3'-UTRs) of all eukaryotic selenoprotein genes, direct/recode a UGA codon within the coding region to serve as Sec [38] by recruiting SBP2 to form a stable SBP2-SECIS complex [39]. Intriguingly, SBP2 is also observed to be stably associated with 28S rRNA of ribosomes in a Sec-independent manner, suggesting that SBP2/SECIS element interaction occurs only during UGA recoding and that SBP2 thus selects

competent ribosomes for Sec insertion [39, 43]. Furthermore, SBP2 binds EF_{sec}, which functions to specifically recruit Sec $tRNA^{[Ser]Sec}$ and insert Sec into nascent polypeptides. Finally, L30 is known to trigger the conformational transition of the SECIS element from an open to kinked form by competing with SBP2, suggesting that L30 and SBP2 act sequentially during UGA recoding to recruit EF_{sec} and deliver Sec $tRNA^{[Ser]Sec}$ to the ribosomal 'A' site during translation of selenoproteins [42, 43]. Recently, two additional proteins, SECp43 and SLA (soluble liver antigen), have been shown to play a role in selenoprotein biosynthesis; they interact with the Sec $tRNA^{[Ser]Sec}$ complex and help in its shuttling across different cellular compartments [44].

Thus Sec is very different from any other of the 20 amino acids in the mode of its biosynthetic machinery and incorporation into proteins. It essentially requires a structural element in mRNA (SECIS) in addition to the UGA codon; it is synthesized on its own tRNA; and it employs additional Sec-specific components. Because of these unique features, it has been proposed that Sec was added to an already existing genetic code to take advantage of the unique redox properties of Sec which are superior to those of Cys for counteracting environmental stress and evolve new biological functions [6, 45]. These intriguing properties include: (1) the superior nucleophilic nature and better leaving group ability of Sec relative to Cys because of a significantly lower pKa (5.2 vs. 8.3), and (2) the superior ability of selenium to accept electrons (electrophilicity) as compared to sulfur [46]. Accordingly, evidence suggests that the essential 'chemico-biological' function why there is a pressure on the genome to maintain such complex Sec-insertion machinery is the ability of selenoenzymes to resist inactivation by irreversible oxidation [46] and control cellular redox status.

Selenoproteins in Scavenging RONS and Regulating Cellular Redox Status

Reactive oxygen and nitrogen species (RONS) are generated as a result of cellular processes like mitochondrial oxidative phosphorylation, and action of enzymes such as NADH/NADPH oxidase, p450 monooxygenases, lipoxygenases (LOX), cyclooxygenases (COX), and xanthine oxidase (XO) [47]. An imbalance between the antioxidant defense system in cells and overwhelming levels of pro-oxidant species leads to the accumulation of RONS resulting in oxidative stress. Several selenoproteins serve as antioxidant enzymes to mitigate the damage caused by RONS. Three major biological reactions that selenoenzymes catalyze are shown in Fig. 2 to demonstrate their roles as redox regulators [48].

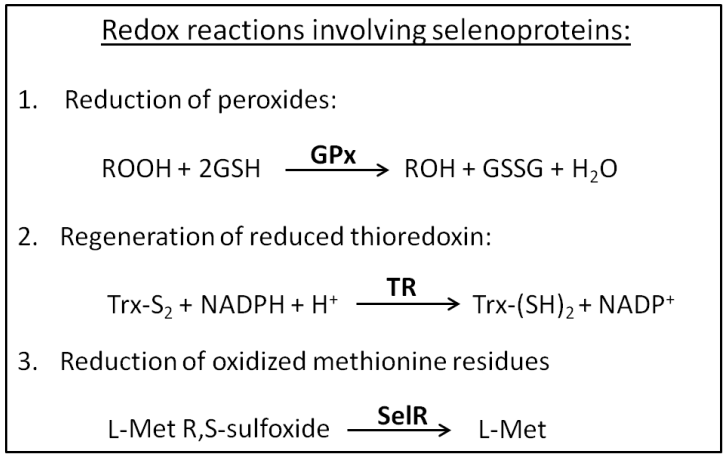

Fig. 2 Examples of important redox reactions catalyzed by selenoproteins. In the first reaction, glutathione peroxidase (GPx) uses glutathione (GSH) as a substrate to reduce different forms of peroxides (R-O-O-H). In the second, reduced thioredoxin (Trx) is regenerated by using NADPH in a reaction catalyzed by thioredoxin reductases (TR). In the third reaction, methionine sulfoxides are reduced back to methionine by selenoprotein R (SelR, also called MsrB1). (Adapted and modified from Reeves & Hoffman, 2009)

Levels of RONS influence the expression of inflammatory genes, where selenoproteins play a pivotal role in regulating the oxidative tone of immune cells thereby affecting the outcome in the form of inflammatory disease phenotypes. For instance, during inflammation, neutrophils

and macrophages are recruited to the site and their “respiratory burst” action leads to a massive generation of RONS. H_2O_2 is a short-lived, but highly toxic ROS produced as a result of NADPH oxidase activity during the respiratory burst [49]. The GPx family of enzymes combats the buildup of RONS by reducing H_2O_2 and other reactive fatty acid hydroperoxides (FAHP) to water and less reactive corresponding alcohols by utilizing GSH, one of the abundant small molecular weight thiol-containing antioxidants synthesized *de novo* in mammalian cells, as an electron donor [48, 50]. Glutathione disulfide, GSSG, produced in the process is reduced back to GSH by glutathione reductase that uses reducing equivalents in the form of NADPH. GSSG is normally maintained as less than 1% of total glutathione [50]. GPxs form a major component of the antioxidant machinery in cells that maintains the optimal GSSG: GSH ratio. Such a mechanism of control of ROS is seen in the gastrointestinal epithelium where GPx2 serves as the first line of defense against oxidative stress induced by ingested pro-oxidants or gut microbes [48]. The substrate specificity of GPx2 is similar to that of GPx1, where H_2O_2, *tert*-butyl hydroperoxide, cumene hydroperoxide, and linoleic acid hydroperoxide, but not phophatidylcholine hydroperoxide serve as preferred substrates [51]. The ability of GPx2 to quench inflammation-related increases in hydroperoxides suggests a protective role for this selenoprotein in gastrointestinal inflammation and cancer development [52]. GPx3 is unique among the GPxs in that it is a secreted enzyme and serves as a local extracellular antioxidant, constituting 20% of the selenium in plasma [53]. GPx3 has an important cardioprotective role suggested by studies, which show impaired metabolism of RONS as a result of reduced GPx3 activity resulting in insufficient nitric oxide (NO) levels, affecting normal platelet homeostasis and predisposing subjects to arterial thrombosis [54]. In contrast to other GPx enzymes, the most preferred substrate for GPx4 is the phospholipid hydroperoxide in membranes [55]. GPx4 has been recently demonstrated to be a critical sensor of oxidative stress and a transducer of cell death

signals [56]. In addition, GPx4 has a fundamental role in sperm maturation and structural integrity of spermatozoa because of its protein-thiol peroxidase activity [52].

The other major cellular redox system is the thioredoxin reductase (TR), thioredoxin (Trx), and NADPH system. Reduced Trx is used by several cellular enzymes as a cofactor in dithiol-disulfide exchange reactions. Mammalian TRs have a conserved N-terminal disulfide domain CysXXXXCys and a C-terminal active site sequence Gly-Cys-Sec-Gly, which also confers mechanistic advantages like the ability to function at an acidic pH [57]. The Trx/TR system catalyzes the reduction of protein disulfides such as in ribonucleotide reductase- an enzyme essential for DNA synthesis [58], thioredoxin peroxidases (peroxiredoxins) – crucial enzymes against oxidative stress [59], protein disulphide-isomerase (PDI) – a major enzyme within the ER [60]. TR also reduces disulphide bonds in NK-lysin, an antibacterial polypeptide naturally produced by T-cells, thus protecting the cell against the cytotoxicity of NK-lysin [61]. Besides macromolecules, many small molecules are efficient substrates of TR. These include vitamin K, alloxan, dehydroascorbate, lipoic acid, and S-nitrosoglutathione (GSNO) [52]. TR plays a part in the metabolism of selenium compounds like selenite, selenodiglutathione, methylselenite, selenocysteine, and ebselen, and in controlling selenoprotein synthesis [62-65]. The essential nature of TR1 and TR2 is seen in mice, which demonstrate that genetic deletion is embryonically lethal due to their role in DNA synthesis and cell proliferation [66]. TR2 is also involved in control of mitochondrial redox processes where the oxido-reductase plays a role in apoptosis signaling by reducing cytochrome *c* [67]. Finally, TR is a negative regulator of HIV-1 encoded transcriptional regulator, Tat, where the disulfide bonds between critical thiol residues are targeted by TR leading to inactivation of Tat [68].

There is a very strong relationship between oxidative stress and inflammation, as RONS function as second messengers to propagate inflammatory signals [69, 70]. This includes activation of pathways like MAPK/AP1 and NF-κB, which are central to inflammation [70, 71]. Through such effects, oxidative stress damages vascular endothelial cells and exacerbates cardiovascular disease processes like hypertension, atherosclerosis and congestive cardiac failure. Moreover, oxidative stress has also been implicated in other pathologies like arthritis, Alzheimer's disease, Parkinson's disease, HIV-AIDS, and many cancers, via its ability to promote chronic inflammation [47]. The fact that numerous epidemiological studies show the predisposition of such diseases in deficiency of selenium and that selenoproteins are the mainstay of the cellular antioxidant defense system suggest that selenium's role in physiology extends beyond an anti-oxidant agent to an element essential for mitigating inflammation.

Inflammation and Arachidonic acid – Cyclooxygenase Pathway

Inflammation [*inflammare* (L.) – to set on fire] is the body's protective reaction to injurious stimuli in the form of infections, trauma, foreign particle, auto-antibodies, ionizing radiation, and physical, chemical or thermal stress. From a pathology standpoint, inflammation is characterized by vascular events such as vasodilation and exudation of plasma, and cellular events in form of infiltration of the affected tissue by cells of the immune system such as neutrophils and macrophages, which are mainly responsible for orchestrating the events of inflammation. Macrophages play a role in appropriately sustaining an inflammatory response and bringing a timely resolution. A tight regulation of macrophage function is thus critical to prevent the progression of a protective, physiological, acute inflammatory response to a destructive, pathological, chronic inflammatory state. Chronic inflammation forms the basis of widely prevalent diseases, which constitute a major healthcare burden world-wide.

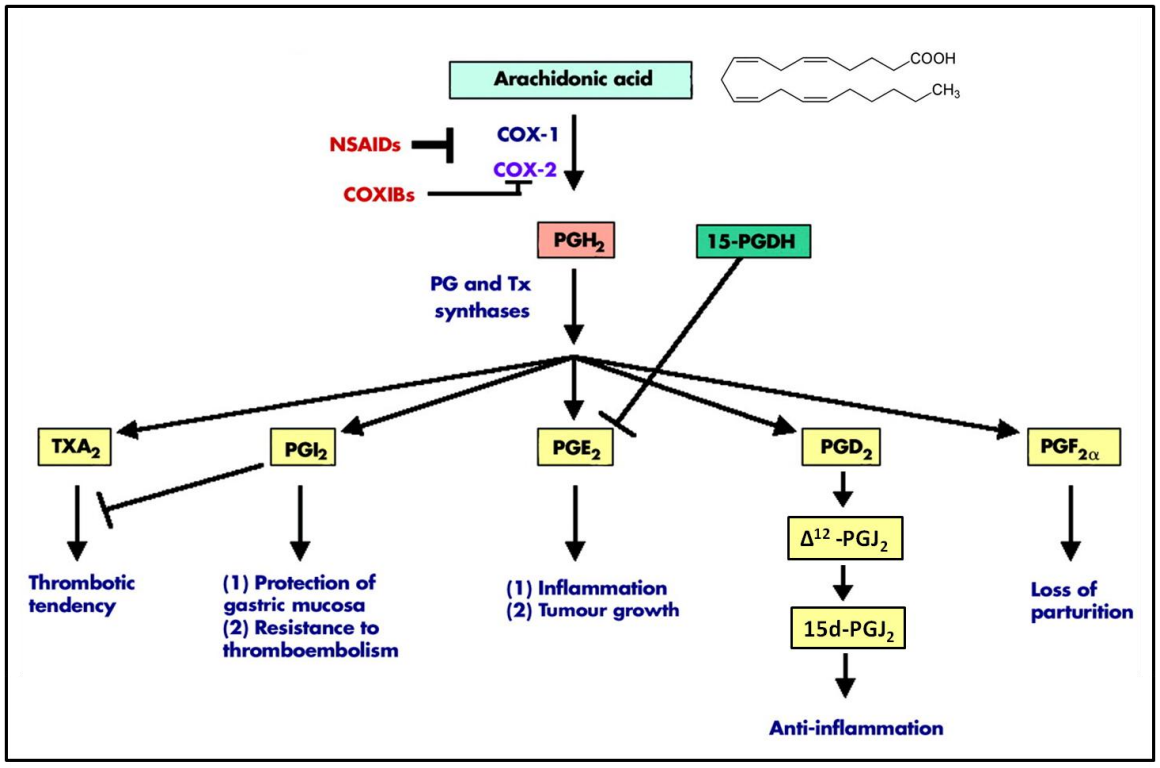

Fig. 3 Arachidonic acid (AA) – cyclooxygenase (COX) pathway. AA is formed by the action of phospholipase A_2 (PLA_2) on cell membrane phospholipids. COX enzymes act on AA to generate prostaglandin H_2 (PGH_2). PGH_2 is acted upon by various PG synthases to give rise to different PGs. 15-prostaglandin dehydrogenase (15-PGDH) is a metabolizing enzyme, predominantly degrading PGE_2. NSAIDS: non-steroidal anti-inflammatory drugs; COXIBs: selective COX-2 inhibitors (Adapted and modified from D. Wang and R. N. DuBois, *GUT*, 2006).

Apart from undergoing the oxidative 'respiratory' burst, 'classical' macrophage activation involves the production of protein mediators like IL-1, IL-6 and TNF-α, as well as lipid mediators like the arachidonic acid (AA)-derived eicosanoids - prostaglandin (PG)E_2, PGD_2, thromboxane (TX)A_2, and 15d-PGJ_2 [72]. The vascular endothelial cells produce PGI_2 (prostacyclin), which plays an important role in mitigating the vascular events of inflammation by counteracting the effects of TXA_2 [73]. The cell membrane-derived, 20-carbon fatty acid, AA, is acted upon by cyclooxygenase (COX)-2, an enzyme that is rapidly induced by inflammatory stimuli. The resultant product, PGH_2, can be converted into a variety of different prostaglandins through

reactions catalyzed by prostaglandin synthase enzymes, namely, microsomal PGE synthase (mPGES-1), hematopoietic PGD synthase (H-PGDS), TXA_2 synthase (TXAS), and prostacyclin synthase (PGIS) depending on the state of inflammation [73]. A crucial role of AA-derived lipid mediators has been demonstrated in the initiation and resolution of acute inflammation by shifting from pro-inflammatory PGE_2 to anti-inflammatory PGD_2 and its metabolite, 15d-PGJ_2, using a wound repair model [74]. Serhan *et al.* revealed the presence of lipid mediators derived not only from AA but also from eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), known as resolvins and protectins, respectively, with effective anti-inflammatory and pro-resolving properties [75]. Given that lipid mediators play a dual role in inflammation, many recent studies have focused on studies related to the regulation of signal transduction pathways where cellular oxidative stress and redox tone of cells act as key factors that modulate inflammation.

Selenium and Inflammation

Epidemiology: Selenoenzymes, especially those belonging to the GPx and TR families, are involved in regeneration of antioxidant systems, maintenance of intracellular redox state and membrane integrity, as well as gene regulation by redox control of binding of transcription factors to DNA [76]. Epidemiological studies show that inadequate levels of selenium and the associated production of RONS is linked with a higher incidence of cardiovascular diseases, progression of viral infections including HIV-AIDs, Alzheimer's disease, and infertility, where inflammation plays a significant role in pathogenesis [76, 77]. Kashin-Beck or 'big bone' disease is a disabling osteoarticular disease, involving the growth plate and joint cartilage, that has affected more than three million people in a small, endemic region starting from lower Siberia,

crossing northern China, and ending in central Tibet, where the soil is known to be extremely low in selenium [78].

Supplementation of selenium improves the health status of patients suffering from inflammatory conditions [79-82]. A randomized, double-blind, placebo-controlled trial in asthma patients showed an improvement in quality of life (QoL) indices of individuals consuming selenium supplementation [83]. In animal studies, adequate selenium supplementation facilitated significant response to ovalbumin challenge in lung in terms of lung cytokine levels, eosinophilia, anti-ovalbumin immunoglobins, goblet cell hyperplasia, STAT-6 levels and airway hyperactivity [84]. In a case-control study nested within a Finnish cohort, serum selenium was observed to be inversely linked to the occurrence of rheumatoid factor-negative (RFn-RA) arthritis [85]. The study carried out on various sub-groups of RA patients indicated that the relative risk of developing RA was significantly higher in RFn-RA patients with low selenium intake, suggesting that selenium might prevent RA in certain patients with rheumatoid-inflammatory diseases. A dramatic reduction in mortality in patients with acute necrotizing pancreatitis was noted when they were administered selenium intravenously, in a small controlled trial in Germany [86]. Similarly, a trial in Manchester, UK, revealed that oral selenium supplementation reduced the pain and frequency of attacks in patients with chronic, recurrent pancreatitis [87]. Ischemia-reperfusion injury is one of underlying mechanisms for the development of fatal arrhythmias. A study in rats showed that oral selenium prevented the occurrence of such reperfusion injury and thus had a cardioprotective role [88]. Deficiency of selenium levels has been observed in patients with septic shock and septic shock-like conditions, and RONS are known to play a critical role in pathology through their destructive actions. Selenium supplementation in high doses has been shown to be a promising candidate to reduce mortality and improve the health status of such severely ill patients [81, 89, 90]. Thus, any

condition that is associated with oxidative stress or inflammation might be expected to be influenced by selenium levels.

Deficiency of selenium is also linked with the occurrence, virulence, or progression of viral infections. Oxidative stress induced by low selenium intake significantly mutates coxsackievirus B leading to development of cardiomyopathy in patients with Keshan disease [91, 92]. One of the earliest studies relating selenium deficiency and HIV infection was the one by Dworkin and colleagues in 1988. They provided evidence that patients with HIV manifested with selenium deficiency as determined by diminished plasma and RBC GPx activity [93]. Many studies have indicated a significant correlation between decreasing CD4 lymphocyte counts and a progressive decline in plasma selenium levels in HIV/AIDS positive patients [94]. Baum *et al.* observed that selenium-deficient HIV patients are nearly 20 times more likely to die from HIV-related causes than those with adequate levels [95]. Selenium supplementation improves the immune status and viral handling (rapid clearance) of live attenuated poliovirus and a lower occurrence of mutations in the virus when given to adults with marginal selenium status [96]. If such effects were applicable to other RNA viruses like influenza and hepatitis, there would be considerable public health implications. The increasing virulence of certain strains of influenza virus in the selenium-deficient belt of China might thus be explained. From many such epidemiological studies, it is clear that the anti-viral effects of selenium reside in the ability of this micronutrient to modulate cellular signaling pathways.

Regulation of eicosanoid/AA pathway: Increasing interest in elucidating the immuno-modulatory and anti-inflammatory mechanisms of selenium has led to studies focusing on the regulation of the AA-pathway metabolism. Maddipati and Marnett [97] showed that the fatty acid hydroperoxide-reducing properties of plasma GPx prevented the deleterious inhibitory

effect of such hydroperoxides on the protective, AA-pathway regulator, PGIS. Separate studies using alveolar macrophages and lung neutrophils isolated from rats maintained on selenium-deficient diets further showed increased production of PGE_2 and TXA_2; these effects were attributed to modulated cellular hydroperoxide levels [98, 99]. In another study on an experimental myocardial ischemia model, rats fed with grains grown in selenium-deficient soil displayed higher activities of phospholipase A_2 (PLA_2), creatine kinase (CK), lactate dehydrogenase (LDH), and higher levels TXA_2, leukotriene C_4 (LTC_4) and lipid peroxides (LPO), with decreased levels of PGI_2 48 hours after ligation of the coronary vessels in rats [100]. Supplementation with selenium or vitamin E in the feed reversed the above effects; best results were seen when both antioxidants were combined. Using human placental explants, Eisenmann *et al.* demonstrated that different forms of selenium variably affected the ratio of TXB_2/6-keto-$PGF_{1\alpha}$, which is a major determinant of pre-eclamptic pregnancies [101]. Furthermore, selenium-deficient bovine mammary endothelial cells (BMEC) produced higher amounts of 15-hydroperoxyeicosatetraenoic acid (15-HPETE) and TXA_2, and lower amounts of PGI_2, leading to vascular dysfunction [102]. The importance of GPx1 was demonstrated for its role in the reduction of lipoxygenase (LOX) products of the AA-pathway, such as 12- HPETE to 12-hydroxyeicosatetraenoic acid (HETE) in platelets, and 5-HPETE to 5-HETE in leukocytes [103, 104]. Compared to their precursors, HETEs have a significantly lower potential in activating pro-inflammatory signals. All these studies support the hypothesis that the AA-pathway is redox sensitive and that selenium can modulate the levels of downstream AA-metabolites chiefly involved in the vascular events of inflammation.

Effects on NF-κB and PPARγ activation: Studies by Zamamiri-Davis *et al.* and Prabhu *et al.* have provided some insight on the regulation of target genes of inflammation as a result of modulation of AA-pathway by selenium [105, 106]. An inverse relationship between the level of

selenium and cellular levels of COX-2 and inducible nitric oxide synthase (iNOS) was demonstrated using a murine macrophage model, where a link between the up-regulation and activation of the nuclear factor-kappa B (NF-κB) family of transcription factors (Fig. 4) and selenium-deficiency was established.

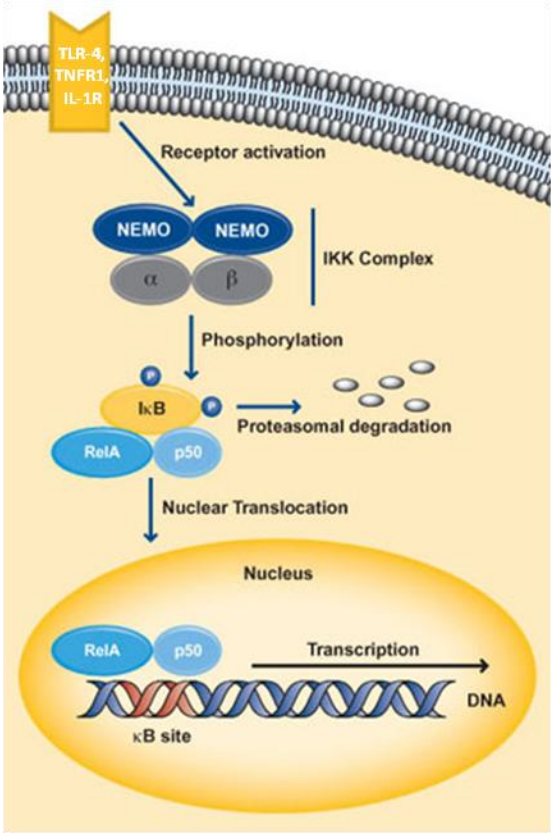

Fig. 4 Canonical (classical) pathway of NF-κB activation. NEMO, NF-κB essential modulator; IKK, inhibitor of kappa B (IκB) kinase; TLR-4, Toll-like receptor-4; TNFR1, TNF-α receptor; IL-1R, interleukin-1 receptor. (Adapted and modified from www.abcam.com)

Vunta *et al.* demonstrated that the anti-inflammatory effects of selenium supplementation on macrophages are due to the increased production of the cyclopentenone eicosanoid mediator, 15d-PGJ_2 [107]. As described later in this chapter, 15d-PGJ_2 inhibits the activation of NF-κB by the covalent modification of critical cysteine thiols of a rate-limiting enzyme, inhibitor of kappa

B kinase (IKK)-β, and also acts as a high-affinity ligand for the transcription factor, peroxisome proliferator-activated receptor (PPAR) γ, which activates a wide range of anti-inflammatory genes (Fig. 5). In addition to directly trans-activating anti-inflammatory genes, PPARγ also trans-represses various inflammatory genes through mechanisms involving sequestration of limited coactviators, or by 'sumolyation' of PPARγ that prevents the necessary exchange between co-repressors and co-activators for NF-κB-driven gene regulation [108, 109].

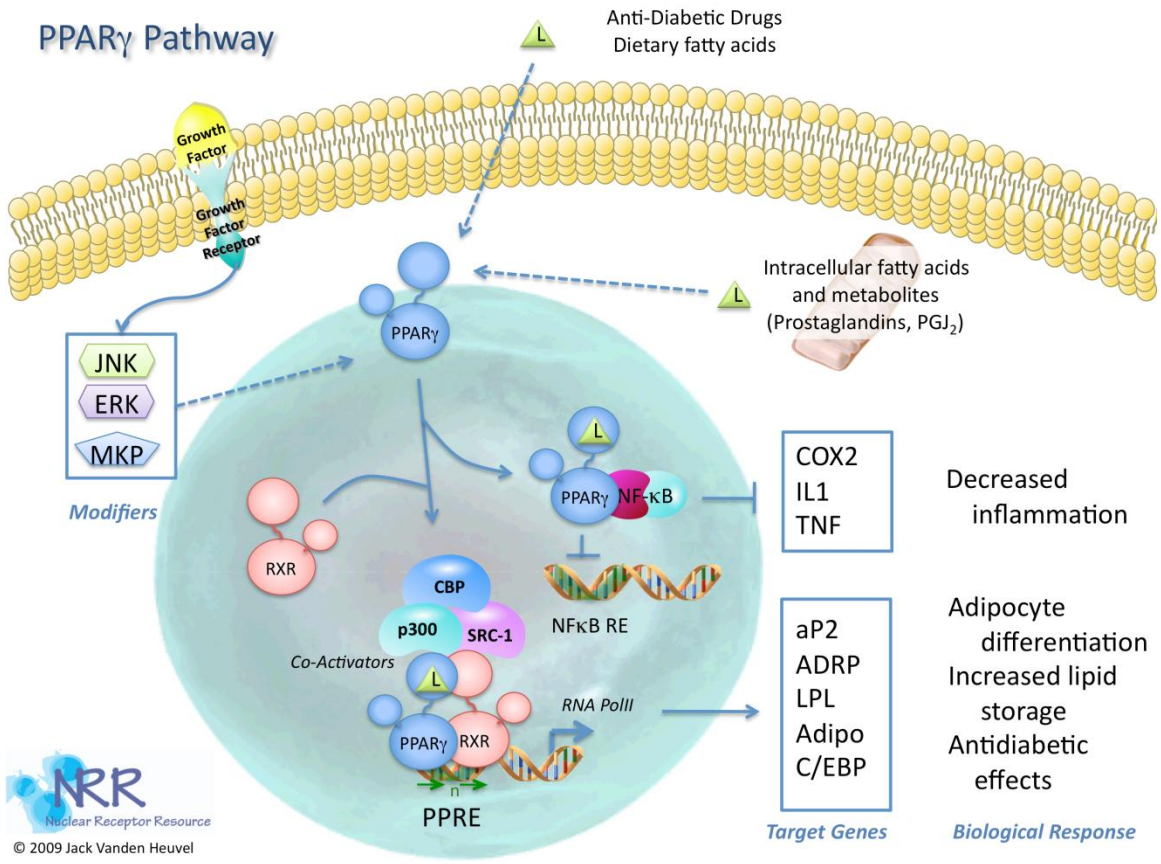

Fig. 5 PPARγ pathway with ligands, mechanisms of action, important target genes, and major effects. (From http://ppar.cas.psu.edu)

15d-PGJ_2 antagonizes the activities of other pro-inflammatory transcription factors like AP-1 and STAT3, while stimulating the anti-inflammatory transcription factor Nrf-1 in various cell types [110-113]. Given that these cyclopentenone PGs have the ability to differentially modulate the

activities of NF-κB, PPARγ as well as other transcription factors linked with inflammation, such a shunting was speculated to be responsible for the switching of macrophage phenotype from the 'classically' M1 activation state to the 'alternative' M2 activation state that favors resolution of inflammation. In fact, Nelson *et al.* demonstrated that effect by showing an increased expression of M2 markers such as *Arg-1*, *Fizz-1* and *Mrc-1* in selenium-supplemented murine macrophages [114]. Taken together, these recent studies indicate the role of selenium in modulating the production of AA-derived lipid mediators that can significantly affect cellular events of inflammation and perhaps facilitate resolution of inflammation.

Effects on WBC recruitment: Another important anti-inflammatory mechanism of selenium is thought to be mediated by its role in monocyte adhesion and migration. Monocytes adhere to endothelial cells and migrate towards tissues where they differentiate into macrophages with varied functions. This adhesion is mediated by expression of L-selectin on their cell surface during an inflammatory response. Selenium induces down-regulation of L-selectin on monocytes as a consequence of metalloproteinase-dependent shedding of this membrane-anchored adhesion molecule [115]. The recruitment of monocytes also involves endothelial expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial-leukocyte adhesion molecule-1 (E-selectin). TNF-α is a strong inducer of these adhesion molecules. One study indicated that selenite significantly inhibited TNF-α-induced expression of each adhesion molecule in a dose-dependent manner in human umbilical vein endothelial cells (HUVECs) [116]. A recent study performed in macrophages lacking the 'selenoproteome' (myeloid-specific *Trsp* disruption) showed that selenoprotein deficiency led to the atypical expression of extracellular matrix (ECM) –related genes, and diminished migration of macrophages in a protein gel matrix. Thus, selenium in form of selenoproteins may also regulate immunity and tissue homeostasis through ECM-related trans-regulation and

macrophage invasiveness. Moreover, the anti-carcinogenic properties of supra-nutritional doses of selenium have been shown through mechanisms such as perturbation of tumor cell metabolism, induction of apoptosis, and inhibition of angiogenesis, which suggests a certain degree of overlap between the anti-inflammatory and anti-proliferative effects of selenium [117].

15d-PGJ_2 – Role in physiology, pathology, and pharmacology

Formation and Structure:

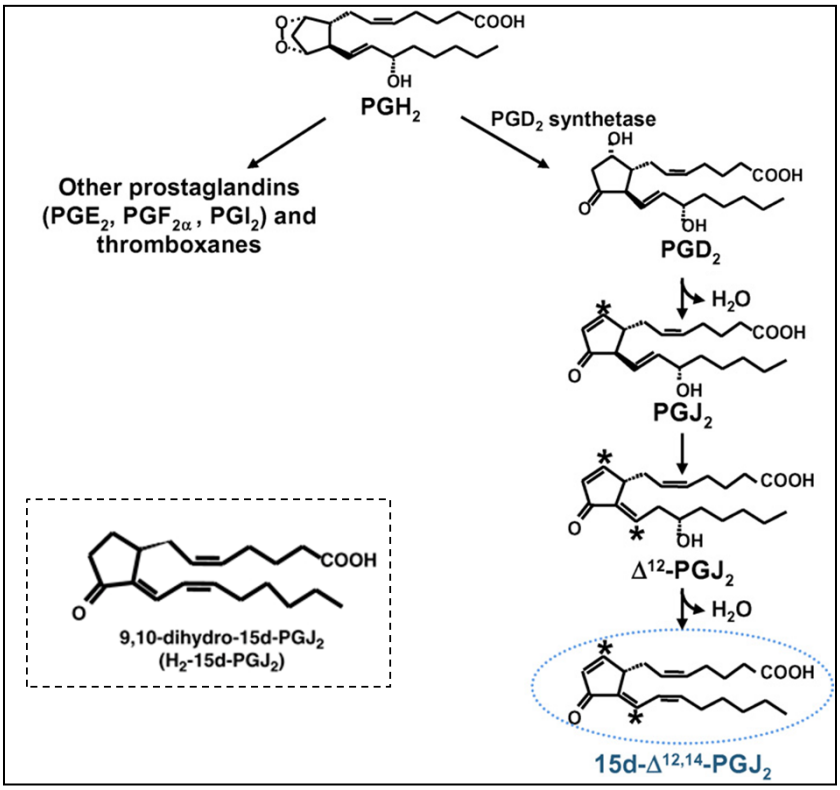

Fig. 6 Structure and formation of 15d-PGJ_2. PGD_2 is formed from PGH_2 in a reaction catalyzed by PGD_2 synthase. The subsequent steps include two non-enzymatic dehydration reactions to form 15d-PGJ_2. The inset shows the structure of the 15d-PGJ_2 analogue, 9, 10-dihydro-15d-PGJ_2, which lacks the reactive α,β-unsaturated carbonyl moiety. The asterisk (*) indicates reactive carbons in the structure. (Adapted and modified from Kim and Surh, Biochemical Pharmacology, 2006).

As described in earlier sections, the first step of PG synthesis is the release of arachidonic acid (AA) from membrane phospholipids by phospholipase A_2. Cyclooxygenase (COX-1, 2) convert AA to PGH_2 followed by the isomerization of the 9, 10-endoperoxide group of PGH_2 to form PGD_2 in a reaction catalyzed by PGD synthases, hematopoietic-PGDS (H-PGDS) and lipocalin-type PGDS (L-PGDS). PGD_2 spontaneously undergoes dehydration to form PGJ_2 (also referred to as Δ^{13}-PGJ_2), which undergoes further dehydration, coupled with a migration of the 13, 14-double bond, giving rise to 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) [118]. PGJ_2 can also convert stoichiometrically into Δ^{12}-PGJ_2 in an albumin-dependent manner [119]; recent studies have shown that eicosapentaenoic acid (C20:5; n=3)-derived PGJ_3 can form Δ^{12}-PGJ_3 even in the absence of albumin [120]. PGJ_2, Δ^{12}-PGJ_2, and 15d-PGJ_2 are collectively referred to as cyclopentenone prostaglandins (CyPGs). The *in vitro* synthesis of 15d-PGJ_2 was shown to be catalyzed by human serum albumin [121]. *In vivo* formation of 15d-PGJ_2 has been demonstrated by immunochemical procedures in different pathological models [119, 122, 123]. The 15d-PGJ_2 immunoreactivity was also detected in LPS-, carrageenan-, and IL-13- activated macrophages [112, 119, 124, 125]. Furthermore, the dependence of 15d-PGJ_2 synthesis on PGD_2 suggests that this formation is delayed as compared to other PGs, and that 15d-PGJ_2 has an important role during the resolution phase of inflammation [126]. However, the validity of immunological methods of detection has been questioned and it is clear that the gold standard for detection of such eicosanoids is mass spectrometry [127]. A highly sensitive liquid chromatography/tandem mass spectrometry assay for 15d-PGJ_2 has been reported by Bell-Parikh *et al.* [128].

15d-PGJ_2 possesses a cyclopentenone ring which readily reacts with molecules containing nucleophilic groups such as the cysteine thiol groups of proteins [129]. Such reactions are termed as Michael addition reactions. The α,β-unsaturated carbonyl group in the structure of 15d-PGJ_2 thus makes it a strong electrophilic molecule [130]. The importance of this reactive

group in some of the receptor-independent actions of 15d-PGJ_2 are evident from the observation that 9, 10-dihydro- 15d-PGJ_2, a 15d-PGJ_2 analogue that lacks the reactive α,β-unsaturated carbonyl group, could not reproduce many of the effects of 15d-PGJ_2 [120, 131, 132].

Biological targets and effects:

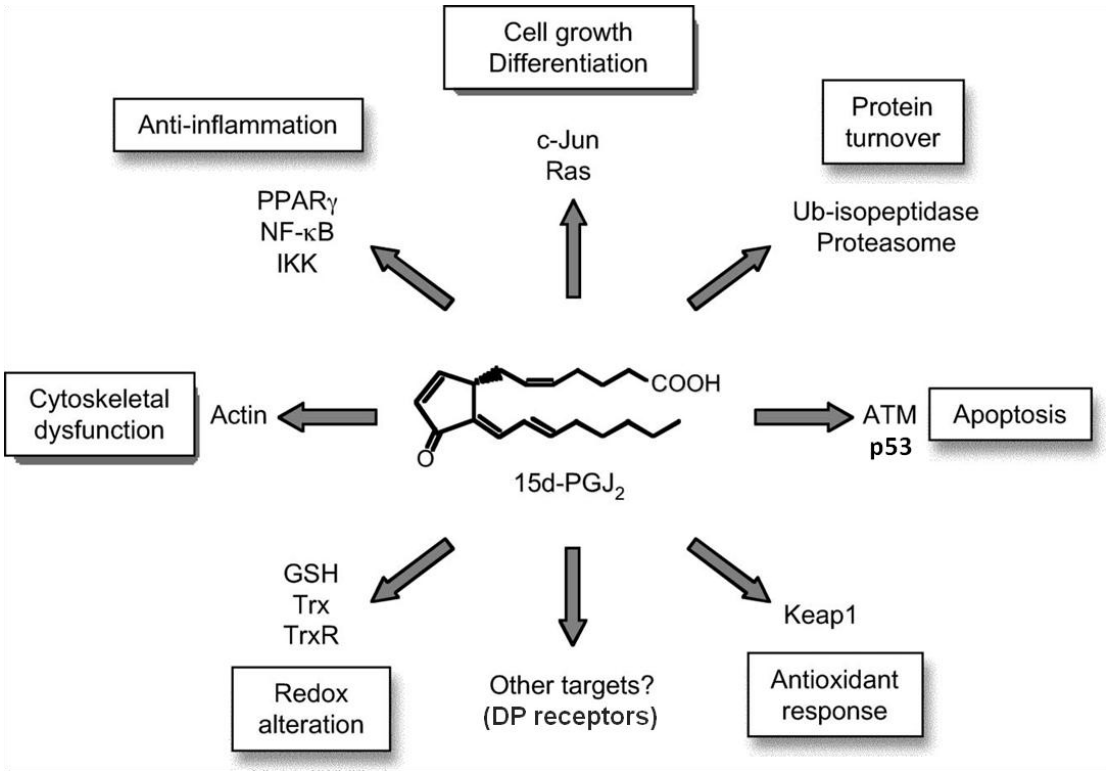

Fig. 7 Biological targets and effects of 15d-PGJ_2. (Adapted and modified from Uchida and Shibata, *Chemical Research in Toxicology*, 2008).

There are various ways how 15d-PGJ_2 exerts its multitude of effects (Fig. 7). Activation of cell surface DP receptors is one such mechanism. 15d-PGJ_2 has been shown to weakly bind DP_1 receptors on natural killer cells, neutrophils and other immune cells, and activate protein kinase A (PKA), which has potential anti-inflammatory effects [133, 134]. DP_2 or $CRTH_2$ (chemotactic receptor on TH_2 cells) on TH_2 cells, eosinophils, and basophils has also been identified as one of

the targets of PGD_2 and 15d-PGJ_2 [135, 136]. $CRTH_2$ is coupled with $G\alpha_i$ and its activation increases intracellular Ca^+ influx [137]. *In vitro* studies have suggested that 15d-PGJ_2 has pro-inflammatory effects in form of a potent chemoattractant of eosinophils at lower concentrations through the $CRTH_2$ receptor, however at higher concentrations, it inhibits eosinophil survival by inducing apoptosis via other mechanisms [138].

The most studied of the intranuclear targets of 15d-PGJ_2 is the peroxisome proliferator-activated receptor (PPAR) family of transcription factors, which regulate gene expression of proteins important in lipid homeostasis, inflammation, cell proliferation, and malignancy [139, 140]. 15d-PGJ_2 is an endogenous ligand for PPARγ [141]. PPARγ is one of the three distinct isoforms of the PPAR family (PPARα and PPARβ/δ being the other two) [142]. It is expressed in adipocytes, hepatocytes, macrophages/monocytes, myocytes, fibroblasts, breast cells, and human bone marrow precursors [143-145]. The electrophilic carbon at position 13, as a part of the core α,β-unsaturated carbonyl moiety of 15d-PGJ_2, reacts preferentially with the sulfur atom of Cys285 in the ligand-binding domain (LBD) of PPARγ [146]. Such a ligation/covalent modification of PPARγ by 15d-PGJ_2 induces the release of PPARγ from its corepressor and the PPAR-RXR heterodimer binds to PPAR response elements (PPREs) in the promoters of various target genes resulting in their transcriptional expression and/or repression [147]. Through this mechanism, 15d-PGJ_2 negatively regulates classical macrophage activation by antagonizing LPS-induced transcription responses of activator protein (AP)-1, NF-κB, and signal transducer and activator of transcription (STAT)-1 [148]. PPARγ shares a number of cofactors like CREB-binding protein (CBP)/p300 and steroid receptor coactivator (SCR)-1 with other transcription factors like AP-1, NF-κB, STATs etc. Expectedly, 15d-PGJ_2-activated PPARγ competes with these pro-inflammatory transcription factors for a limited quantity of CBP/p300 for the transactivation of IFN-γ promoter [149, 150]. PPARγ-dependent anti-inflammatory actions of 15d-PGJ_2 are

documented in animal models of arthritis [151, 152], ischemia-reperfusion [153], inflammatory bowel disease [154, 155], Alzheimer's disease [156], and lupus nephritis [157]. Similarly, 15d-PGJ_2 has cytoprotective actions from ischemic-reperfusion in the brain [158], apoptotic effects on endothelial cells [159] and breast cancer cells [160], as well as anti-angiogenic effects [161] through PPARγ-dependent mechanisms. Moreover, 15d-PGJ_2 also upregulates the expression [162, 163], DNA-binding activity [161, 164], and transcriptional activity of PPARγ [118, 165, 166].

Another major molecular mechanism through which 15d-PGJ_2 exerts its biological effects is direct covalent modification of transcriptional activities of redox-sensitive transcription factors like NF-κB, AP-1, p53, nuclear factor erythroid 2p45 (NF-E2)-related factor 2 (Nrf2), STAT, and hypoxia inducible factor (HIF)-1 [118]. This is possible due to the ability of 15d-PGJ_2 to form Michael adducts with cysteine thiols in various proteins (Fig. 8). NF-κB is a family of five transcription factors that exert pleotropic effects via numerous signaling pathways involved in the induction of pro-inflammatory genes like iNOS, COX-2, adhesion molecules, and cytokines [167]. 15d-PGJ_2 blocks the activation of NF-κB by covalent modification at multiple levels [168]. Cys179 in the active site of inhibitor of kappa B (IκB) - kinaseβ (IKKβ) is one such target [169]. In addition, 15d-PGJ_2 modifies the DNA-binding domains of NF- κB subunits: Cys62 of p50 or Cys38 of p65 [168]. It has also been shown to blunt NF- κB signaling by inhibiting IκBα degradation, nuclear translocation of NF-κB, activation of PI3K-Akt, and recruitment of p300 by p65 [170], where modification of Ser276 of p300 is critical [171]. Covalent binding of 15d-PGJ_2 to cysteine residues of Keap1 leads to increased nuclear translocation of the Nrf2 that result in anti-oxidant responses such as transcriptional activation of glutathione S-transferase (GSTs), NAD(P)H: quinone oxidoreductase 1 (NQO1), and heme oxygenase (HO-1) [112, 172, 173]. Similarly, 15d-PGJ_2 disrupts AP-1 activation by covalently modifying Cys269 of c-Jun and directly inhibiting the

DNA-binding activity of AP-1 [174]. This results in the down-regulation of AP-1-driven expression of COX-2, iNOS, eNOS, VEGF, and matrix metalloproteinases [118].

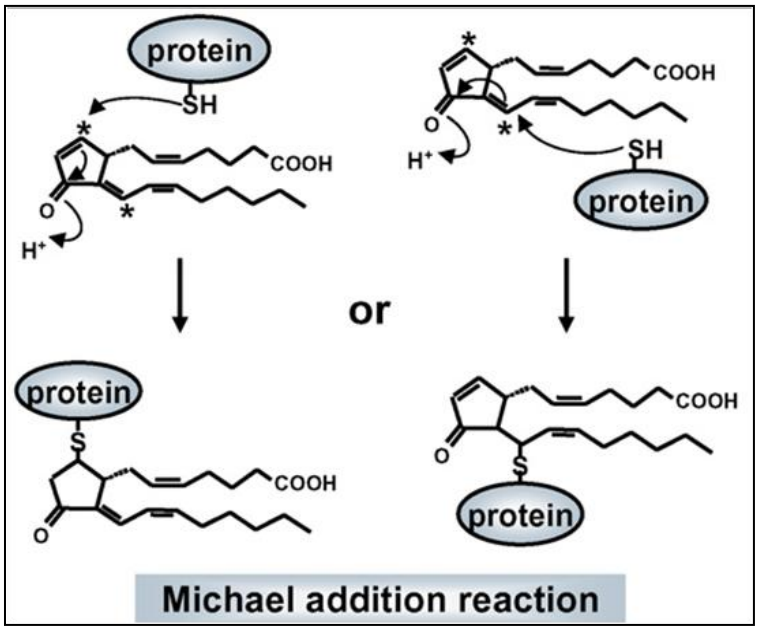

Fig. 8 Mechanism of covalent modification by 15d-PGJ$_2$ through Michael adduct formation. The α,β-unsaturated carbonyl group is responsible for some of the effects of 15d-PGJ$_2$ on intracellular proteins. The asterisks (*) indicate positions of reactive, electrophilic carbon atoms. (From Kim and Surh, *Biochemical Pharmacology*, 2006).

15d-PGJ$_2$ plays a role in resolution of inflammation by inducing apoptosis of activated macrophages [175]. With regards to its ability to induce apoptosis of tumor cells, 15d-PGJ$_2$ increases the expression, nuclear translocation, phosphorylation, and DNA binding activity of the pro-apoptotic, tumor suppressor p53 [123, 162, 176, 177]. Moreover, the presence of redox-regulated Cys277 in the DNA-binding domain of p53 [178] raises the probability of 15d-PGJ$_2$ interacting with p53 through that mechanism. 15d-PGJ$_2$ activates ATM (ataxia-telangiectasia mutated) through cysteine modification, leading to p53 activation and apoptosis [179]. 15d-PGJ$_2$ also downregulates the expression of anti-apoptotic proteins, cyclin B1 and survivin, which leads to inhibition of the G$_2$-M phase progression [180]. Nakata *et al.* showed that 15d-PGJ$_2$ has the

ability to induce death receptor 5 (DR5) expression through mRNA stabilization and promote tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis [181]. Reports also suggest that 15d-PGJ_2 can induce apoptosis of tumor cells via ROS generation [182, 183]. So far, the anti-apoptotic activity of 15d-PGJ_2 has been demonstrated in numerous cancer cells or cell lines including those associated with colon cancer [183, 184], ovarian cancer [185], non-small-cell lung cancer [123, 186], neuroblastoma [123], and leukemia [183, 187].

Thus, the biological effects of the cyclopentenone prostaglandin 15d-PGJ_2 are mediated not only through receptors such as PPARγ- and DP- mediated mechanisms but also through direct covalent modification of critical thiol groups in a variety of proteins, which vary depending on the concentration, cell type, intracellular redox status, and, most importantly, the accessibility of the thiolate anion group [118].

Leukemia and Leukemic Stem Cells

Leukemia is a cluster of malignant conditions of the hematopoietic system characterized by the uncontrolled proliferation of leukocytes (white blood cells). Leukemias are broadly classified as acute (immature blast cells) or chronic (terminally differentiated) depending on the degree of differentiation of the cells, and myeloid (granulocytic, monocytic, erythroid, megakaryocytic origin) or lymphoid (B-cell, T-cell, natural killer-cell origin) depending on the lineage of the predominant cell type involved. Accordingly, acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), chronic myeloid leukemia (CML), and chronic lymphocytic leukemia (CLL) are the four major types of leukemia. Each type has been further divided into subtypes based on other pathological criteria. The medical treatments for each of these are different and mainly consist of long periods of combined chemotherapy. However the success rates are variable. Allogenic hematopoietic stem cell (HSC) transplantation, commonly known as bone marrow

transplantation, is the only means of curing the diseases; the availability of a suitable donor and the cost of this method make it the last resort of treatment in most cases. Studies over the past few decades have focused on understanding the molecular mechanisms involved in the pathogenesis of different forms of leukemias for developing more efficient, targeted therapeutic approaches.

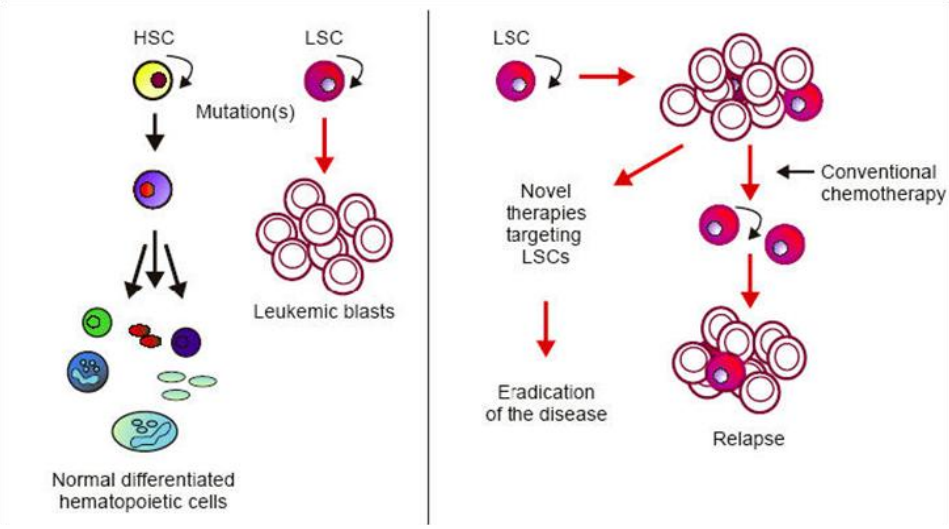

Fig. 9 Leukemic stem cell model of leukemia and the need to develop LSC-specific treatment strategies. HSC – normal hematopoietic stem cell; LSC – leukemic stem cell. (From Guzman and Jordan, *Cancer Control*, 2004.)

Leukemic stem cells: Normal stem cells are characterized by their ability for self-renewal, multi-lineage potential and extensive proliferative capacity. Certain mutations in normal stem cells give rise to cancer stem cells (CSCs) that drive the formation and growth of tumors [188]. There is enough evidence to suggest that myeloid leukemias are a result of such transformation events which force certain populations of hematopoietic stem cells (HSCs) to become leukemic stem cells (LSCs) [188-190]. Earlier, Park *et al.* showed that when mouse myeloma cells obtained from mouse ascites, separated from normal hematopoietic cells, were put in clonal *in vitro* colony-forming assays, only 1 in 10,000 to 1 in 100 cancer cells were able to form colonies [191]. Even when leukemic cells were transplanted in vivo, only 1–4% of cells could form spleen colonies

[192, 193]. These clonogenic leukemic cells were thought of as LSCs. Later, Bonnet and Dick demonstrated that most leukemic cells were unable to proliferate extensively and only a small, defined subset of cells was consistently clonogenic [194]. In that study, LSCs for human AML were identified and purified as $CD34^+$ $CD38^-$ cells from various patient samples. Despite representing a small proportion of the total AML cells (0.2–1%), LSCs were the only cells capable of transferring AML from human patients to NOD/SCID (non-obese diabetic/severe combined immunodeficiency) mice [194, 195]. These SCID-leukemia initiating cells (SC-IC) were exclusively $CD34^+CD38^-$, regardless of the FAB subtype of AML or the heterogeneity of lineage markers expressed by leukemic blasts, suggesting that normal primitive cells i.e. HSCs, rather than committed progenitor cells, were the target for leukemic transformation [194].

Chronic myelogenous leukemia (CML) is a myeloproliferative disease of clonal origin that is characterized by a chronic indolent phase (CP) of gradual mature myeloid cell expansion, and a terminal acute phase known as blast crisis (BC), where there is a massive rise of undifferentiated blasts. Nowell and Hungerford gave major insight into the clonal origin of CML through their finding of a common chromosomal abnormality in CML patient samples [196]. This unique chromosome, known as the Philadelphia (Ph) chromosome, is the result of a reciprocal translocation between long arms of chromosomes 9 and 22 [t(9:22)(q34;q11)] which leads to the fusion of breakpoint cluster (BCR) and human ABL1 genes. The resultant BCR-ABL protein is a constitutively active tyrosine kinase that induces transformation [197, 198]. The identification of BCR-ABL as the molecular entity responsible for CML led to the discovery of tyrosine kinase inhibitor imatinib mesylate (Gleevec^{TM}) as a drug that was extremely effective at inhibiting the proliferation of BCR-ABL expressing cells both *in vitro* and *in vivo* [199]. However, more than 95% of patients who achieve a complete remission with imatinib retain detectable levels of BCR-ABL mRNA, and patients who discontinue treatment almost invariably relapse [200, 201]. The

inevitable conclusion is that while imatinib is successful at eliminating the bulk tumor, it is unable to eradicate a certain small population of persistent cells that give rise to relapses. The residual BCR-ABL transcripts are found entirely in the HSC compartment, indicating that Ph^+ HSCs represent the CML LSCs [201]. Thus, the present consensus about the molecular pathogenesis for both AML and CML is that LSCs possess unique features and are quite different from more mature leukemic blasts just like normal stem cells, which are biologically distinct from their more differentiated progeny, with specific cellular and molecular mechanisms that control their behavior. This makes it critical to identify therapies that specifically target LSCs.

Selenium and Leukemias

Extensive studies have shown the protective effect of selenium in a variety of cancers [76, 202-204]. In addition to case-control and cohort observational studies, interventional trials carried out with supplemental selenium have demonstrated significant anti-carcinogenic effects against prostate, colon, lung, and liver cancer [205, 206]. However, there is only limited evidence providing a causal relation between selenium and leukemia. Asfour *et al.* found significantly low levels of selenium in serum from patients with AML; patients who achieved complete remission after induction had higher selenium levels [207]. The same group also demonstrated that high-dose sodium selenite treatment downregulated Bcl-2 and improved clinical outcome in patients with non-Hodgkin's lymphoma [208]. Anti-proliferative effects of sodium selenite have been shown on leukemic cells from patients with AML - M1, M3, M5, ALL (acute lymphocytic leukemia)- L1, L2, and CML, and were thought to be due to Se-dependent inhibition of DNA replication and expression [209]. In vitro studies suggest that sodium selenite induces apoptosis in human acute promyelocytic leukemia (AML-M3) cell line NB4 by a caspase-3-dependent mechanism and a redox pathway [210]. Interestingly, high doses of sodium selenite switch

signaling from pro-survival to pro-apoptotic pathways in NB4 and HL-60 cells by inducing severe ER stress [211]. Moreover, selenium inhibited DNA methylase enzyme, resulting in hypomethylated DNA and subsequent anti-carcinogenic activity in Friend erythroleukemia cells [212]. These reports collectively fail to provide any clear mechanism(s) for the anti-proliferative effects of selenium in leukemia. All studies thus far have only looked at either leukemic cells isolated from peripheral blood of human patients or leukemic cell lines. There is no data on the effects of selenium on LSCs.

Knowing the significance of LSCs on the pathogenesis of leukemia and the inadequate response to conventional treatments available, it is necessary to study the effects of selenium specifically on LSCs and elucidate the mechanisms of anti-proliferative action on this key population of leukemic cells. It is speculated that the constitutively activated NF-κB in primary AML LSCs is a crucial regulator of survival [213]. Parthenolide, a natural sesquiterpene lactone with IKK-inhibitory action, and MG-132, a proteasomal inhibitor that stabilizes IκB, have shown pro-apoptotic effects in AML LSCs [214, 215]. Parthenolide also leads to p53 induction and increased ROS production exclusively in LSCs [214]. However, because of unfavorable pharmacokinetic properties, this potential drug candidate could not achieve the required plasma concentrations when subjected to phase I clinical trials [216]. In addition, molecular pathway studies on the CML LSCs show therapies that target their cellular ROS production and phosphorylation status might be genuinely effective [217, 218]. Selenium inhibits the activation of NF-κB via increased production of 15d-PGJ_2 [219]. CyPGs of PGA series and $\Delta^{12,14}$-PGJ_2 have anti-proliferative activity towards L1210 murine leukemia cells [220]. Recently, 15d-PGJ_2 has also been shown to induce apoptosis in HL-60 leukemia xenograft model in nude mice [183]. It is worth noting that these studies utilized these eicosanoids at very high concentrations (micromolar range), which are not likely to be physiologically relevant.

Current strategies for eradication of LSCs

As described earlier, the chemotherapeutic agents used currently in clinical practice effectively eradicate the leukemic blast cells in most patients; but they have shown to be inactive against the LSCs, which are biologically distinct from blast cells as well as normal hematopoietic stem cells (HSCs) [190]. Since the discovery of LSCs about a decade ago, several of their critical and distinct biological characteristics have been demonstrated, including their unique self-renewal properties, cell surface markers, drug resistance mechanisms, and growth-promoting chromosomal and genetic events. The quiescent, non-cycling state of LSCs is one of the major reasons responsible for their resistance to conventional drugs and subsequent low rates of long-term remission [221]. Additionally, LSCs essentially acquire mutations that allow them to proliferate and persist in the presence of chemotherapeutic drugs, unlike a majority of the leukemic cells. Dysregulation of many molecular pathways has been implicated for such properties of LSCs and many strategies affecting these pathways are thus being investigated.

Cytoplasmic targets: Wnt/β-catenin signaling pathway is important for the self-renewal of HSCs, and its dysregulation has been demonstrated in LSCs in AML and CML [222-224]. Binding of Wnt to cell surface protein Frizzled leads to inactivation of Multiprotein destruction complex (MDC) and stabilization of β-catenin, which translocates to the nucleus and activates transcription of growth-promoting genes like cyclin D1, c-Myc, and SALL4 [221]. The self-renewal capability of CML LSCs has been shown to be decreased significantly by conditional deletion of β-catenin. So Wnt pathway inhibitors might be beneficial in CML patients. Recently, one such Wnt/ β-catenin inhibitor, AV65, was shown to inhibit growth of imatinib-resistant human CML cells *in vitro* [225]. Similarly, activating mutations in *Notch1* are found in more than half of all human T-cell ALLs. Treatment with a γ-secretase inhibitor that blocks ligand-induced Notch proteolysis and

signaling causes apoptosis of T-ALL cells [226], although effects on LSCs have not been studied. However, it is speculated that treatments directed against such self-renewal (Wnt and Notch) pathways may affect normal HSCs too, especially when in combination with other chemotherapy.

The PI3K/PTEN/Akt/mTOR pathway is another plausible target in AML LSCs. *Pten* (a lipid phosphatase that negatively regulates PI3K signaling) deletion leads to increased activation of Akt and mTOR in human AML blasts. Rapamycin, an mTOR inhibitor, eliminates LSCs in *Pten*-deleted mice, while rescuing the depletion of *Pten*-deficient HSCs [227]. Rapamycin and other mTOR inhibitors are being tested in clinical trials. The Ras/Raf/MEK/ERK kinase cascade has also been shown to be overexpressed in >70% of cases of AML [228]; the role of inhibitors of this pathway has been recently reviewed in *Leukemia* [229].

Nuclear targets: It has been known for more than a decade that the transcription factors of NF-κB family are constitutively activated in most AML LSCs [213]. Molecules like parthenolide and proteasome inhibitor, MG-132, drive apoptosis of these LSCs by downregulating NF-κB [213, 214]. The proteasome inhibitor, bortezomib, is already in phase I/II clinical trials [230]. TZDZ-8, a non-ATP competitive inhibitor of glycogen synthase kinase-3 beta (GSK-3β), also has significant NF-κB inhibitory activity and induces death of primary AML progenitor cells [190]. The successful addition of all-trans retinoic acid (ATRA) to anthracycline chemotherapy in curing acute promyelocytic leukemia (PML; or AML-M3) [231] implies that ATRA, which targets the oncogenic transcription factor PML-RARα (retinoic acid receptor-α), must be eliminating LSCs. Other transcription factors like HoxB4, HoxA9, Bmi-1, WT-1, PU.1, and JunB are also thought to be potential targets [221].

Membrane targets: LSCs may also develop drug resistance through decreased drug uptake and/or increased drug efflux. The ATP-binding cassette (ABC) transporters ABCB1, ABCG2, and ABCC1 are the three major multidrug resistance transmembrane proteins shown to be overexpressed on LSCs and associated with poor treatment outcome in AML patients [232]. Multi-drug resistance protein (MDR1) overexpression is another such event commonly observed. Unfortunately, the current generation MDR modulators are not as effective because these transporters are not unique to the LSCs [232].

Another strategy to eradicate LSCs is to target specific cell surface molecules expressed on LSCs using cytotoxic antibodies. Many likely candidate proteins have been investigated. Gemtuzumab ozogamicin, a recombinant humanized anti-CD33 monoclonal antibody conjugated with a cytotoxic antibiotic calicheamicin, has been shown to induce remissions in relapsed $CD33^+$ AML cases [233]. Similarly, a diphtheria toxin-IL-3 fusion protein used to target CD123, the receptor for IL-3, was toxic specifically for AML progenitors in NOD-SCID mice models [234]. CD44, CLL-1 (C-type lectin-like molecule-1), and VLA-4 ($\alpha_4\beta_1$ integrin) are some other targeted surface molecules [230].

Chapter 2

Selenoprotein-dependent upregulation of hematopoietic prostaglandin D_2 synthase in macrophages is mediated through the activation of peroxisome proliferator-activated receptor (PPAR) γ

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[**Contributions:** This study was co-performed by Naveen Kaushal (Figs. 11, 12B, C, D, E, and 16A, B), Kodihalli Ravindra (Figs. 1C, 12F, and 16C), Shakira Nelson (Fig. 15), and Hema Vunta (Fig. 13A, B)]

Abstract

The plasticity of macrophages is evident from their dual role in inflammation and resolution of inflammation that are accompanied by changes in the transcriptome and metabolome. Along these lines, we have previously demonstrated that micronutrient selenium (Se) increases macrophage production of arachidonic acid (AA)-derived anti-inflammatory 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), while decreasing the proinflammatory PGE_2. Here we hypothesized that Se modulated the metabolism of AA by a differential regulation of various prostaglandin (PG) synthases favoring the production of PGD_2 metabolites, Δ^{12}-PGJ_2 and 15d-PGJ_2. A dose-dependent increase in the expression of hematopoietic-PGD_2 synthase (H-PGDS) by Se and a corresponding increase in Δ^{12}-PGJ_2 and 15d-PGJ_2 in RAW264.7 macrophages and primary bone marrow-derived macrophages was observed. Studies with organic non-bioavailable forms of Se and the genetic manipulation of cellular Se incorporation machinery indicated that selenoproteins were necessary for H-PGDS expression and 15d-PGJ_2 production. Treatment of Se-deficient macrophages with rosiglitazone, a peroxisome proliferator-activated receptor (PPAR)γ ligand, upregulated H-PGDS. Furthermore, electrophoretic mobility shift assays indicated the presence of an active PPAR-response element (PPRE) in murine H-PGDS promoter suggesting a positive-feedback mechanism of H-PGDS expression. Alternatively, the expression of nuclear factor-κB-dependent thromboxane synthase and microsomal PGE_2 synthase was downregulated by Se. These results suggest the importance of Se in the shunting of AA metabolism towards the production of PGD_2 metabolites that may have clinical implications.

Introduction

The essential trace nutrient selenium (Se) plays a major role as a key component of the antioxidant machinery in all cell types. This ability of Se is attributed, in part, to the co-translational incorporation of amino acid selenocysteine (Sec) into the active site of selenoproteins, many of which have important enzymatic functions [5]. Proteins of the glutathione peroxidase (GPX) and thioredoxin reductase (TR) families are well characterized selenoenzymes that are involved in regeneration of antioxidant systems, and maintenance of intracellular redox state and membrane integrity, as well as gene regulation by redox control of binding of transcription factors to DNA [76]. Epidemiological studies suggest that inadequate Se levels and associated reactive oxygen and nitrogen species (RONS) production is linked to increased incidence of cardiovascular diseases, viral infections including progression of HIV-AIDS, Alzheimer's disease, infertility, and most notably, cancer [7, 76, 77, 235]. Supra-nutritional doses of Se have demonstrable anti-carcinogenic effects in a variety of cancers through mechanisms such as perturbation of tumor cell metabolism, induction of apoptosis, and inhibition of angiogenesis [117]. Also, supplementation of Se has been shown to improve the health status of patients suffering from inflammatory conditions such as septic shock, autoimmune thyroiditis, pancreatitis, allergic asthma, and rheumatoid arthritis [76, 79, 81, 82]. These anti-inflammatory actions can be partly explained by the antioxidant role of various selenoproteins, but the precise mechanisms in the context of specific cell types involved in inflammation need to be explored.

Macrophages are cells of the immune system that are central to the progression of a physiological, protective inflammatory response into a pathological, tissue destructive inflammatory state. An imbalance between the production of RONS and the scavenging anti-oxidant mechanisms forms the basis for such progression [236]. In addition to the oxidative

('respiratory') burst reaction, classical macrophage activation is also characterized by the production of several mediators such as IL-1, IL-6, TNF-α, prostaglandin E_2 (PGE_2), and thromboxane A_2 (TXA_2) [72]. PGE_2, TXA_2, PGD_2, and PGD_2 downstream products, Δ^{12}-PGJ_2 and 15d-PGJ_2, are the major eicosanoids derived from the fatty acid arachidonic acid (AA) in macrophages [73], while PGI_2 is predominantly produced by vascular endothelial and smooth muscle cells that express high levels of PGI_2 synthase (PGIS) [237]. The initial step of prostaglandin (PG) synthesis involves formation of PGH_2 from AA by the action of cyclooxygenase (COX)-1 and COX-2, which is subsequently acted upon by specific PG synthases, microsomal PGE synthase-1 (mPGES-1), thromboxane synthase (TXAS), and PGD synthase (PGDS), to form PGE_2, TXA_2, and PGD_2, respectively [73]. Studies have shown that during the resolution phase of inflammation, the AA metabolism shifts from the production of PGE_2 towards that of PGD_2 and its downstream double-dehydration product, 15d-PGJ_2 (13-14). This warrants further research into the regulation of the AA-COX metabolism pathway, which includes the various PG synthase enzymes. Oxidative stress in cells activates transcription factor nuclear factor-κB (NF-κB). NF-κB serves as a key transcription factor for mPGES-1 and TXAS, leading to the upregulation of PGE_2 and TXA_2, respectively [238, 239]. Our laboratory has previously shown that Se supplementation of macrophages downregulated NF-κB with a corresponding increase in the activation of PPARγ, thus affecting the production of PGE_2 [105, 106, 219].

Lipocalin-type PGDS (L-PGDS) and hematopoietic PGDS (H-PGDS) are two isoforms that catalyze the conversion of PGH_2 to PGD_2. While the former is expressed mainly in the CNS, the latter is found in immune cells like macrophages and mast cells, suggesting a role of H-PGDS in the regulation of inflammation [240, 241]. PGD_2 and 15d-PGJ_2 have been shown to possess anti-inflammatory properties, which are conferred via different mechanisms including the activation

of DP receptors, PPARγ activation, and covalent modification of cysteine thiols in target proteins such as IKKβ and p65 of the NF-κB signaling axis [118, 241]. However, not much is known about the molecular regulation of the glutathione (GSH)-dependent sigma-class glutathione S-transferase, H-PGDS. Studies from multiple laboratories suggest that AA pathway is redox sensitive [70, 97, 106]. Selenium has been shown to modulate the expression of TXAS and prostacyclin synthase (PGIS) in endothelial cells [102]. Furthermore, it has been previously reported that Se exerts its anti-inflammatory effects in activated macrophages via the enhanced production of 15d-PGJ_2 in a COX-1 dependent manner [107]. This led to the hypothesis that H-PGDS is regulated by the redox state of macrophages, which depends largely on their Se status as well as the differential modulation of PPARγ and NF-κB. Here it is demonstrated that Se supplementation of macrophages differentially regulates the expression of mPGES-1, TXAS, and H-PGDS to favor the shunting of AA through the H-PGDS pathway where selenoproteins are indispensable.

Methods and Materials

Reagents: Sodium selenite, methylseleninic acid (MSA), L-selenomethionine (Se-Met), bacterial lipopolysaccharide (*E. coli* 0111:B4), ebselen (2-phenyl-1, 2-benzoselenazol-3-one), indomethacin and DMSO were obtained from Sigma-Aldrich (St. Louis, MO). 1, 4-phenylene bis(methylene) selenocyanate (*p*-XSC) was provided by Dr. S. Amin, Penn State College of Medicine (Hershey, PA). CAY10526, HQL-79, 15d-PGJ_2**,** and rosiglitazone were purchased from Cayman Chemicals (Ann Arbor, MI). Recombinant L-methioninase (rMETase) and parthenolide (PTN) were from Wako Chemicals (Richmond, VA) and Calbiochem (San Diego, CA), respectively.

Cell Culture and Animals: Murine macrophage-like cell line RAW264.7 (ATCC, Manassas, VA) was cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) containing 5 % fetal bovine serum (ATCC), 2 mM L-glutamine (Cellgro, Manassas, VA), penicillin-G (100 U/ml), and streptomycin (100 μg/ml; Invitrogen). For routine culture, the cells were passaged every three days at a ratio of 1:10 as per the recommendations by ATCC. For experiments, the cells were seeded at a density of ~ 500,000/well of a 6-well plate and cultured for 2-4 days depending on the treatments. Selenite and Se-Met were dissolved in sterile water. *p*-XSC, ebselen, parthenolide, CAY10526, rosiglitazone, and 15d-PGJ_2 were dissolved in DMSO. The final concentration of DMSO in the cell culture medium was 0.1 % v/v. Human embryonic kidney (HEK293T) cells were used for extracting nuclear proteins for the EMSA. They were cultured in DMEM containing 10% FBS, 2 mM L-glutamine, and penicillin-streptomycin. Primary murine macrophages (BMDMs) were prepared by extracting femoral bone-marrow plugs from ~3-month old C57BL/6 mice maintained on Se-deficient (0.01 ppm selenite), Se-adequate (0.1 ppm selenite) and Se-supplemented (0.4 ppm selenite) diets for 12 weeks. These cells were then cultured in the same medium as described with the addition of 20% L929-fibroblast conditioned medium (as a source of granulocyte-macrophage colony-stimulating factor) for one week.

Macrophages from Se-deficient, Se-adequate, and Se-supplemented mice were cultured in defined media containing 7 nM, 100 nM, or 250 nM Se (as selenite). The special diets were formulated based on the American Institute of Nutrition recommended rodent diets, and purchased from Harlan-Teklad (Madison, WI). All animals for experimental use were approved by the institutional animal care and use committee of The Pennsylvania State University.

Glutathione Peroxidase Activity Assay: Cells cultured under different concentrations of sodium selenite (0-250 nM) were harvested, lysed with the mammalian protein extraction reagent (M-PER; Pierce) containing protease inhibitor cocktail and 1 mM PMSF. Whole cell lysates were used for the GPX assay. The GPX activity was measured using H_2O_2 and NADPH, and the activity was expressed as nmoles of NADPH oxidized per minute per milligram of protein in a spectrophotometric assay as described earlier [242].

Arginase Activity Assay: Arginase activity, assessed by a colorimetric assay that detects urea production generated by arginase hydrolysis of L-Arg, has been previously described [243]. Selenium -deficient and Se-adequate RAW264.7 macrophages assayed were cultured as described earlier. Optical density at 560 nm was recorded on a Packard plate reader. A urea standard calibration curve (0-1 µmoles; $y = 9E\text{-}05x + 0.0007$, $R^2 = 0.99$) was used to calculate the arginase activity. Enzyme activity is expressed as µmoles of urea produced/mg of protein [243].

SDS-PAGE and Immunoblotting: Whole cell lysates were prepared from RAW264.7 cells or BMDMs using M-PER containing protease inhibitors, and protein concentration was estimated by BCA reagent. Equal amount of protein (ranging from 5-25 µg) was electrophoresed on a 12.5% discontinuous SDS-polyacrylamide gel. Proteins were then transferred to a nitrocellulose membrane. The following primary antibodies were used to probe the membrane: anti-H-PGDS, anti-TXAS, anti-mPGES-1 (Cayman), anti-GPX1, anti-TR1 (Abcam, Cambridge, MA), and anti-

GAPDH (Fitzgerald, Concord, MA). Appropriate secondary antibodies were used. Immunoreactive bands were visualized using the West Pico and/or West Femto chemiluminescence reagents obtained from Thermo Scientific (Rockford, IL). GAPDH was used as an internal control for normalization. Bands were evaluated by densitometry using the Image J software program (National Institutes of Health).

Real-time (quantitative) and Semi-quantitative RT-PCR: RNA was isolated from RAW264.7 cells or BMDMs using the Isol-RNA lysis reagent (5 Prime, Gaithersberg, MD) as per its protocol. RNA purity and concentration was determined using UV-spectroscopy. 2 µg of DNAse (New England Biolabs) - treated RNA was used for reverse transcription reaction using High Capacity cDNA Reverse Transcriptase kit, per the manufacturer's instructions (Applied Biosystems, Foster City, CA). Pre-validated TaqMan probes for *Hpgds*, *Mpges-1*, *Txas* and *Pgis* were purchased from Applied Biosystems (Foster City, CA). *Gapdh* probe was used as an internal control to normalize the data. Amplifications were performed using PerfeCTa qPCR SuperMix Master Mix (Quanta Biosciences) in a 7300 Real-time PCR system (Applied Biosystems). ΔCt ($Ct_{Gene} - Ct_{Gapdh}$) was calculated for each sample and used for analysis of transcript abundance with respect to the untreated negative control as described [244]. For the semi-quantitative RT-PCR, the sequences of the primers are: mouse *Hpgds* sense 5′-ATGCCTAACTACAAACTGCTT-3′ and antisense 5′-CTAGAGTTTTGTCTGTGGCCT-3′; mouse *Gpx1* sense 5′-ACAGTCCACCGTGTATGCCTTC-3′ and antisense 5′-CTCTTCATTCTTGCCATTCTCCTG-3′; mouse *β-actin* sense 5′-TGGAATCCTGTGGCATCCATGAAAC-3′ and antisense 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′. PCR was carried out using 0.2 µM of primers, 2.5 mM $MgCl_2$, 0.2 mM of each deoxyribonucleotide triphosphate, 1.25 U of GoTaq DNA Polymerase (Promega, Madison, WI) and 2.5 ng of template cDNA. After initial denaturation at 95°C for 2 min, PCR was continued with amplification cycles of 30 sec at 95 °C, 30 sec at 56 °C and 1 min at 72 °C, followed by elongation for 5 min at 72 °C.

The yield of PCR products was tested to be in the linear range and optimal cycle number was chosen. The PCR products were separated by electrophoresis on a 1% agarose gel and visualized by UV-transillumination. *β-actin* was used as an internal control for normalization. Bands were evaluated by densitometry using the Image J program.

Transfections and Knockdowns: siRNA for ^{Sec}tRNA synthetase was obtained as ON-TARGET *plus* SMART pool (Thermo Scientific Dharmacon). 2 µg siRNA was transfected into 2 x 10^6 RAW264.7 cells using Nucleofector Solution V according to the manufacturer's specifications (Amaxa Biosystems). AllStars Negative Control siRNA (Qiagen) was used in equal amounts as a control. Selenite was added 12 h after transfection at a final concentration of 100 nM, and cells were harvested at 36 h after transfection for analysis by immunoblotting. The pU6-TetO4m4 vector containing shRNA target sequence, 5′-GACGTAGAGTTGGCATACC-3′ (nucleotides 694–712) for selenophosphate synthetase 2 (SPS2) was obtained from Dr. Dolph L. Hatfield National Cancer Institute, National Institutes of Health, Bethesda, MD. The control vector was designated pU6Tet control, which consisted of the same construct minus the target sequence. For transfection, RAW264.7 macrophages were seeded into 6-well plates (1 x 10^6 cells/well) without antibiotics. After 24 h, cells were transfected with either shSPS2 or empty vector using TransIT®-LT1 (Mirus Bio Corp.) according to the manufacturer's specifications. The constructs were used at a final concentration of 2.5 µg/well. Forty-eight hours after transfection, cells were left in the media with or without 250 nM selenite for 48 h followed by 12 h of 1 µg/ml LPS stimulation. Cells and media supernatants were collected for 15d-PGJ_2 estimation, while the corresponding cell lysates were used for Western immunoblotting analyses. SPS2 knockdown stable RAW264.7 cells were also prepared by transfecting the shSPS2 or the empty vector as described above. Resistant clones were selected in 100 µg/ml hygromycin and then expanded, followed by culturing them in a selenite-containing media to examine the knockdown of selenoproteins. Western blot

analysis of SPS-2 confirmed ~80 % knockdown. Approximately 60% confluent HEK293T cells in a 60 mm culture dish were transfected with 6 µg pCDNA3.1 containing human PPARγ full length cDNA (provided by Dr. Jack Vanden Heuvel, Penn State University) using TransIT®-293 (Mirus) transfection reagent. Rosiglitazone was added 24 h later to a final concentration of 2 µM for the expressed PPARγ to translocate into the nucleus. After 24 h, cells were harvested to prepare nuclear extracts.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay: Cytoplasmic fraction was separated from nuclei by lysing the HEK293T cells in a buffer containing 10 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 % NP-40, 0.1 mM DTT, 1 mM PMSF, 5 µg/mL aprotinin, 5 µg/mL leupeptin and 1 µg/mL pepstatin, followed by centrifugation at 10000 x g for 10 min at 4 ^{o}C. The nuclear pellet was then lysed with a buffer containing 20 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA and protease inhibitors (see mentioned above). The lysates were sonicated (3 pulses) at 60% power on ice and centrifuged at 14000 x g for 10 min at 4 ^{o}C. Concentrations of proteins in this nuclear extract were determined using BCA reagent (Pierce). The DNA sequences for the sense strand of the oligonucleotides included: consensus PPRE (cPPRE) 5'-GGTGAGGAGGGGAAGGGTCAGTGTG-3'; PPRE1 5'-CTTATTAGGGTTAAAGGTCTAAA-3'; PPRE2 5'-TGAGGTGAGGTTAATGGACACAG-3'; PPRE3 5'-TGTAGAAGGGAGAAAGCTCAAAA-3'; PPRE4 5'ATGCACGAGTCCAAAGTTCAGCC-3'. Complementary strands were annealed and 4 pmol/µl of the resultant double-stranded oligonucleotide was labeled with [γ-^{32}P] ATP (3000 Ci/mol at 10 mCi/ml) using T4-polynucleotide kinase (New England Biolabs). ^{32}P-labeled oligonucleotides were separated using gel filtration G-25 macro spin columns (The Nest Group Inc. Southborough, MA). For the binding reaction, 10 µg of nuclear extracts were incubated for 20 min at room temperature with 5x gel-shift binding buffer (50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 2.5 mM DTT, 2.5 mM EDTA, 250 mM NaCl, 20%

glycerol, 500 ng/mL BSA), 50 µg/ml poly dI-dC, and ^{32}P-labeled PPRE oligonucleotides (20000 cpm). In addition, a competitive binding assay between labeled PPRE1 and unlabelled consensus PPRE (at different ratios) was also performed. 2 µg anti-PPARγ antibody (Cayman) was used for the supershift reaction. The samples were analyzed by polyacrylamide (% T= 4) gel electrophoresis at 120 V for 45-50 min in TBE buffer. After drying, the gel was exposed to X-ray film overnight at -80 ^{o}C. The PPARγ bands were confirmed by competition using a >100-fold excess of corresponding unlabeled oligonucleotide.

PG Quantitation: The concentrations of PGE_2 and TXB_2 in culture media supernatants of BMDMs and RAW264.7 cells treated with Se as indicated and treated with 1 µg/ml LPS were determined by enzyme immunoassay according to the manufacturer's instructions (Cayman) and normalized to total cellular protein. 15d-PGJ_2 was quantitated from supernatants from RAW264.7 cells transiently transfected with shSPS2 by using enzyme immunoassay kit from Assay Designs (Ann Arbor, MI). In most cases, the values were normalized to total cell protein.

LC-MS Analysis of 15d-PGJ_2 and Δ^{12}-PGJ_2 Production: The RAW264.7- or BMDM- cell culture supernatants were acidified with HCl (1N) to pH 3.0 and extracted with ethyl acetate. The compounds 15d- PGJ_2 and Δ^{12}-PGJ_2 were analyzed using an LC-MS/MS system comprising of Shimadzu LC20AD UFLC pumps and API2000 triple quadruple mass spectrometer set to negative mode with a temperature of 200^{o}C for the confirmation molecular ions. The solvent system used was methanol: H_2O (70:30) with 0.1 % acetic acid at a flow rate of 0.15 ml/min. The MS analysis performed on API2000 set to scan mode (*m/z* 100-350) for authentic standards, where as molecular ions *m/z* 315.6 (M-H^{+}) for 15d PGJ_2 and 333.5 (M-H^{+}) for Δ^{12}-PGJ_2 respectively, were used for quantitation. As an internal control to calculate extraction efficiency, 200 ng of deuterated 15d-PGJ_2 (d4) (Cayman Chemicals) was used, which showed a 70 % extraction

efficiency. Standard calibration curves for 15d PGJ_2 and Δ^{12}-PGJ_2 were set up for the quantitation and calculations were performed based on the following equations: y = 76.831x – 608.42; r^2 = 0.9927 (for 15d PGJ_2) and y = 7.2516x + 172.34; r^2 = 0.9871 (for Δ^{12}-PGJ_2). As described earlier, the values were normalized to total cell protein.

Statistical Analysis: The results are expressed as mean ± s.e.m. The differences between groups were analyzed by Student's *t* test using GraphPad Prism. The criterion for statistical significance was $P < 0.05$.

Results

Selenium upregulates H-PGDS expression and its downstream metabolites in RAW264.7 macrophages

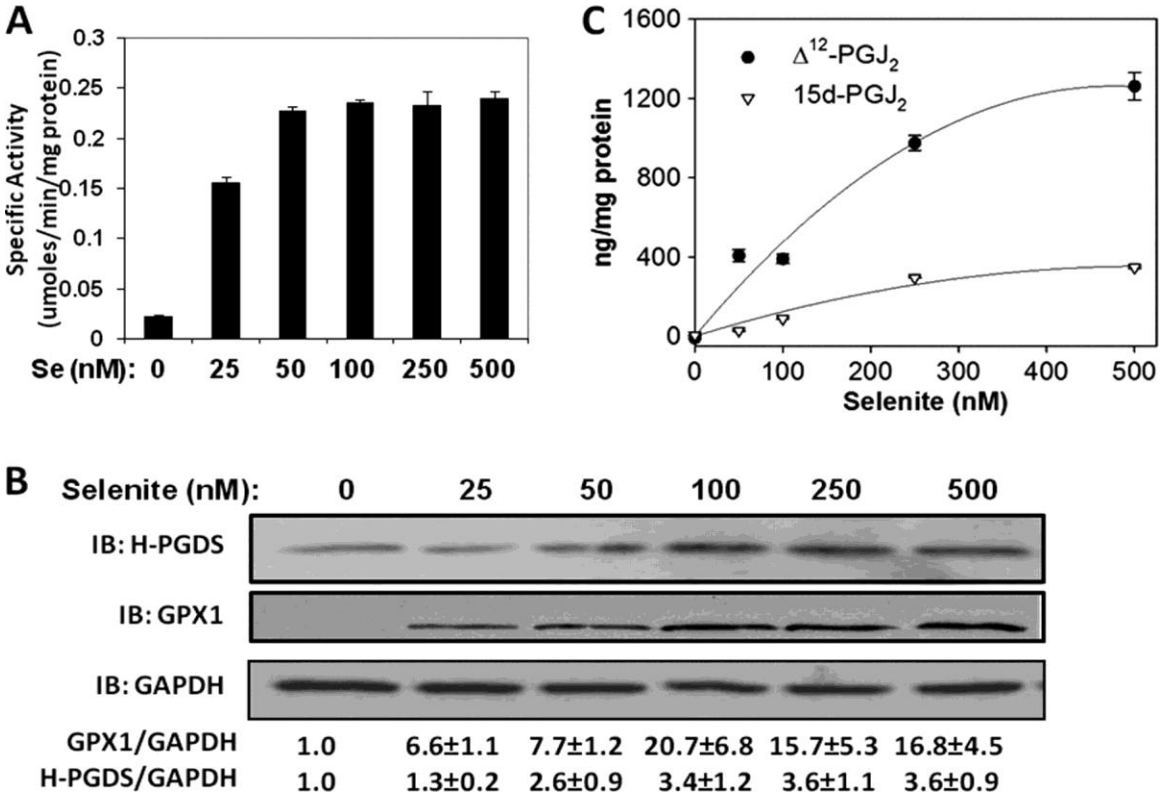

Fig. 10 Sodium selenite upregulates H-PGDS expression and its downstream metabolites in RAW264.7 macrophages. RAW264.7 cells were cultured for 3 days in different concentrations of sodium selenite and the cell lysates were used for GPX1 activity assay (Panel **A**) and representative immunoblot of n= 3 (Panel B). Densitometric ratios normalized to GAPDH are shown below for GPX1 and H-PGDS in form of mean fold changes with respect to 'Se 0' ± s.e.m. are shown. Panel **C** represents a LC-MS quantitation of Δ^{12}-PGJ_2 and 15d-PGJ_2 from cell culture supernatants, normalized to total cell protein (n=3).

To examine the regulation of H-PGDS by Se, RAW264.7 macrophages cultured for three days in various concentrations of exogenously added Se (0-500 nM Se as selenite) were used. Analysis of the cell lysates displayed a dose-dependent increase in the cytosolic GPX enzyme activity (Fig.

10A). While the level of GPX was very low in Se-deficient conditions, 100 nM Se induced a ~10-fold increase in the GPX activity and protein expression (Fig. 10A, B). Using this model, we examined the ability of Se to upregulate expression of H-PGDS. As seen with GPX activity, the expression of H-PGDS also increased as a function of Se concentration, with saturation at 150-250 nM Se. We observed a ~3-fold increase in protein levels of H-PGDS at 250 nM (Fig. 10B). In concert with the increased levels of H-PGDS, downstream metabolites of PGD_2, Δ^{12}-PGJ_2 and 15d-PGJ_2, also showed a similar dose-dependent increase with selenite in the cell culture supernatants using quantitative LC/MS-based assays (Fig.10C). At all the doses of Se tested, the concentration of Δ^{12}-PGJ_2 was much higher than its dehydrated metabolite, 15d-PGJ_2.

Selenite differentially regulates the expression of PG synthases in RAW264.7 cells and BMDMs

To investigate the role of Se in the shunting of the AA-COX pathway, the expression of two other additional PG synthases, mPGES-1, and TXAS, which are highly expressed in macrophages, were examined by immunoblotting and real-time RT-PCR. With an increase in Se, the expression of mPGES-1 and TXAS showed a dose-dependent decrease at both protein and transcript levels. However, transcript levels of *Hpgds* increased as a function of Se, in agreement with the protein level as described earlier (Fig. 11A, B). Analysis of the levels of *Pgis* expression showed an interesting biphasic regulation in that an initial increase in the transcript was seen with 50 and 100 nM Se (Fig. 11B). However, further addition of Se (>100 nM) decreased the levels of *Pgis*. Analysis of protein expression of PG synthases in primary BMDMs isolated from the bone marrow of mice on diets containing different Se concentrations a dose-dependent increase in H-PGDS; while mPGES-1 and TXAS expression was decreased at 100 and 500 nM of Se relative to Se-deficient cells (Fig. 11C).

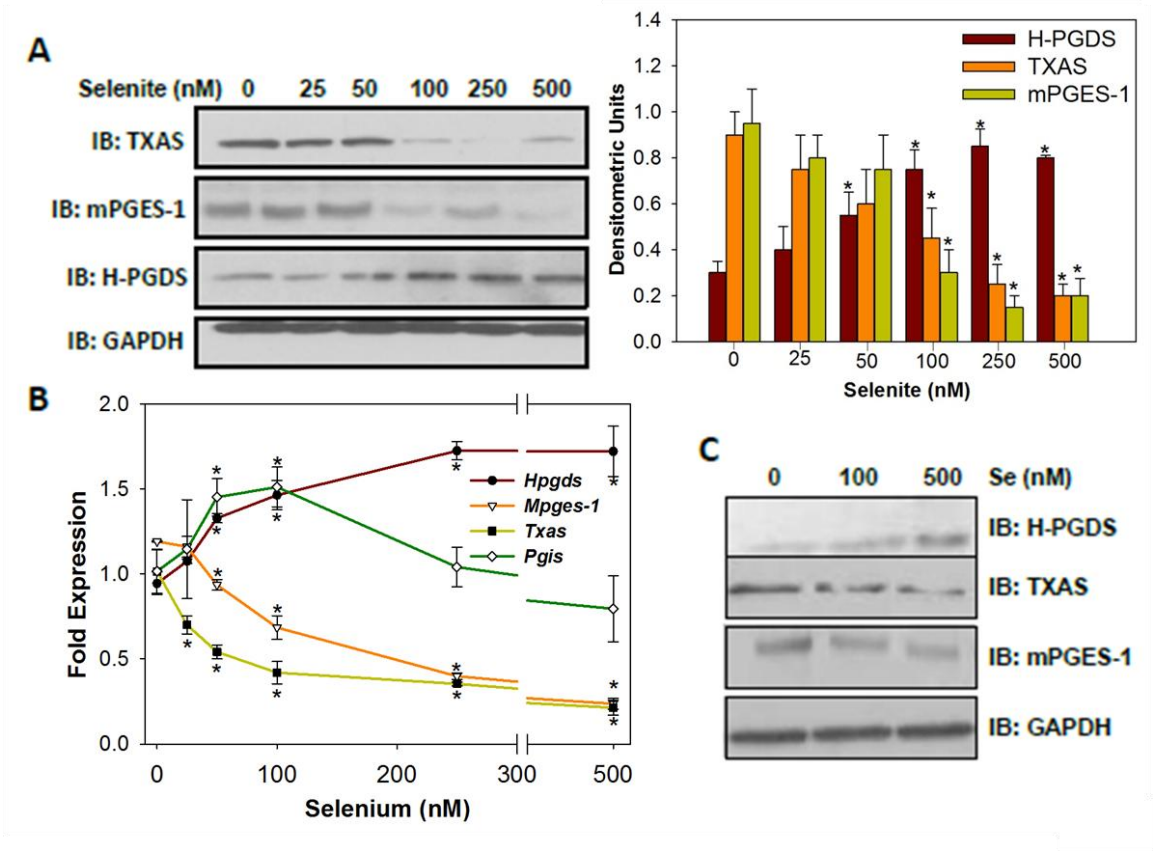

Fig. 11 Selenite differentially regulates the expression of PG synthases in RAW264.7 cells and BMDMs. BMDMs isolated from Se-deficient mice and RAW264.7 cells were treated with different concentrations of sodium selenite for 3 days. Panels A and B represent immunoblot and real-time RT-PCR results, respectively. Densitometric ratios from the immunoblots are shown as mean ± s.e.m. of n=3. Panel C shows the dose-response of protein expression of PG synthases on BMDMs from C57BL/6 mice maintained on Se-deficient (0.01 ppm), Se-adequate (0.1 ppm), and Se-supplemented (0.4 ppm) diets. * $p<0.05$

Different forms of Se have variable effects on expression of PG synthases in RAW264.7 cells and BMDMs

To further understand the Se-dependent regulation of AA-COX pathway, the effects of four commonly used forms of Se were compared. RAW264.7 cells were incubated with 0, 100 and 500 nM of Se in form of sodium selenite, MSA, p-XSC or Se-Met for three days and analyzed for protein and transcripts of H-PGDS and other PG synthases. GPX1 levels were also examined as a

marker of selenoproteins status of these cells. Fig. 12A, B show that MSA upregulated *Hpgds* in a manner comparable to sodium selenite treatment, but p-XSC and Se-Met did not lead to a significant change in H-PGDS or GPX1 from the baseline levels. On the other hand, expression of *Mpges-1* and *Txas* was downregulated with MSA, but not Se-Met and p-XSC. Real-time RT-PCR analyses on BMDMs (from Se-deficient mice) treated with different forms of Se *ex vivo* showed similar patterns of expression of PG synthases (Fig. 12C), an exception being the downregulation of *Txas* by Se-Met.

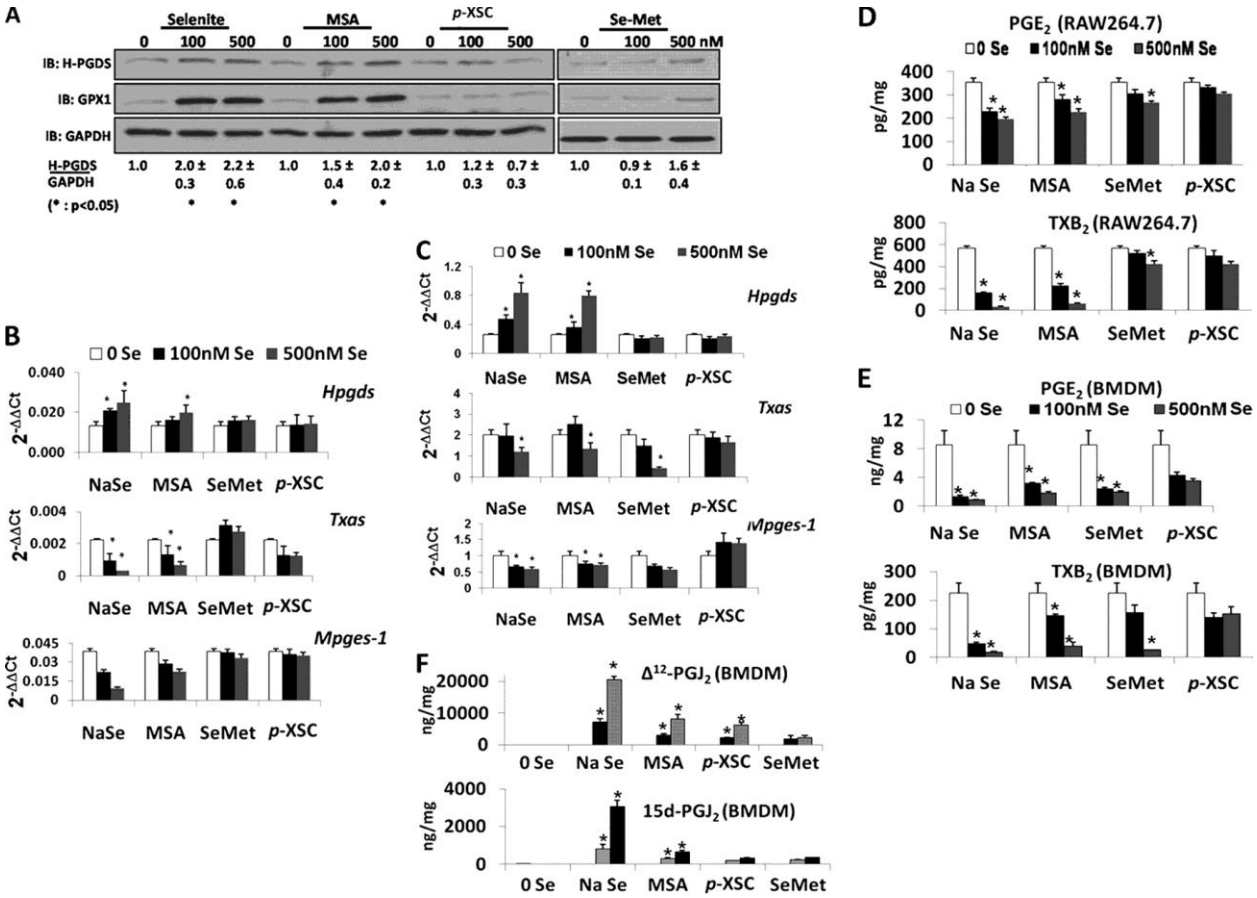

Fig. 12 Regulation of PG synthases by different forms of Se. Panel A represents the immunoblot of lysates from RAW264.7 cells treated with indicated concentrations of sodium selenite, MSA, p-XSC, or Se-Met for 3 days. Panel B and C represent real-time RT-PCR analysis of transcripts of *Hpgds*, *Txas*, and *Mpges* in RAW264.7 and BMDMs treated with different forms of Se, respectively. Panels D and E show the levels of PGE_2 and TXB_2 in the cell culture

supernatants from RAW264.7 and BMDMs, respectively, upon treatment with different forms of Se. Panel F represents mass-spectrometric analysis of 15d-PGJ_2 and Δ^{12}-PGJ_2 on supernatants from BMDMs treated with different forms of Se as shown. All data shown is mean ± s.e.m. of three independent observations. * $p<0.05$ when compared with the corresponding '0 Se' sample.

Quantification of the levels of PGE_2 and TXB_2 in RAW264.7- and BMDM- cell culture supernatants by ELSIA confirmed the results obtained from the expression analyses of PG synthases (Fig. 12D, E). Analyses of the BMDM supernatants by LC/MS for Δ^{12}-PGJ_2 and 15d-PGJ_2 revealed significantly high levels with the 500 nM selenite treatment, and minor increases within the other groups as compared with the Se-deficient samples (Fig. 12F). These results suggest that increased bioavailability of Se, perhaps in the form of selenoproteins, is a critical determinant of Se-dependent regulation of PG synthases in macrophages.

Selenoprotein synthesis is critical to regulate the expression of H-PGDS by Se

To examine if incorporation of Se into selenoproteins was essential for the upregulation of H-PGDS and subsequent formation of 15d-PGJ_2, a genetic knockdown approach was used. Se-deficient RAW264.7 macrophages were transfected with either shSPS2 or control constructs and then maintained under Se-deficient and Se-supplemented (250 nM) conditions for two days followed by 1 µg/mL LPS stimulation for 12 h. The cell lysates were used for the analysis of the expression of SPS2, GPX1, and TR1 by immunoblotting. While the expression of GPX1 and TR1 was significantly knocked down in shSPS2-transfected cells in spite of the presence of Se, no change in the expression of these proteins in cells transfected with the vector control construct (Fig. 13A). To confirm that SPS2 knockdown cells cannot produce 15d-PGJ_2 due to the lack of selenoproteins, the cell supernatants were utilized for the analysis of 15d-PGJ_2 production. The production of 15d-PGJ_2 was almost completely inhibited in SPS2 knockdown cells even after supplementing with 250 nM Se. In contrast, we observed a significant increase in the production

of 15d-PGJ$_2$ in those cells that were transfected with control constructs followed by selenite treatment (Fig. 13B). Evaluation of H-PGDS expression using the shSPS2 stably transfected macrophages that were cultured with 100 nM Se, showed a clear downregulation of GPX1 as well as H-PGDS only in shSPS2 knockdown cells, but not in the vector control (Fig. 13C).

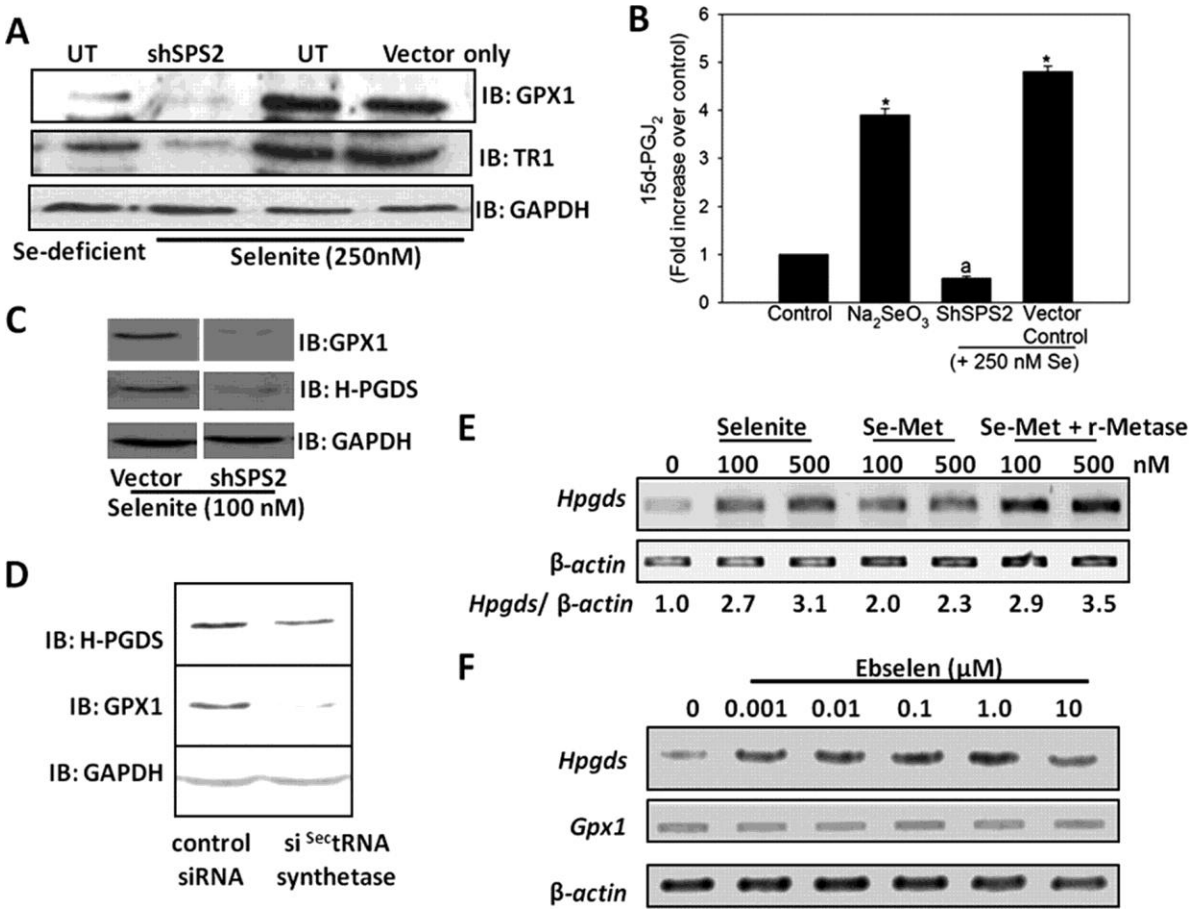

Fig. 13 Selenoprotein synthesis is critical to regulate the expression of H-PGDS by Se. Panel A shows the protein expression of selenoproteins GPX1 and TR1, while panel B shows the amounts of 15d-PGJ$_2$ in cell culture supernatants upon transient knock-down of selenophosphate synthetase 2 (SPS2) in RAW264.7 cells using shSPS2, respectively. UT: untransfected cells. Panel C shows the protein levels of GPX1 and H-PGDS in RAW264.7 cells stably transfected with shSPS2 or empty vector control. Panel D represents the effects of transiently knocking down selenocysteine tRNA (^{Sec}tRNA)-synthetase in selenite supplemented RAW264.7 cells. Panel E shows the effect on *Hpgds* transcript levels upon adding 0.1 U/mL recombinant methioninase (r-Metase) along with selenomethionine to RAW264.7 cells. The

effect of 24 h treatment with the GPX-mimetic ebselen on *Hpgds* transcripts is shown in Panel F. All data shown are representative of at least three independent experiments.

Furthermore, we genetically manipulated the expression of ^{Sec}tRNA synthetase, an enzyme that is critical in the pathway of Sec incorporation into selenoproteins [245]. Western immunoblotting of cells that were subjected to siRNA mediated knockdown of ^{Sec}tRNA synthetase clearly showed a substantial downregulation of H-PGDS and GPX1 (Fig. 13D). Interestingly, treatment of Se-Met in presence of r-METase, an L-Met α, γ-lyase that releases Se for incorporation into selenoproteins, led to the upregulation of H-PGDS expression (Fig. 13E). To understand if the activity of GPX, one of the most abundantly expressed selenoproteins, was pivotal to upregulate H-PGDS, ebselen, a small molecular mimetic of GPX1 [246] was used. RAW264.7 cells treated with different concentrations of ebselen showed a dose-dependent increase in levels of H-PGDS transcripts in absence of any increase in GPX1 (Fig. 13F). Taken together, these results provide further evidence in support of the important role of selenoproteins in the upregulation of H-PGDS and subsequent shunting of the AA-COX pathway in macrophages.

PPARγ-dependent transcription of H-PGDS

We speculated the presence of a positive-feedback regulation of H-PGDS by the endogenous PPARγ ligand, 15d-PGJ_2. Examination of the murine *Hpgds* promoter for the presence of PPAR-response elements (PPREs) using MatInspector (Genomatix Software GmBH, Munich, Germany) resulted in at least four putative binding sites at -1250, -3401, -4478 and -4896 bp for PPARγ found within 5 kb upstream of the transcription start site of *Hpgds*, which have a matrix similarity score of >0.75 when compared with a consensus PPRE (cPPRE) (Fig. 14A).

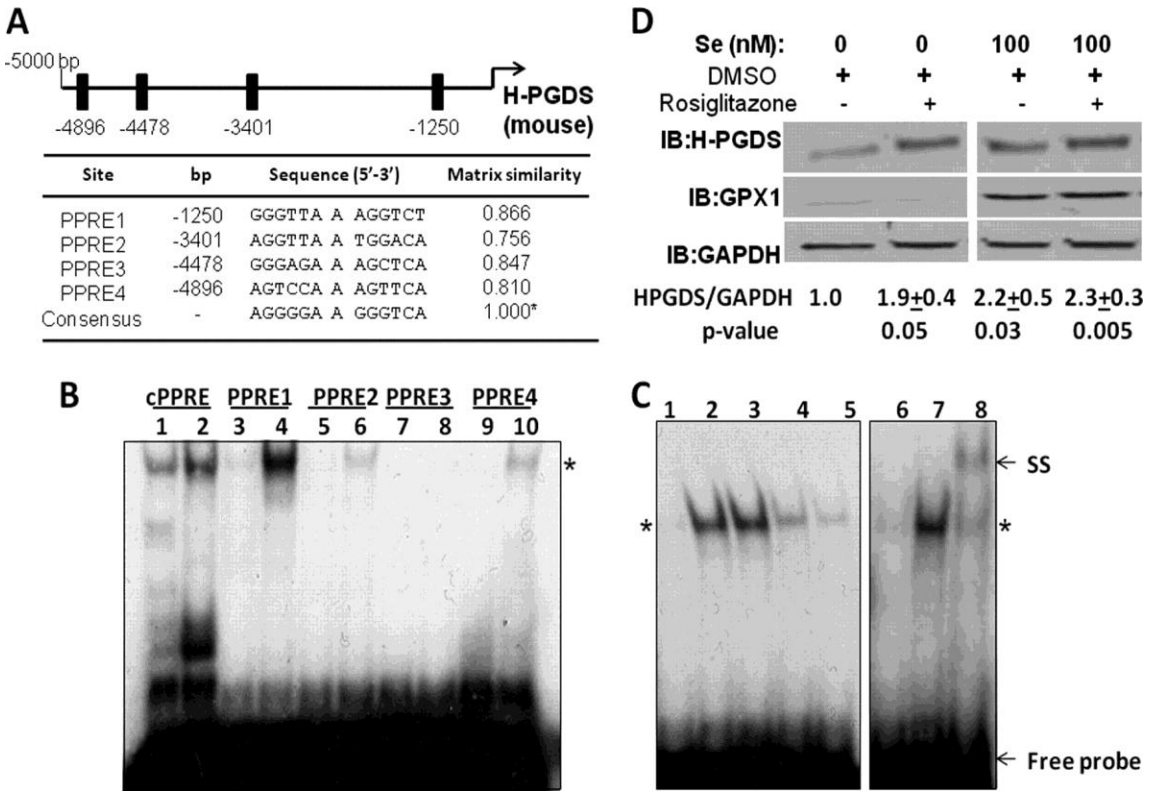

Fig. 14 The role of PPARγ in the expression of murine H-PGDS. Panel A shows the comparison of the murine H-PGDS promoter for putative PPAR response elements (PPREs) using *MatInspector* and their matrix similarity scores with that of consensus PPRE. Panel B shows electrophoretic gel-shift assay demonstrating the binding of PPREs in murine H-PGDS promoter. Nuclear extract was prepared from HEK293T cells transfected with full length PPARγ expression construct and stimulated with 2 μM rosiglitazone. Lanes 1, 3, 5, 6, 7, 9 represent ^{32}P- labeled PPREs with their corresponding 'cold' competitor in a ~100 fold excess; while lanes 2, 4, 6, 8, 10 represent binding (*) with ^{32}P-labeled PPREs alone, respectively. Panel C shows EMSA with competitive binding of labeled PPRE1 with unlabeled consensus PPRE as well as anti-PPARγ-induced supershift. Lanes 1-8 represent, PPRE1 with excess 'cold' PPRE1, PPRE1, PPRE1 and 'cold' cPPRE (1:5), PPRE1 and 'cold' cPPRE (1:25), PPRE1 and 'cold' cPPRE (1:125), binding of PPARγ to PPRE1 in presence of 'cold' PPRE1, absence of 'cold' competitor, and supershift (SS) of PPARγ-PPRE1 complex in presence of anti-PPARγ antibody, respectively. Panel D shows immunoblot on lysates from RAW264.7 cells treated with or without selenite and 1 μM rosiglitazone. Densitometric ratios from four representative Western blots are shown as mean of fold change ± s.e.m.

To examine the binding of these PPREs to PPARγ within the context of the promoter, we analyzed the binding of oligonucleotides containing each of these PPREs to HEK293T-expressed PPARγ. Gel shift analysis clearly showed that PPRE1 (-1250) interacted strongly with PPARγ, somewhat similar to the cPPRE; while PPRE3 (-4478) was relatively ineffective and PPRE2 (-3401) and PPRE4 (-4896) showed minimal binding (Fig 14B). We confirmed the specificity of PPARγ binding to PPRE1 by competing ^{32}P-labeled PPRE1 oligonucleotide with varying amounts of unlabeled cPPRE oligonucleotide as well as by displaying a supershift using anti-PPARγ antibody (Fig. 14C). Having located functionally active PPREs (-1250 and -4896) in the murine *Hpgds* promoter, we studied the regulation of H-PGDS expression upon treatment of RAW264.7 macrophages with rosiglitazone, a bona fide agonist of PPARγ. Rosiglitazone significantly increased H-PGDS protein expression in Se-deficient cells (Fig. 14D). These studies indicate that PPARγ plays a critical role in the regulation of H-PGDS expression, which is facilitated by Se.

Inhibition of H-PGDS blocks Se-mediated upregulation of PPARγ-dependent Arginase-1 production

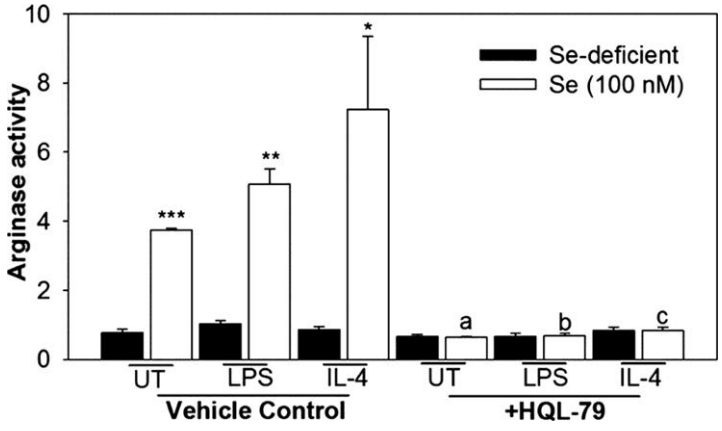

Fig. 15 Inhibition of H-PGDS blocks the Se-mediated production of Arg-1 in macrophages. RAW 264.7 cells in Se-deficient conditions or 100 nM selenite were pretreated with 25 μM HQL-79 for 2 h followed by stimulation with IL-4 (5 ng/μl; 20 h) or LPS (1 μg/mL; 12 h). Arginase activity was determined. Values are means ± s.e.m of n=3. * p< 0.05,

** p<0.01, *** p<0.001 indicate significant differences when compared with their Se-deficient counterparts. a, b and c represent significant differences (p<0.05) between vehicle and HQL-79 treated groups.

Having previously shown that there is increased nuclear translocation of PPARγ in presence of Se in macrophages [107], and knowing that macrophage alternate activation marker arginase-1 (Arg-I) is induced by PPARγ [247] and increase in CyPGs in Se-treated cells, we studied the effect of Se on the activation of arginase-1 and the role of H-PGDS in this process. Treatment of RAW264.7 macrophages with 100 nM Se increased the arginase-1 activity up to ~ 7-fold compared with the Se deficient cells in presence of IL-4, which is a Th2 cytokine essential for alternate activation. Interestingly, pretreatment of cells with an H-PGDS inhibitor, HQL-79, completely blocked the Se-mediated increase in arginase-1 (Fig. 15).

Control of NF-κB activation shunts the AA pathway towards PGJ_2 production

Given that NF-κB is known to regulate the expression of mPGES-1 and TXAS [238, 239], we examined whether inhibition of NF-κB in Se-deficient cells provides an alternate route of metabolism of PGH_2, possibly through H-PGDS, which provides sufficient 15d-PGJ_2 to upregulate H-PGDS. To test this potential feedback activation, RAW264.7 macrophages were treated with 1 μM parthenolide (PTN) for 1 h before stimulation with 1 μg/mL LPS. Inhibition of NF-κB with PTN upregulated H-PGDS expression (transcripts and protein) under Se-deficient conditions. Increase in H-PGDS was accompanied by a decrease in TXAS and mPGES-1 expression (Fig. 16A, B). Similarly, inhibition of mPGES-1 by CAY10526 in these cells, followed by LPS stimulation, resulted in an increase in H-PGDS expression (Fig 16A, B) as well as increased Δ^{12}-PGJ_2 and 15d-PGJ_2 levels in the supernatants, when analyzed by LC-MS (Fig 16C).

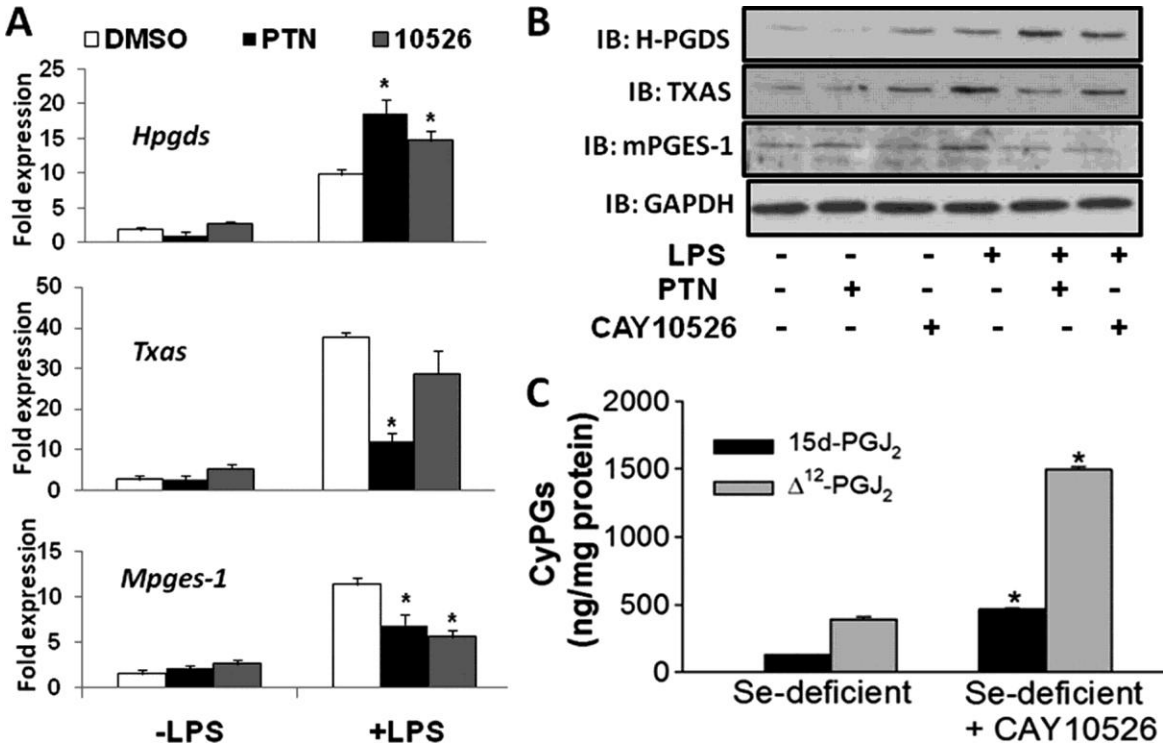

Fig. 16 Inhibition of NF-κB shunts the AA pathway towards PGJ_2 production. Panels A and B represent real-time PCR and immunoblot analyses, respectively, for *Hpgds*, *Txas*, and *Mpges-1* expression in Se-deficient RAW264.7 cells were treated with 1 μM parthenolide (PTN) or 20 μM CAY10526 for 1 h followed by stimulation with 1 μg/mL LPS for 2 h. Panel C shows the effect of CAY10526 on the production of 15d-PGJ_2 and Δ^{12}-PGJ_2 in Se-deficient RAW264.7 cells. All data are representative of n= 3, mean ± s.e.m. shown.

Discussion

Epidemiological evidence suggests that the benefits of essential micronutrient selenium in optimal health are, in part, contributed by the ability of selenium to alleviate inflammatory signaling pathways [76, 77]. Selenium might impart these anti-inflammatory properties via different selenoproteins, but the mechanisms have not been understood completely. These selenoproteins form an important component of the cellular redox regulatory machinery, especially in oxidative stress - prone macrophages. Inhibition of the activation of the NF-κB signaling axis and its effect on the downregulation of its pro-inflammatory gene products such as COX-2, mPGES-1 and TXAS is well known. It is shown earlier that selenium can inhibit NF-κB activation in macrophages [105, 106, 219]. Given the fact that macrophages, among other immune cells, have the enzymatic machinery to metabolize AA to all PG classes including TXA_2, and that PGD_2 metabolites produced by these cells have the ability to modulate NF-κB and PPARγ pathways [118, 240, 241], we explored the possibility of selenium regulating the production of different PGs at the transcription level. Here, we report that bioavailability of selenium for incorporation into selenoproteins is essential to upregulate the expression of H-PGDS and downregulate the expression of mPGES-1 and TXAS. The resultant increase in H-PGDS shunts the metabolism of AA-derived COX-product, PGH_2, from pro-inflammatory PGE_2 and TXA_2 towards the anti-inflammatory and pro-resolution product 15d-PGJ_2.

The dose-dependent increase in the activity and expression of GPX1 with sodium selenite reinforces the fact that GPX1 is a reliable marker of cellular selenium status in macrophages. The saturation of GPX1 activity and expression at 100 nM Se in the form of selenite added to Se-deficient cells demonstrates the sensitivity of the selenoprotein synthesis machinery to bioavailable selenium. Therefore, we subjected Se-deficient primary BMDMs and RAW264.7

cells to different doses (0-500 nM) of selenite to examine if there was an effect on the transcription of H-PGDS, which is the rate limiting step for the formation of PGD_2 and its stable metabolites, Δ^{12}-PGJ_2 and 15d-PGJ_2. The dose-dependent upregulation of H-PGDS along with the increased production of its downstream products by selenite, in both primary and immortalized macrophages, demonstrates that cellular selenium status plays a pivotal role in regulating the expression of proteins outside the selenoproteome. Higher levels of Δ^{12}-PGJ_2 as compared to 15d-PGJ_2 in the cell culture supernatant of primary cells than those in the immortalized cells are consistent with those reported for eicosanoids in general by Rouzer *et al.* [248]. Also, the increased accumulation of Δ^{12}-PGJ_2 than its dehydrated product (15d-PGJ_2) is also in agreement with the reports on the thermodynamically unfavorable conversion of Δ^{12}-PGJ_2 to 15d-PGJ_2 in the presence of proteins [249, 250]. On the other hand, the dose-dependent downregulation of mPGES-1 and TXAS and their pro-inflammatory products PGE_2 and TXA_2 at similar concentrations of selenite indicate a favorable skewing of AA-COX pathway metabolites. This regulation by selenium could be a direct consequence of the change in the redox state of the cell affecting the transcription of many diverse redox-sensitive proteins, or an indirect consequence of the activation of signaling pathway(s), which eventually regulates H-PGDS expression. Taken together, the data argues that selenium in the form of selenoproteins appear to be crucial for this effect. Evidence that selenium can modulate PGIS expression in endothelial cells [102], prompted us to look at the regulation of this enzyme in our macrophage model. We found that PGIS is expressed in insignificant quantities, which is consistent with the fact that PGIS is predominantly expressed in endothelial cells and smooth muscle cells [237].

Interestingly, our studies demonstrate that selenium in different forms variably affects the expression of PG synthases. In cell culture models, sodium selenite and MSA are known to make selenium easily bioavailable for the formation of Sec, which is incorporated into selenoproteins

during translation [7]. However, Se-Met and *p*-XSC do not release selenium for selenoprotein synthesis as reflected by their inability to increase GPX1 expression above the baseline levels (Fig. 12A). The expression of H-PGDS follows the same pattern as GPX1 for all of these forms of selenium, which suggests that selenoproteins appear to be involved in the upregulation of H-PGDS expression. Similar observations obtained from primary BMDM at the level of transcripts and end-products suggest that the regulation of PG synthases by selenium, in the form of selenoproteins, is not just a feature of immortalized cell line such as RAW264.7 macrophage-like cell line. Apart from using genetic methods to knockdown the expression of selenoproteins, we have taken the absence of γ-lyase in our *in-vitro* culture system to our advantage to provide support for the role of selenoproteins in AA metabolism. This may not be the case at the organismal level where γ-lyase is expressed mostly in the kidney to metabolize these compounds to contribute, in part, to the anti-carcinogenic properties of such compounds as *p*-XSC [251, 252]. Alternatively, it is also possible that these compounds may target signaling pathways even without involving selenoproteins to impart their beneficial actions. Compounds like Se-Met require an enzymatic reaction involving a γ-lyase to release selenium. Thus, when the macrophages were treated with Se-Met and r-METase, there was a dose-dependent increase in H-PGDS, which continues to support the involvement of selenoproteins to play a key role in the expression of H-PGDS. Furthermore, the genetic knockdown of two critical enzymes in the selenoprotein incorporation pathway, SPS-2 and ^{Sec}tRNA synthetase, also led to the downregulation of H-PGDS even in the presence of selenium. These results make a compelling case to identify the selenoprotein(s) that are crucial for the regulation of H-PGDS expression and subsequent production of the potent anti-inflammatory mediator, 15d-PGJ_2. Based on our studies with NF-κB inhibitors, we speculate that changes in the redox status of cells, as a result of the modulation of selenoprotein expression, could likely be the underlying mechanism. The

ability of GPX-mimetic ebselen to upregulate H-PGDS in Se-deficient macrophages also indicates that redox modulatory pathways are likely to contribute to the shunting of AA. These are very interesting aspects that justify exploration in the future.

The regulation of H-PGDS at the transcriptional level and the already-documented finding from our laboratory that Se-supplementation of macrophages leads to an increase in activation of PPARγ-mediated by the increased levels of an endogenous PPARγ-ligand, 15d-PGJ_2 [107], led us to examine the PPARγ-dependent regulation of the murine H-PGDS gene. Results from the PPARγ gel-shift assays clearly indicate that PPARγ binds to the promoter of H-PGDS at one or more of the four PPREs, with the PPRE at -1250 bp (PPRE1) being the strongest. Likewise, the upregulation of H-PGDS by prototypic PPARγ ligand, rosiglitazone, in the absence of selenium, indicates that H-PGDS is indeed a PPARγ-dependent gene. These findings also open an intriguing possibility of feedback regulation of H-PGDS by 15d-PGJ_2 in the Se-supplemented macrophages. It is possible that in addition to PPARγ, PPARβ could also play a critical role in the upregulation of H-PGDS since PPARβ is highly expressed in macrophages and is known to possess a variety of anti-inflammatory properties [253, 254]. However, preliminary promoter analyses for PPARβ binding sites and the inability of PPARβ-specific synthetic agonists to activate H-PGDS expression suggest that the binding activity is perhaps restricted to PPARγ (data not shown).

Besides being a PPARγ ligand, 15d-PGJ_2 has been shown to inhibit the activation of pro-inflammatory genes by covalently binding specific cysteine residues in IKKβ, a pivotal enzyme in the canonical pathway of NF-κB activation [168]. Thus, in Se-supplemented macrophages, the upregulation of H-PGDS leads to the enhanced production of 15d-PGJ_2, which not only acts in a positive-feedback loop to upregulate H-PGDS transcription in a PPARγ-dependent manner, but also inhibits NF-κB target genes, including mPGES1 and TXAS enzymes, in LPS-stimulated Se-

supplemented macrophages. To provide more evidence for the shunting of the PG synthesis pathway, we used parthenolide (PTN), a well-known NF-κB inhibitor, to inhibit mPGES-1 and TXAS expression to create an increase in the availability of PGH_2 for H-PGDS. Alternatively, inhibition of mPGES-1 by CAY10526 also mimicked the effect of PTN with respect to increased H-PGDS expression and subsequent Δ^{12}-PGJ_2 and 15d-PGJ_2 production. Taken together, the subsequent upregulation of H-PGDS under Se-deficient conditions strongly supports the possibility of a feed-forward loop involving H-PGDS →15d-PGJ_2 → PPARγ → H-PGDS (Fig. 17).

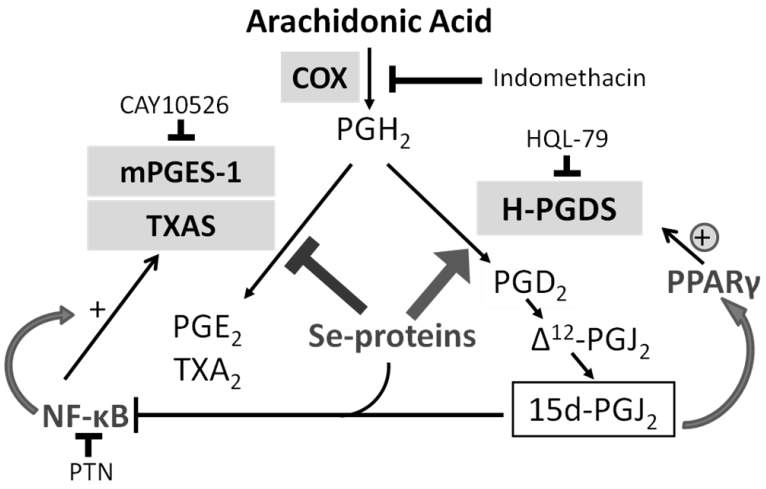

Fig. 17 Schematic representation of the selenoprotein-dependent shunting of AA metabolism in macrophages. Treatment of macrophages with bioavailable Se shunts the pathway of AA metabolism to produce higher levels of PGJ_2 metabolites, while lowering the levels of PGE_2 and TXA_2 via the differential regulation of the respective synthases. CAY10526, PTN, and HQL-79 represent inhibitors of mPGES-1, NF-κB, and H-PGDS, respectively.

In summary, our studies demonstrate a critical role for selenium in anti-inflammation, which is particularly relevant since macrophages are implicated in both the initiation and resolution of inflammation. Thus, it is important to understand how exactly their functions are so tightly regulated. While the initial phases of inflammation involve increases in levels of pro-inflammatory mediators like PGE_2 and TXA_2, a switch towards the pro-resolving and anti-

inflammatory mediators like 15d-PGJ_2 during the latter stages suggests the presence of a critical regulator being involved. As seen with the increased activity of arginase in macrophages treated with, shunting of the AA pathway, particular in macrophages, may have many implications, the most notable being the switch from classically activated 'M1' macrophage to alternatively activated 'M2' macrophage phenotype that have wound-healing and resolving properties.

Chapter 3

Selenium supplementation eradicates leukemic stem cells (LSCs) in murine models of leukemia through the modulation of arachidonic acid–cyclooxygenase (AA-COX) pathway metabolism

[**Contribution:** Shailaja Hegde helped with transplantation, blood work, and flow cytometry analysis for all experiments; Naveen Kaushal contributed to Figs. 18A, 20A, and 21A.]

Abstract

The micronutrient selenium (Se) has been demonstrated to have anti-leukemic effects through several redox mechanisms. However, there are no studies showing the ability of Se to affect leukemia stem cells (LSC), which are chiefly responsible for maintenance and relapse of the disease. We hypothesize that Se can specifically target LSCs via their ability to generate cyclooxygenase (COX)-derived endogenous pro-apoptotic metabolite, 15d-prostaglandin (PG) J_2 *in vivo*. We have used three well characterized murine models of leukemia – BCR/ABL-transduced LSC transplant model, MLL-AF9-transduced LSC transplant model, and Friend virus infected erythroleukemia model. In each of these three models, we have observed that the mice on Se-deficient (0.01 ppm selenite) or Se-adequate (0.1 ppm selenite) diets succumbed to leukemia, unlike the mice maintained on Se-supplemented (0.4 ppm selenite) diets which showed normal blood counts and spleen size, and an absence of LSCs (Kit^+Sca^+). These effects of Se were blocked when the mice were treated with a non-specific COX-inhibitor, indomethacin, which means that COX metabolites play a pivotal role. This was confirmed by rescuing the indomethacin-treated mice by administration of 15d-PGJ_2. Upon comparing the effect of the conventionally used chemotherapeutic agent, imatinib (Gleevac), with that of Se supplementation, the mice in the former group showed relapse of disease upon discontinuation of therapy. Furthermore, e*x vivo* treatment of LSCs with antagonists for the cell surface prostanoid DP receptors prevented Se-induced apoptosis. All these studies show that Se taken in supplemental quantities can potentially cure leukemia by selectively eradicating LSCs via anti-proliferative COX metabolites.

Introduction

Leukemia is a term for a group of malignant conditions characterized by uncontrolled proliferation of white blood cells, well-differentiated or poorly-differentiated, myeloid or lymphoid in origin. There is enough evidence indicating that most leukemias are stem cell diseases obeying the cancer stem-cell (CSC) hypothesis, formulated on the basis of studies performed by Dick *et al.* and other investigators [255, 256]. In 1994, Dick and colleagues showed that leukemic stem cells (LSCs) resided within the $CD34^{+}CD38^{-}$ sub-population of acute myeloid leukemia (AML) cells, and formed only a small fraction of the total leukemic blasts. Importantly, these LSCs were capable of transmitting AML to non-obese severe combined immune-deficiency (NOD-SCID) mice and repopulate the marrow of irradiated recipient mice [255]. The CSC hypothesis states that tumors are maintained by a small population of stem-like cancer cells that have the capacity of indefinite self-renewal [194]. Whereas a normal hematopoietic stem cell (HSC) differentiates into functional cells performing their biological functions and normally dying by apoptosis after an appropriate time period, LSCs arise from mutations in HSCs that allow them to grow and persist in the presence of chemotherapeutic drugs, unlike the majority of the leukemic cells [221]. In AML, conventional chemotherapy induces remission in >70% patients, but the majority relapse within five years despite continued treatment [257]. In chronic myeloid leukemia (CML), the BCR-ABL inhibitor, imatinib mesylate, induces clinical remission in almost all patients during the chronic phase of CML, however most patients relapse upon stopping of the drug [258]. Moreover, there is rarely a molecular remission observed at any stage [259]. Hence, the eradication of LSCs is considered as a cure for leukemia.

Eradication strategies for LSCs have been reviewed in detail in Chapter 1. One of the compounds that have been found to be potent in ablating LSCs is parthenolide (PTL), which inhibits NF-κB

and induces oxidative stress [214]. Although *in vitro* effects have looked very promising, the poor solubility and bioavailability in terms of attaining optimum plasma concentrations in animal models have made PTL a pharmacologically suboptimal compound [260]. This limitation led to the development of *in silico* gene-expression based screens using the PTL transcriptional signature as a means to identify other compounds with anti-LSC activity. The result of this bioinformatics study was the discovery of agents such as celestrol, 4-hydroxy 2-nonenal, and 15d-PGJ_2 for the potential eradication of AML LSCs [261]. The anti-apoptotic properties of the cyclopentenone prostaglandin, 15d-PGJ_2, are well known [123, 151, 180, 183]. It has been observed that prostaglandins of PGA series and $\Delta^{12,14}$-PGJ_2 have anti-proliferative activity towards L1210 murine leukemia cells [220], and that 15d-PGJ_2 has also been shown to induce apoptosis in HL-60 leukemia xenograft model in nude mice [183]. However, the concentrations of exogenously administered prostaglandins used in these studies have been very high. It is getting increasingly clear that 15d-PGJ_2 in nanomolar concentrations can exert many important biological effects in experimental settings [262]. Recently, a study from our laboratory has demonstrated the pro-apoptotic effect of 15d-PGJ_2 on LSCs *ex vivo* from Friend-virus- and BCR-ABL transplanted- leukemic mice, at concentrations as low as 25 nM [120]. In the same study, Hegde *et al.* concluded that 15d-PGJ_2 or other CyPGs acted through mechanisms that were independent of NF-κB and PPARγ. Thus, one of the possibilities that were considered to study was the role of DP receptors, DP1 and DP2, in activating pathways of apoptosis. Two types of G protein–coupled receptors for PGD_2 and its metabolites have been cloned: D-prostanoid receptor (DP1), and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2; DP2) [263]. DP receptors are expressed not only on a variety of hematopoietic cells but also on vascular smooth muscle cells and bronchial epithelial cells. They initiate downstream signaling events by modulation of cAMP levels leading to activation of protein kinases A or C.

Besides emerging as potential targets for allergic diseases, the DP receptors and their downstream signaling pathways play important roles in cell proliferation events [264, 265]. Moreover, there is a need to explore the potential of endogenously generated 15d-PGJ_2 in appropriate models of leukemia.

As described in Chapter 2, selenium has been shown to shunt the metabolism of the arachidonic acid – cyclooxygenase (AA-COX) pathway in macrophages to favor increased production of 15d-PGJ_2 [107], by differential modulation of the expression of prostaglandin synthases where PPARγ-dependent mechanisms are involved [266]. 15d-PGJ_2 production was detected in the sera of LPS (lipopolysaccharide)-treated selenium supplemented mice in nanomolar range [107]. On the other hand, the anti-carcinogenic property of selenium supplementation has been a major topic of research over the last few decades. In addition to case-control and cohort observational studies, interventional trials carried out with supplemental selenium have demonstrated significant anti-carcinogenic effects against prostate, colon, lung, and liver cancer [205, 206]. However, there is only limited evidence providing a relation between selenium and leukemia. One epidemiological study found significantly low levels of selenium in serum from patients with AML; patients who achieved complete remission after induction had higher selenium levels [207]. Anti-proliferative effects of sodium selenite have been shown on leukemic cells from patients with AML - M1, M3, M5, ALL (acute lymphocytic leukemia)- L1, L2, and CML, and were thought to be due to selenium -dependent inhibition of DNA replication and expression [209]. In vitro studies suggest that sodium selenite induces apoptosis in human acute promyelocytic leukemia (AML-M3) cell line NB4 by a caspase-3-dependent mechanism and a redox pathway [210]. However, there is no general consensus on the exact mechanism(s) involved in these effects of selenium. More importantly, none of these studies have looked at the effects of selenium on LSC populations *in vivo*.

Having understood the need to examine potential treatment strategies that can eradicate LSCs as a 'cure' for myeloid leukemias, *it is hypothesized that supplemental selenium has the potential to induce apoptosis of LSCs via the modulation of arachidonic acid pathway in favor of increased endogenous 15d-PGJ$_2$ production, which has demonstrable anti-proliferative and pro-apoptotic actions.* The two main models used for the project are: (i) Friend-virus (FV or FVP) erythroleukemia model and (ii) BCR-ABL LSCs transplantation model, which closely represent the pathologies of AML and chronic-phase CML, respectively. A third model, the MLL-AF9 LSCs transplantation model, which resembles a subtype of human AML, is used for a few major experiments. The results indicate that dietary supplementation of mice with selenium prevents the development of leukemia in all three models by eradicating the LSCs through mechanisms where AA-COX pathway metabolites such as 15d-PGJ$_2$ are of prime importance. Furthermore, data also suggests that CyPGs initiate pathways of apoptosis in LSCs via the action of DP receptors, which has not been previously described in literature.

Methods, Models, and Materials

Reagents: Sodium selenite, methylseleninic acid (MSA), L-selenomethionine (Se-Met), bacterial lipopolysaccharide (*E. coli* 0111:B4), indomethacin and DMSO were obtained from Sigma-Aldrich (St. Louis, MO). 1, 4-phenylenebis(methylene) selenocyanate (*p*-XSC) was provided by Dr. Shantu Amin, Penn State College of Medicine (Hershey, PA). CAY10526 (mPGES-1 inhibitor), HQL-79 (H-PGDS inhibitor), 15d-PGJ_2**,** 11-deoxy-16,16 -dimethyl PGE_2 (PGE_2 analog), imatinib mesylate, CAY10471 (DP2 antagonist), MK0524 (DP1 antagonist) , PGD_2 methyl ester (DP agonist), and ZK118182 (DP agonist) were purchased from Cayman Chemicals (Ann Arbor, MI). PKI 14-22 amide myristoylated (PKA inhibitor) and GF109203X (PKC inhibitor) were purchased from Tocris Biosciences (Minneapolis, MN), while KU55933 (ATM kinase inhibitor) was purchased from Calbiochem.

Friend-virus infection erythroleukemia model: The murine Friend erythroleukemia represents a powerful, working model of a disease whose evolution is very similar to that of human AML. The Friend virus is a retroviral complex of a replication-defective spleen focus forming virus (SFFV) that is the pathogenic component, and a replication-competent Friend murine leukemia virus (F-MuLV) [267]. FV induces an acute erythroleukemia that proceeds through a characteristic two-stage progression. The initial stage is marked by a polyclonal expansion of infected cells in the bone marrow and spleen of susceptible mice, while in the second stage, a clone of infected cells acquires new mutations, specifically mutations of p53 [268] and proviral insertional activation of *Spi/Pu.1* [269], which leads to the emergence of a leukemic clone and eventually erythroleukemia. The presence of these LSCs, now identified as $Kit^+Sca1^+M34^+$ cells [270], has been shown in this leukemic clone by performing colony assays and *in vivo* experiments. Supernatant of FP63 cells, which constitutively produce the polycythemia-causing strain of FV

(FVP), is used to inject FVP in Balb/c mice to induce leukemia. Once the mice develop disease in about two weeks after FV-infection, they are euthanized and spleens are used for analysis.

BCR-ABL-GFP-transduced-HSC transplantation model: The murine bone marrow (BM) retroviral transduction and transplantation model of CML is the most extensively studied and widely used model; it recapitulates many of the central features of human CML [271]. The BCR-ABL fusion oncoprotein is the result of a chromosomal translocation between long arms of chromosomes 9 and 22 [t(9:22)(q34;q11)]. MIGR-p210-BCR/ABL-IRES-GFP vector and control MSCV-IRES-GFP retroviral vector were a gift from Dr. Warren Pear (University of Pennsylvania, Philadelphia, PA). Retroviral stocks are produced in HEK293 cells using FuGENE 6 (Roche) transfection reagent. Virus-containing supernatant medium is collected after 1-2 days, concentrated by ultracentrifugation, and used for transducing hematopoietic stem cells (HSCs) [272]. 5-flurouracil-primed donor C57BL/6 mice (CD45.1 background) are used to obtain HSC-enriched BM cells. 24 hours after transducing these HSCs with the retrovirus, GFP^+ cells are sorted and 5 x 10^5 cells are injected into each lethally-irradiated recipient C57BL/6 mice (CD45.2 background). All recipient mice developed a fatal granulocytosis and splenomegaly within two weeks, a disease termed the murine CML-like myeloproliferative disorder [271, 273].

MLL-AF9-GFP-transduced-HSC transplantation model: The mixed lineage leukemia (*MLL*) gene is found in >60 different chromosomal translocations resulting in a number of different MLL-fusion proteins (MML-FPs). MLL-AF9 is one such fusion oncoprotein that is a result of t(9;11)(p21-22;q23) translocation, which is the most commonly observed chromosomal abnormality in the acute monocytic leukemia subtype of human AML (AML-M5) [274]. The model used here is also a bone marrow retroviral transduction and transplantation model similar to the one used for BCR-ABL (see above). As with the BCR-ABL model, the fusion oncoprotein MLL-AF9 was

expressed by the MSCV retroviral system that also expresses GFP. The transplanted C57BL/6 mice develop disease in ~5-6 weeks.

Animals, selenium diets, and indomethacin treatments: For the FV-leukemia model, Balb/c mice (~3 months old), maintained on Se-deficient (0.01 ppm selenite), Se-adequate (0.1 ppm selenite), or Se-supplemented (0.4 ppm selenite or 3 ppm methylseleninic acid) for 12 weeks, were retro-orbitally injected with FV as described previously [275]. These mice were dissected on day 15 post-infection and signs of splenomegaly as well as WBC count in the peripheral blood, characteristic of leukemia in this model [276, 277], were examined. For the BCR-ABL- and MLL-AF9- transplantation models, C57Bl/6 mice (~3 months old), maintained on Se-deficient (0.01 ppm selenite), Se-adequate (0.1 ppm selenite), or Se-supplemented (0.4 ppm selenite) for 12 weeks were lethally irradiated (950 rad), followed by transplantation of BCR-ABL- or control MSCV- transduced HSCs through their retro-orbital sinus. Hematological analysis was performed on an Advia 120 hematology auto analyzer equipped with a veterinary software program. COX-inhibitor indomethacin was administered to the mice at a concentration of 0.00325 % (w/v) in drinking water as described [278], from two weeks prior to FV injection or BCR-ABL transplantation until two weeks post-infection/transplantation, when the animals were euthanized. 15d-PGJ_2 or 11-deoxy- 16, 16-dimethyl-PGE_2 (PGE_2 analog) were exogenously administered at a concentration of 0.025 mg/kg/day (dissolved in PBS) intra-peritoneally from day 7 to day 14 post-infection (FV model) to the respective groups. The special diets were formulated based on the American Institute of Nutrition recommended rodent diets, and purchased from Harlan-Teklad (Madison, WI). All animals for experimental use were approved by the institutional animal care and use committee of The Pennsylvania State University.

***Ex vivo* BCR-ABL cell culture:** Two weeks post - BCR-ABL transplantation in Se-deficient mice, the mice were euthanized and spleen and/or bone marrow isolated. Spleens were mashed with a syringe plunger in sterile PBS. The tissue homogenate was passed through a 70 µm sterile cell strainer (BD Falcon) and the flow-through cells were suspended in a RBC lysis buffer. The resultant splenocytes were plated in culture dish with medium containing IMDM, 15 % FBS, 1 % BSA, penicillin-G (100 U/mL), streptomycin (100 µg/mL), 10 mg/mL insulin, 200 mg/mL transferrin, 1 mM β-mercaptoethanol, 2 mM L-glutamine, 50 ng/mL SCF (stem cell factor), 15 ng/mL BMP4 (bone morphogenetic protein 4), and 200 ng/mL sHH (sonic hedgehog). To these *ex vivo* culture of cells, various chemical treatments were done as described in the experiments for duration of ~24 hours, followed by FACS analysis for LSC populations based on GFP^+ cells and/or other stem cell markers.

Preparation of lipid extracts from cell culture supernatants: Murine macrophage-like cell line RAW264.7 (ATCC, Manassas, VA) was cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) containing 5 % FBS (ATCC), 2 mM L-glutamine (Cellgro, Manassas, VA), penicillin-G (100 U/ml), and streptomycin (100 µg/ml; Invitrogen). For the experiment, ~2 x 10^6 cells were plate in a 10 cm^2 culture dish. The cells were pre-stimulated with 100 ng/mL LPS for 2 hours and washed twice. Cells were treated with indomethacin (10 µM), HQL-79 (25 µM), or CAY10526 (20 µM), followed by addition of sodium selenite (250 nM) for three days. Supernatants (5 mL) were collected every 24 hours for three days. Finally the pooled supernatant (15 mL total) was used for extraction of lipid fraction by phase separation using an equal volume of hexane - ethyl acetate mixture (1:1). Lipid extracts were stored in ethyl acetate (5 mL) at -20 ^oC until further use. For treatments, 100 µL of lipid extract was dried using argon gas and reconstituted in sterile PBS, which was added to the *ex vivo* cultured LSCs (sorted) or splenocytes (unsorted).

Flow cytometry and FACS (fluorescence-activated cell sorting): For the experiments involving FV-model, spleen cells were labeled with anti-Sca1, anti-Kit (BD Biosciences) and anti-M34 (from Dr. Paulson's lab) antibodies for flow cytometry. M34 is a monoclonal antibody that recognizes gp55 from SFFV (spleen focus-forming virus) component of FV [279]. For the BCR-ABL-transplantation experiments, BCR-ABL- or control MSCV- transduced HSCs were sorted by FACS for GFP^+ cells using the FITC channel. For the post-transplantation analysis of LSC populations in spleen or bone marrow, anti-Sca1, anti-Kit antibodies were used for labeling the cells, besides gating the cells for GFP^+ (FITC). For the MLL-AF9 model, LSC populations were determined in spleen or bone marrow based on GFP^+ cells. For apoptosis studies on LSCs, Annexin V FITC (BD Biosciences) was incubated with cells for 15 min on ice followed by flow cytometric analysis. FC500 (Beckman Coulter) and Influx Cell Sorter (BD Biosciences) were the instruments used for flow cytometry and sorting, respectively. Data was analyzed using FlowJo software (Treestar, San Carlos, CA)

Western blotting: Spleen lysates were prepared by mashing spleen with the homogenizer and lysing the tissue with mammalian protein extraction reagent (M-PER, Thermo Scientific) containing protease inhibitors. Protein concentration was estimated by BCA reagent. Equal amount of protein (25 µg) was electrophoresed on a 12.5% SDS-PAGE followed by immunoblotting. The following primary antibodies were used to probe the membrane: anti-p53, anti-GPX1 (Abcam, Cambridge, MA), anti-DP2 (Santa Cruz Biotechnology, CA) and anti-GAPDH (Fitzgerald, Concord, MA). Appropriate secondary antibodies were used. Immunoreactive bands were visualized using the West Pico and/or West Femto chemiluminescence reagents obtained from Thermo Scientific (Rockford, IL). GAPDH was used as an internal control for normalization.

Statistics: The results are expressed as mean ± SE from replicate experimental readings. Differences between the groups were analyzed by either one-way ANOVA followed by Tukey's post-hoc test (using GraphPad Prism Version 5, or vassarstats.net), or Student's *t* test (using GraphPad Prism). The criterion for statistical significance was $P<0.05$, unless state otherwise.

Results

Selenium supplementation prevents development of splenomegaly

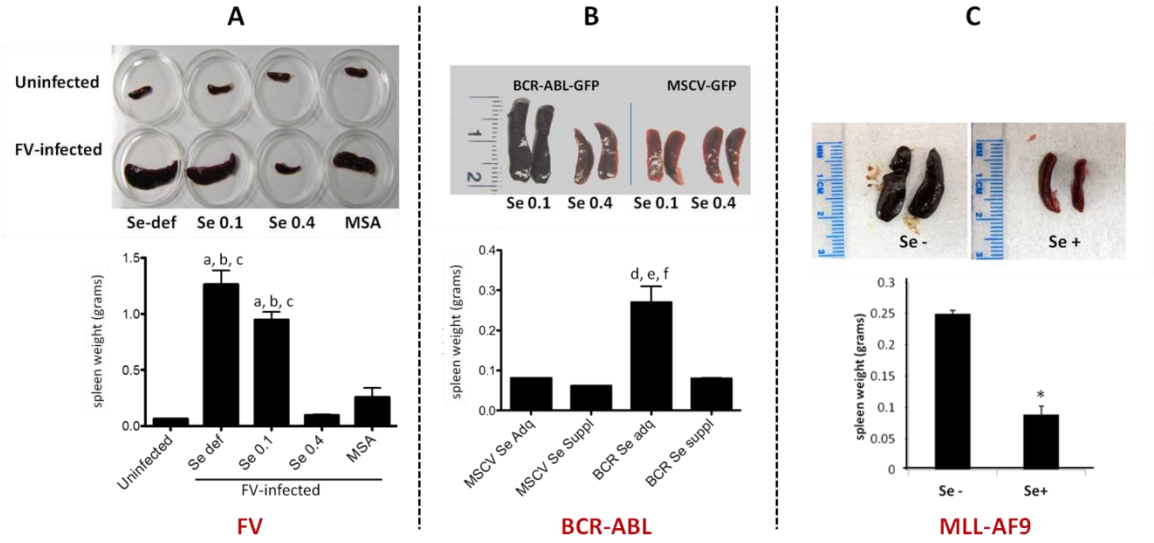

Fig. 18 Selenium supplementation prevents splenomegaly. Panel A shows spleens from Friend virus (FV)-infected or uninfected Balb/c mice on Se-deficient, Se-adequate (0.1 ppm selenite), Se-supplemented (0.4 ppm selenite), or MSA (4 ppm methyl seleninic acid) diets at two weeks post-infection. Spleen weights (n=4 per group) are plotted as means ± SE where statistical significance (a – compared with Uninfected, b – compared with Se 0.4, and c – compared with MSA) was set at $P<0.05$, as determined by one-way ANOVA followed by Tukey's post-hoc test. Panel B shows spleens from Se-adequate and Se-supplemented C57Bl/6 mice transplanted with MSCV-GFP- (control vector) or BCR-ABL-GFP-transduced HSCs at two weeks post-transplantation. Spleen weights (n=3 per group) are plotted as means ± SE where statistical significance (d – compared with MSCV Se Adq, e – compared with MSCV Se Suppl, and f – compared with BCR Se Suppl) was set at $P<0.05$, as determined by one-way ANOVA followed by Tukey's post-hoc test. Panel C shows spleens from Se-def or Se-supplemented C57Bl/6 mice transplanted with MLL-AF9-GFP- transduced HSCs at five weeks. Spleen weights (n=3 per group) are plotted as means ± SE where statistical significance (* - compared with Se-) was set at $P<0.01$, as determined by Student's t-test.

The sizes of spleens from Se-deficient FV-infected mice increased as compared to those of Se-def uninfected controls, mainly due to erythroid hyperplasia. Se-adequate (0.1 ppm selenite) status was also not sufficient to prevent splenomegaly. However, the sizes of spleens from Se-

supplemented (0.4 ppm selenite) FV-infected mice were nearly the same as those from the controls (Fig. 18A). MSA diet also significantly reduced the size of spleen, but the difference was not as noticeable as 0.4 ppm selenite. For the BCR-ABL transplantation, only Se-adequate and Se-supplemented (0.4 ppm selenite) mice were used. Empty MSCV vector transplantation was used as a negative control. As observed in the FV experiment, the spleens from Se-adequate BCR-ABL^{+} mice were enlarged, but Se-supplementation prevented splenomegaly when compared with their respective controls (Fig 18B). Similar results were obtained when spleens from Se-supplemented and Se-adequate MLL-AF9 LSC transplanted mice were studied (Fig. 18C). H&E-stained splenic sections from FV experiment are shown in Fig 19. The splenic architecture was grossly disrupted in Se-deficient mice upon FV infection (Fig. 19C); there was no distinction between the white pulp and the red pulp. The spleen section from Se-supplemented FV-infected mice appeared similar to that from an uninfected control (Fig. 19A, B).

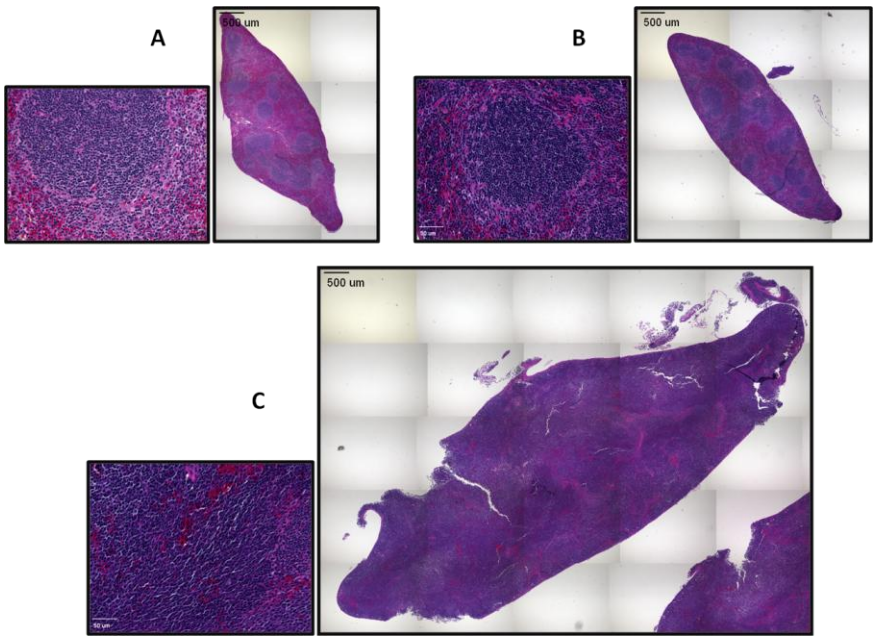

Fig. 19 Selenium supplementation prevents disruption of splenic architecture. Panel A, Se-deficient uninfected; Panel B, Se-supplemented FV-infected; Panel C, Se-deficient FV-infected. Lower and higher magnifications of H&E stained sections of spleens are shown, representative of three spleens in each group.

Selenium supplementation prevents leukemic increase of WBC count

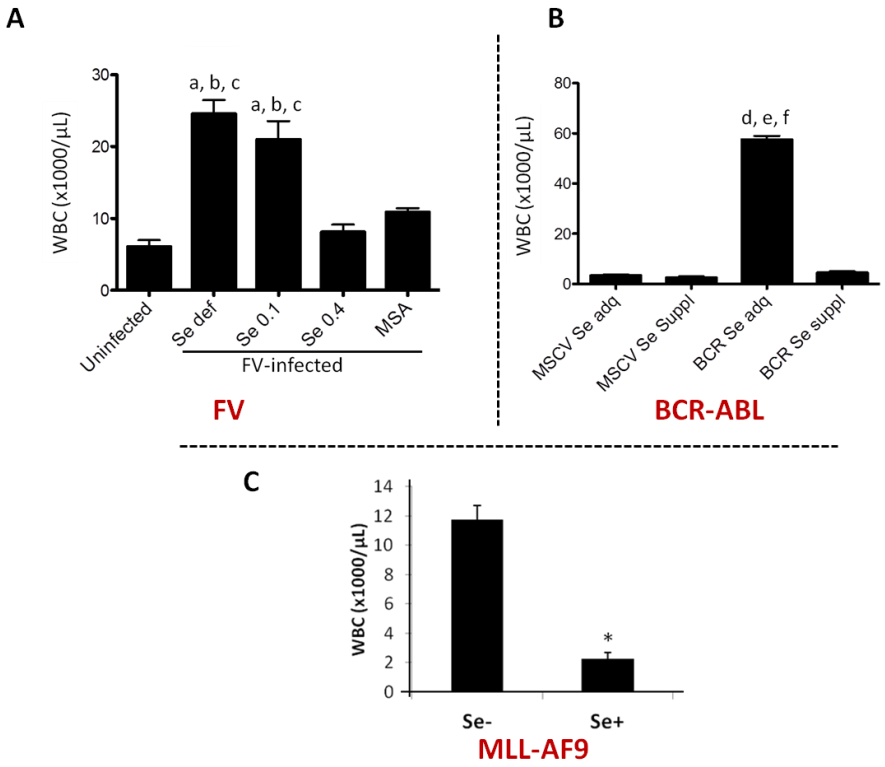

Fig. 20 Selenium supplementation prevents rise in WBC counts. Panels A, B, and C show the WBC levels in mice from the FV- (n=4), BCR-ABL- (n=3), and MLL-AF9- (n=3) models of leukemia, respectively. Blood was collected from the retro-orbital sinus before the mice were sacked for obtaining spleen and/or bone marrow. In Panels A and B, data represents the mean ± SE where statistical significance (a – compared with Uninfected, b – compared with Se 0.4, c – compared with MSA, d – compared with MSCV Se Adq, e – compared with MSCV Se Suppl, f – compared with BCR Se Suppl) was set at $P<0.05$, as determined by one-way ANOVA followed by Tukey's post-hoc test. In Panel C, * represents statistical significance ($P<0.05$) in the mean ± SE, using Student's t-test.

The WBC levels were found to be elevated in Se-deficient and Se-adequate groups of FV-infected mice, while they were normal in the Se-supplemented group (Fig. 20A). The MSA group showed counts in the normal range, but were still higher than those in the uninfected and Se-supplemented FV-infected groups. In the BCR-ABL LSC transplantation experiment, WBC counts in the BCR-ABL transplanted Se-adequate group were elevated significantly, while those in the

BCR-ABL-transplanted Se-supplemented group were in the normal range (Fig. 20B). The MSCV-transplanted groups showed counts in normal range (negative controls). The WBC counts were significantly elevated in the MLL-AF9 – transplanted mice on Se-deficient diets as compared with those on Se-Supplemented diets (Fig. 20C).

Selenium supplementation eradicates LSCs

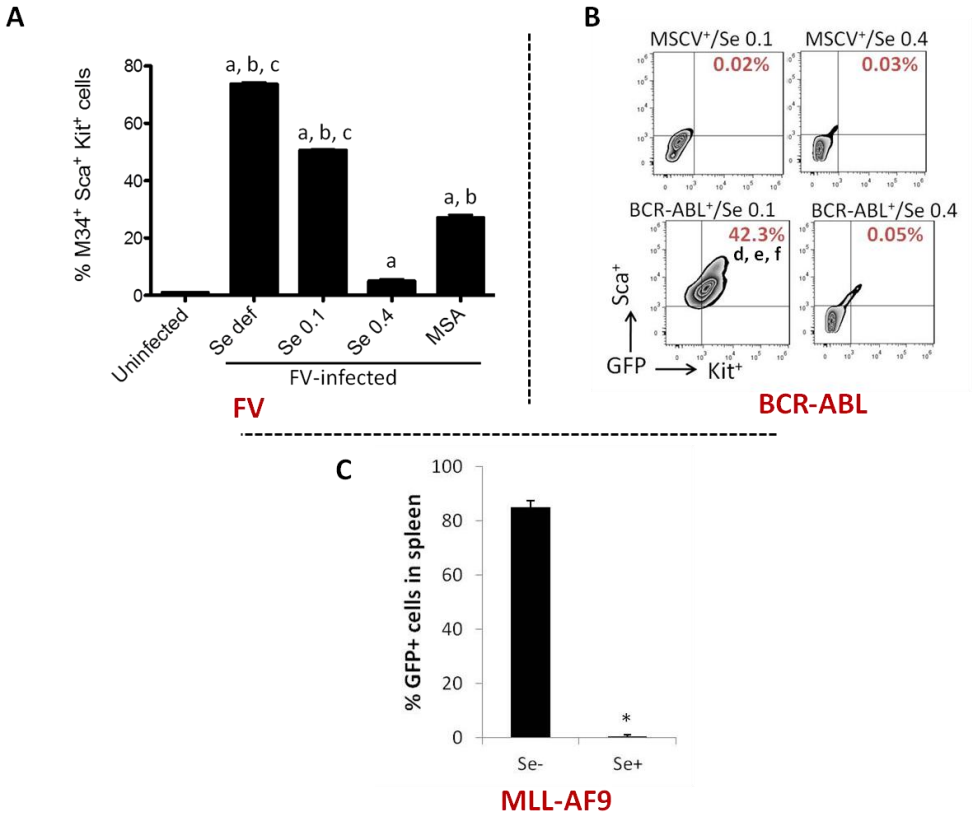

Fig. 21 Selenium supplementation eradicates leukemic stem cells (LSCs). Panel A shows LSC populations from spleens of FV-infected mice (n=4 per group) on different diets compared with uninfected controls (UI). Cells were counted by flow cytometry using M34, Sca, and c-Kit as the markers. Panel B shows number of GFP^+, Sca^+, and $c\text{-}Kit^+$ cells from spleens of MSCV- or BCR-ABL- transplanted mice (n=3 per group) on Se-adeq (0.1 ppm selenite) or Se-supplemented (0.4 ppm selenite) diets. Panel C shows numbers of GFP^+ cells from spleens of MLL-AF9- transplanted mice on Se-deficient or Se-supplemented diets (n=3 per group). In Panels A and B, data represents the mean ± SE where statistical significance (a – compared with Uninfected, b – compared with Se 0.4, c – compared with MSA, d –

compared with MSCV Se Adq, e – compared with MSCV Se Suppl, f – compared with BCR Se Suppl) was set at $P<0.05$, as determined by one-way ANOVA followed by Tukey's post-hoc test. In Panel C, * represents statistical significance ($P<0.05$) in the mean ± SE using Student's t-test.

Flow-cytometry analysis of the spleen using LSC-specific markers (Sca1 and c-Kit) indicated that Se-supplementation significantly ablated the LSCs, when compared to the Se-deficient mice (Fig 21A). We consistently observed selenite to be more effective than MSA. Although there was a decrease in the levels of LSCs in Se-adequate mice, it was not statistically significant. These results correlate to the extent of splenomegaly and WBC counts in mice infected with FVP. Similarly, Se supplementation (0.4 ppm selenite) significantly eradicated the transplanted BCR-ABL GFP^+ LSCs and MLL-AF9 GFP^+ LSCs, when compared to the Se-adequate mice (Fig 21B, C).

Selenium supplementation prevents disease in secondary LSC-transplanted mice

BCR-ABL -transduced LSCs (from donor mice with CD45.1 background) were transplanted into Se-adequate mice (primary transplants). These mice developed disease in two weeks as described earlier. GFP^+ LSCs were sorted from spleens and transplanted into lethally irradiated Se-adequate or Se-supplemented mice (secondary transplants). Fig. 22A shows the schematic outline of the experiment. The secondary transplants were followed up for signs of disease by periodic WBC counts. The Se-adequate group showed signs of disease during fifth week post-transplantation. Spleens, WBC counts, and LSC populations from spleens and bone marrow from both groups of secondary transplants are shown (Fig. 22B, C, D, and E). Mice on Se-supplemented diet failed to develop disease.

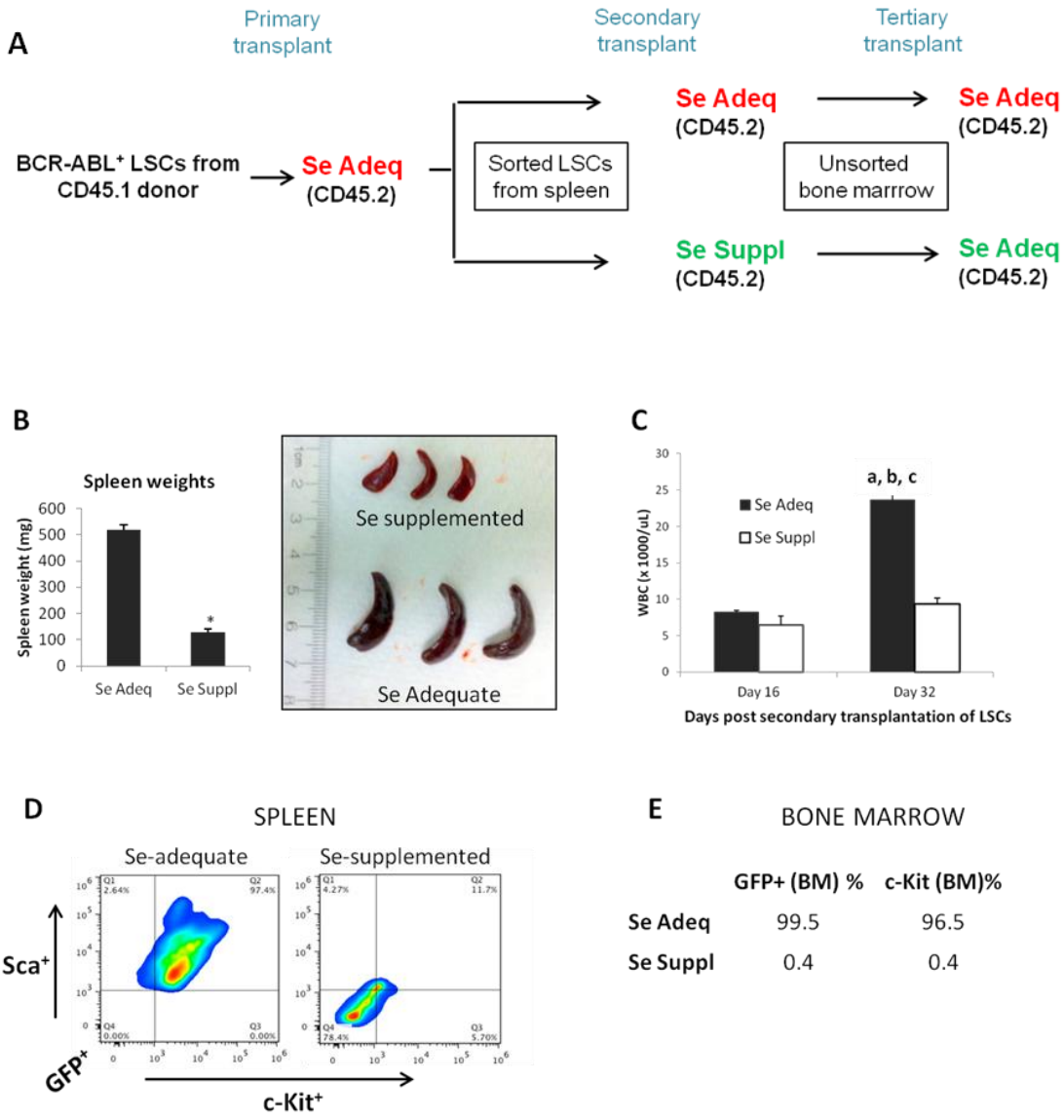

Fig. 22 Selenium supplementation prevents BCR-ABL leukemia in secondary-transplants. Panel A represents the schematic for serial transplants. BCR-ABL-GFP^{+} LSCs from spleens of Se-adequate mice (primary transplants) were sorted by flow cytometry and transplanted into sub-lethally irradiated recipients on Se-adequate or Se-supplemented diets (secondary transplants). Panel B shows spleens from secondary transplants at ~4.5 weeks post-transplantation. * P<0.05, determined by Student's t-test. Panel C shows WBC counts as means ± SE where statistical significance (a – compared with Se Adeq day 16, b – compared with Se Suppl day 16, and c – compared with Se Suppl day 32) was set at P<0.05, as determined by one-way ANOVA followed by Tukey's post-hoc test. Panels D and E show the number of LSCs in spleen and bone marrow, respectively, counted using flow cytometry.

Selenium supplemented secondary-transplant donors prevent disease in selenium adequate tertiary-transplant recipients

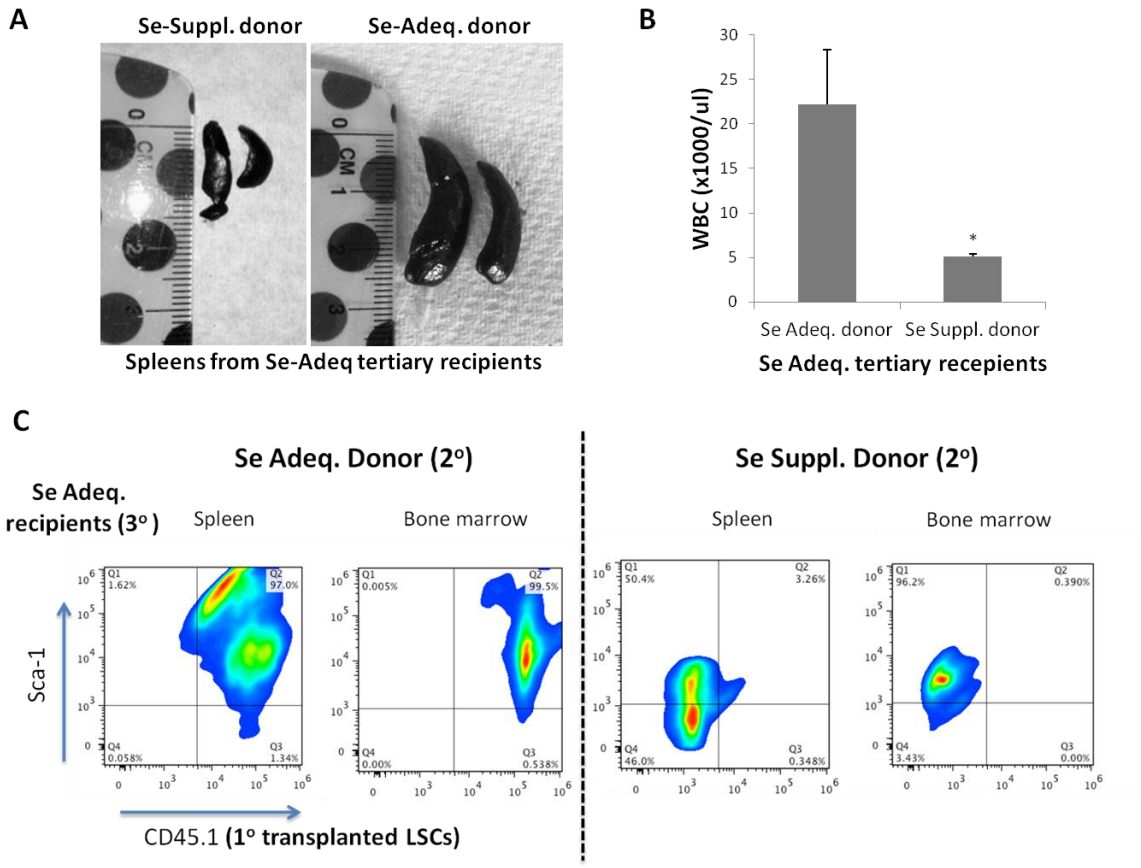

Fig.23 Selenium supplemented donors prevent disease in selenium-adequate recipients. Whole bone marrow from Se-adequate or Se-supplemented BCR-ABL secondary transplants (refer Fig. 22A) were transplanted into lethally irradiated Se-adequate recipient mice (tertiary transplants). After ~three weeks, blood was collected and analyzed for WBC counts as shown in Panel B. * indicates statistical significance ($P<0.05$), as determined by Student's t-test. Panel A shows the spleens, while Panel C shows the numbers of LSCs in spleens and bone marrow of the tertiary transplants on basis of Sca1 and CD45.1 markers. The HSCs used for the primary transplantation were derived from mice with a CD45.1 background, while all the recipient mice used for transplantations were from a CD45.2 background. n=3 for each group of tertiary transplants.

As a continuation of the secondary transplantation experiment described above, whole bone marrow cells from Se-adequate and Se-supplemented secondary transplants were isolated and

transplanted into Se-adequate recipients (tertiary transplants). The aim of this experiment was to confirm the eradication of LSCs from Se-supplemented mice by investigating the 'transmissibility' of the disease to susceptible (Se-adequate) recipients. Se-adequate tertiary transplant group of mice that were transplanted with bone marrow from Se-supplemented secondary transplants showed no disease after ~three weeks, unlike the group that received bone marrow transplantation from Se-adequate secondary transplants, as shown in Fig. 23.

Selenium supplementation prevents relapse in imatinib-treated mice

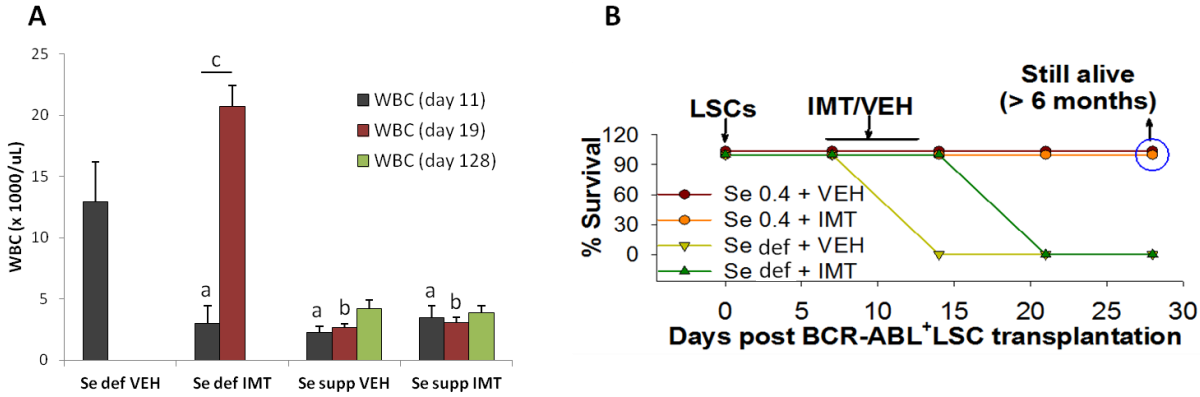

Fig. 24 Selenium supplementation prevents relapse of BCR-ABL leukemia in imatinib-treated mice. Lethally-irradiated Se-supplemented or Se-adequate mice were transplanted with BCR-ABL LSCs. Imatinib (75 mg/kg/day) or vehicle treatment was administered for a week starting from day 7 post-transplantation. The mice were followed for signs of disease and WBC counts. Panel A shows WBC levels at different time points. Data represents means ± SE where statistical significance was set at $P<0.05$, as determined by either one-way ANOVA followed by Tukey's post-hoc test (a – day 11 counts compared with Se def VEH, and b – day 19 counts compared with Se def IMT), or Student's t-test (c). Panel B shows the survival curve for the mice. n=3 in each of the four groups.

To determine if selenium supplementation was effective in preventing relapse after treatment with conventional chemotherapeutic agent, imatinib (Gleevac), BCR-ABL LSCs were transplanted into Se-adequate and Se-supplemented mice. A week later, imatinib mesylate or vehicle control (PBS) was administered intra-peritoneally at a dose of 75 mg/kg/day for 7 days. The mice were

followed up thereafter for signs of disease and periodic WBC counts. Vehicle-treated Se-adequate mice succumbed to disease by the end of the second week, as seen in earlier experiments. Imatinib-treated Se-adequate mice showed increased WBC counts (Fig. 24A) a week after stopping imatinib, suggesting relapse of disease, and died by the end of third week post-transplantation. However, imatinib- and vehicle-treated Se supplemented mice showed normal WBC counts even after 6 months of the BCR-ABL LSC transplantation and were surviving (Fig. 24B).

Indomethacin and HQL-79 block effects of selenium supplementation *ex vivo*

To examine the role of the arachidonic acid – cyclooxygenase (AA-COX) pathway in the selenium-mediated apoptosis of LSCs, an *ex vivo* model was established as described in the methods. Selenite was added exogenously to LSCs in culture plates at different concentrations ranging from 0 to 500 nM for ~24 hours. BCR-ABL-GFP$^+$ HSCs started showing death with increasing selenite concentrations above 100 nM, while the MSCV-GFP$^+$ HSCs (controls) were unaffected by selenite (Fig. 25A). When BCR-ABL-GFP$^+$ HSCs were pre-treated with 10 µM indomethacin (non-specific COX inhibitor) or 25 µM HQL-79 (H-PGDS inhibitor) for 1 hour, treatment with selenite (250 nM) did not kill the pre-treated cells (Fig. 25B). However, selenite killed the vehicle-treated cells. Similarly, when other forms of selenium (all at 250 nM) were tried in the *ex vivo* model of BCR-ABL-GFP$^+$ cells, MSA decreased the number of GFP$^+$ cells significantly, but not to the same extent as selenite. Se-Met and p-XSC were unable to affect the GFP$^+$ cells (Fig. 25C). Furthermore, LPS (100 µg/mL) -pre-stimulated RAW264.7 macrophages were pre-treated with indomethacin, HQL-79, or CAY10526 (PGES inhibitor), and treated with or without 250 nM selenite for three days. Cell culture supernatants were collected from which lipids were extracted and used as a source of PGs. *Ex vivo* BCR-ABL-GFP$^+$ HSCs were then

subjected to these lipid extracts for ~24 hours. Lipid extracts from selenite treated RAW264.7 macrophages killed the GFP$^+$ cells, but those from indomethacin or HQL-79 pre-treated RAW264.7 macrophages did not kill the GFP$^+$ cells (Fig. 25D).

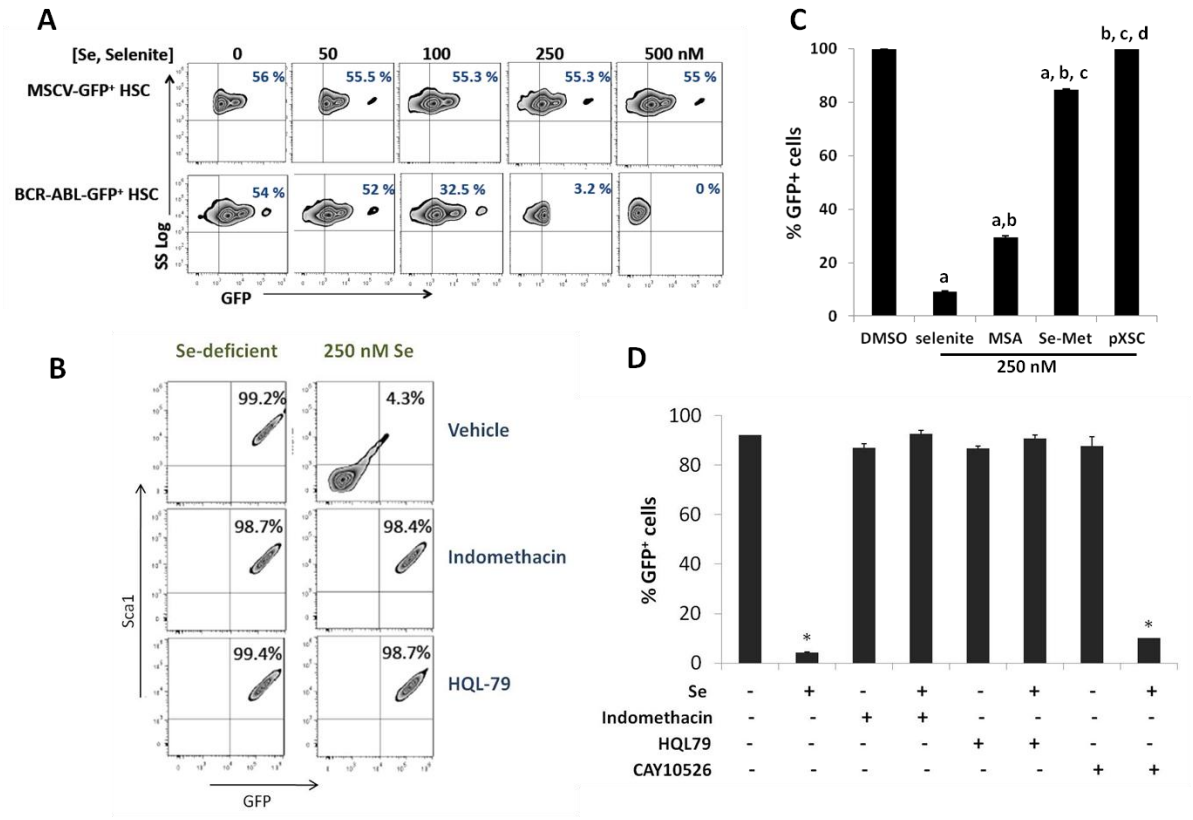

Fig. 25 Indomethacin and HQL-79 block effects of selenite *ex vivo* on BCR-ABL-GFP$^+$ cells. Splenocytes from Se-deficient MSCV-GFP- or BCR-ABL-GFP- transplanted mice were plated in a culture dish. Selenite in different concentrations was added and GFP$^+$ cells were counted after 24 hours (Panel A). In panel B, the flow cytometry results show the effect of 1 hour pre-treatments with 10 µM indomethacin or 25 µM HQL-79 before adding 250 nM selenite. Panel C shows the number of GFP$^+$ cells upon treatment with different forms of selenium at 250 nM. Data represents means ± SE where statistical significance (a – compared with DMSO control, b – compared with selenite, c – compared with MSA, and d – compared with Se-Met) was set at P<0.01, as determined by one-way ANOVA followed by Tukey's post-hoc test. Effect of adding lipid extracts derived from cell culture supernatants of LPS-stimulated RAW264.7 macrophages with various treatments (250 nM Se, 10 µM indomethacin, 25 µM HQL-79, or 20 µM CAY10526) to plated BCR-ABL-GFP splenocytes is shown in Panel D. * indicates statistical significance (P<0.01) between means ± SE when compared with the untreated control group, using Student's t-test.

Indomethacin blocks effects of selenium supplementation *in vivo*; 15d-PGJ_2 rescues

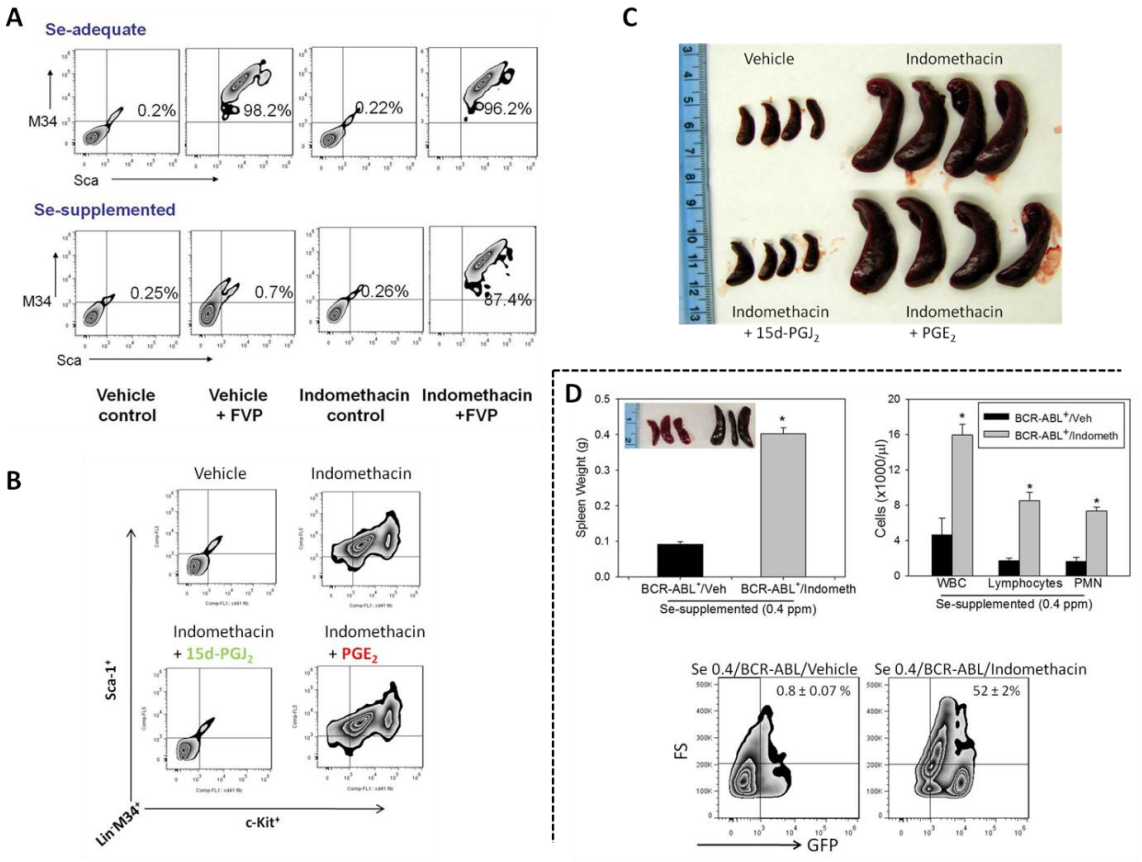

Fig.26 Indomethacin blocks effects of selenium supplementation *in vivo*. Indomethacin was administered to Se-adequate or Se-supplemented Balb/c mice (n=4 per group) through drinking water (0.00325% w/v) for two weeks prior to FV-infection until two weeks later. The LSC populations in their spleens at two weeks post-infection are shown in Panel A. 15d-PGJ_2 or PGE_2 analog was administered to a group each at 0.025 mg/kg/day of indomethacin-treated Se-supplemented mice from day 7 to day 14. Panels B and C show LSC populations on flow cytometry and spleen sizes, respectively. Panel D shows spleens, WBC counts, and LSC numbers in spleens from indomethacin-treated Se-supplemented mice with BCR-ABL-GFP transplantation (n=3 for each group). * P<0.05 when compared with respective vehicle controls, using Student's t-test.

To see if the AA-COX pathway is active *in vivo* to mediate the effects of selenium, indomethacin was administered to the mice (see methods). In the FV model, we found that indomethacin blocked the effects of selenium supplementation in FV-infected mice as compared to the

vehicle-treated Se-supplemented FV-infected mice. The $Sca1^+$ and $M34^+$ cell populations (LSCs) were as high in the indomethacin-treated selenium-supplemented FV-infected mice as in the selenium-adequate FV-infected mice, which developed the disease (Fig. 26A). When 15d-PGJ_2 was administered to one such group of indomethacin-treated selenium supplemented FV-infected mice for 7 days at 0.025 mg/kg/day intra-peritoneally, the mice were rescued of the effects of indomethacin, and they did not show signs of disease as evident from the spleens sizes and LSC populations (Fig. 26B, C). As a negative control, 11-deoxy 16, 16-dimethyl-PGE_2, a PGE_2 analog, was administered to a similar group, which failed to rescue the mice. In the BCR-ABL model, we used only selenium supplemented BCR-ABL LSC transplanted mice, and compared the effects of indomethacin and vehicle treatments. The indomethacin-treated mice showed increased WBC counts and LSC populations as compared with the vehicle-treated mice (Fig. 26D).

DP receptors mediate the effects of 15d-PGJ_2 in LSCs; role of PKA, PKC

As 15d-PGJ_2 and other PGD_2 metabolites act through cell surface DP receptors, we pre-treated whole spleen cells (*ex vivo*) from BCR-ABL transplanted Se-adequate mice with 10 nM MK0524 (DP1 antagonist) or 10 nM CAY10471 (DP2 antagonist) for 1 hour. 250 nM selenite was then added and GFP^+ cells were counted after 24 hours. Both, MK0524 and CAY10471 blocked the selenium-mediated killing of BCR-ABL-GFP^+ cells (Fig. 27A). Whole spleen cells were similarly pre-treated with MK0524 or CAY10526, and instead of adding selenite directly to these cells, lipid extracts from supernatants of Se-deficient (Se-) or Se-supplemented (Se+) LSP-stimulated RAW264.7 macrophages were used as a source of CyPGs. Se+ supernatants eliminated most of the cells as shown by Annexin V staining, while MK0524 and CAY10471 blocked apoptosis (Fig. 27B).

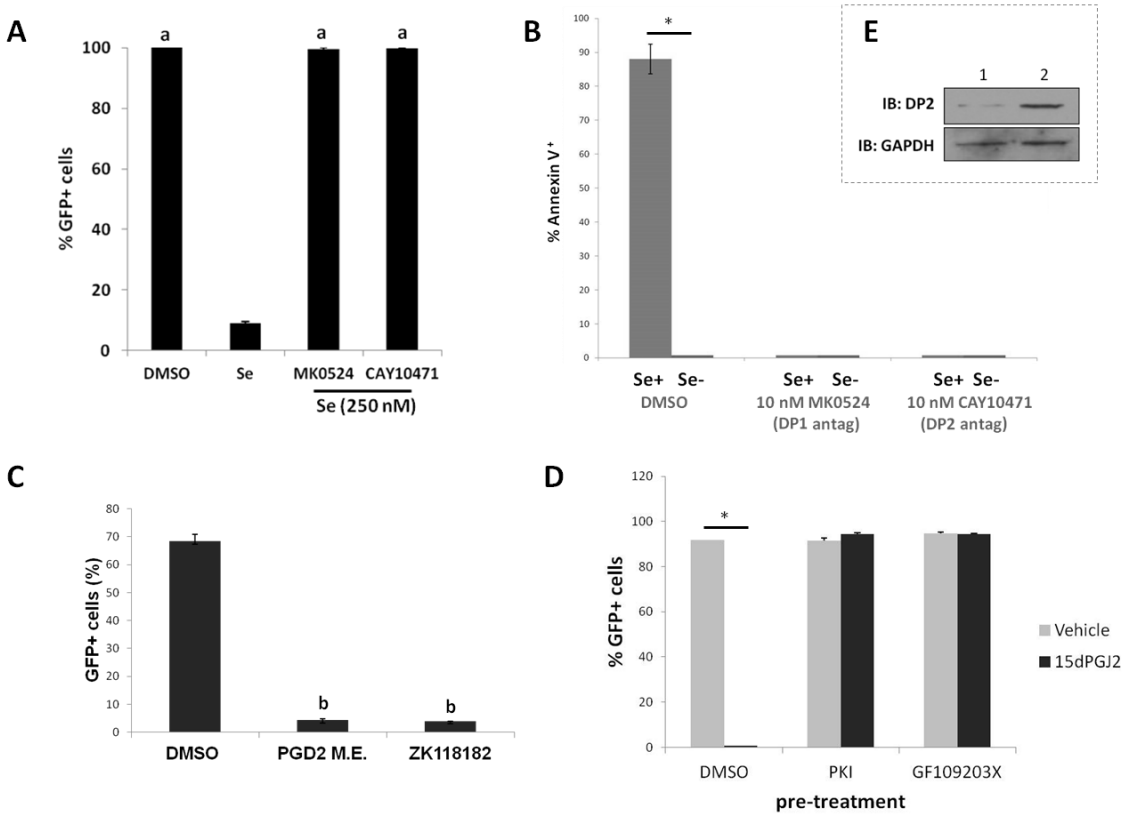

Fig.27 DP receptors mediate the effects of selenite/15d-PGJ$_2$. Panel A shows the GFP$^+$ cell populations when BCR-ABL-GFP splenocytes were pre-treated with 10 nM MK0524 or 10 nM CAY10471 before addition of 250 nM selenite *ex vivo*. Panel B shows the effect of adding lipid extracts derived from supernatants of LPS-stimulated Se-deficient or Se- supplemented RAW264.7 macrophages on BCR-ABL-GFP splenocytes pre-treated with MK0524 or CAY10471. Annexin V staining was done to count the cells undergoing apoptosis. Panel C shows the number of GFP$^+$ cells upon *ex vivo* treatment of sorted BCR-ABL-GFP cells with DP agonists, 25 nM PGD$_2$ methyl ester and 100 nM ZK118182. Panel D shows GFP$^+$ populations after pre-treatment of sorted BCR-ABL-GFP cells *ex vivo* with protein kinase A inhibitor (1 µM PKI 14-22 amide myristoylated) or protein kinase C inhibitor (1 µM GF109203X), followed by treatment with 25 nM of 15d-PGJ$_2$ or vehicle control for 24 hours. Panel E shows Western blot for DP2 expression in unsorted BCR-ABL splenocytes (1), and sorted BCR-ABL LSCs (2). GAPDH expression represents internal loading control. Statistical significance was set at $P<0.01$ as determined by either one-way ANOVA followed by Tukey's post-hoc test (a – compared with Se, b – compared with DMSO control), or Student's t- test (indicated by *).

The eradication of BCR-ABL-GFP$^+$ cells by PGD_2 agonists was examined. Two agonists, PGD_2 methyl ester and ZK118182, were used at 25 nM and 100 nM concentrations, respectively and cells were treated for 24 hours. Flow cytometry for GFP$^+$ populations showed that both PGD_2 agonists significantly lowered GFP$^+$ cells, and the decrease was blocked by CAY10471, but not MK0524 (Fig. 27C). Finally, knowing that signaling downstream of DP receptors involves protein kinases A and C (PKA, PKC), inhibitors of PKA (PKI 14-22 amide, myristoylated) and PKC (GF109203X) were used at 1 µM concentrations for pre-treatments. PKA and PKC inhibition blocked the eradication of BCR-ABL GFP$^+$ cells by 25 nM 15d-PGJ_2 (Fig. 27D).

p53 is involved in selenium - 15d-PGJ_2 – mediated LSC apoptosis

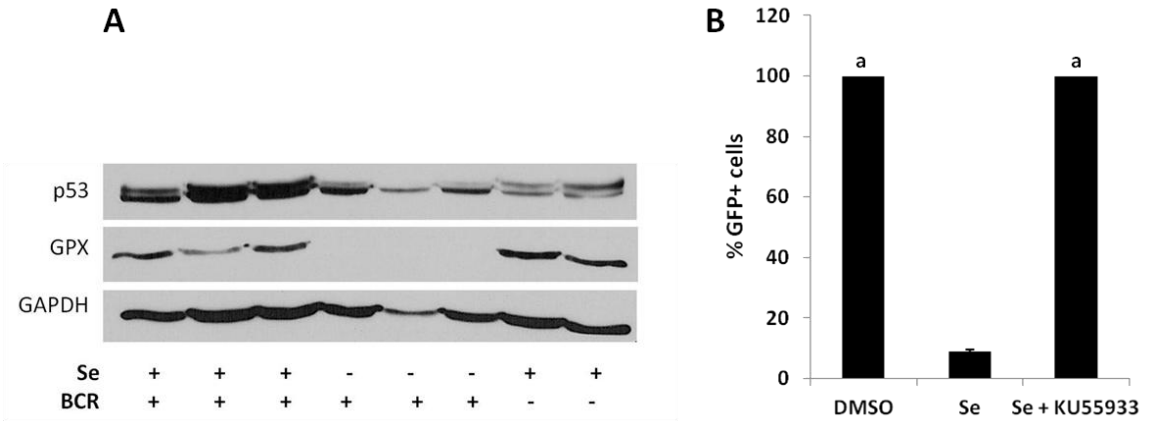

Fig. 28 Involvement of p53 in selenium-mediated apoptosis. Panel A: 25 µg protein from whole spleen lysates from BCR-ABL-GFP transplanted Se-deficient or Se-supplemented mice (n=3) were analyzed for p53 and GPx levels by Western blotting. GAPDH was used as internal loading control. The last two lanes show levels from un-transplanted mice. Panel B shows the GFP$^+$ cell populations after BCR-ABL-GFP splenocytes were pre-treated with ATM kinase inhibitor, KU55933 (50 nM), followed by 250 nM selenite *ex vivo.* Statistical significance (a- compared with Se) was set at $P<0.01$, as determined by one-way ANOVA followed by Tukey's post-hoc test.

p53 activation is a very well known pathway leading to the cascade of apoptosis. We examined at p53 protein levels on spleen lysates from BCR-ABL transplanted Se-supplemented (Se+) or Se-

deficient (Se-) mice. p53 levels were increased in spleen lysates from Se-supplemented mice when compared with Se-deficient mice and non-transplanted controls (Fig. 28A). GPx levels show the selenium status of the mice. 15d-PGJ_2 activates ATM (ataxia telangiectasia mutated) kinase activity by covalent binding with sulfhydryl groups [179], which leads to p53 activation and apoptosis subsequently. We used KU55933, an inhibitor of ATM kinase, to pre-treat whole spleen cells from BCR-ABL transplanted mice, followed by treatment with selenite. 50 nM KU55933 blocked the selenite-mediated apoptosis of GFP^+ cells (Fig. 28B).

Discussion

The anti-carcinogenic effect of selenium has been a major topic of research over the past few decades. Adequate levels of selenium in the body are easily achieved by a daily intake of ~55-70 µg Se/day, which corresponds to 0.1 ppm (mg/kg) Se in diet [252]. Although adequate dietary intake of selenium has been shown to be effective in preventing disorders like Keshan's cardiomyopathy or Kashin-Beck's osteoarthropathy that arise as a direct consequence of prolonged selenium deficiency (<0.01 ppm Se in diet) [7, 78], adequate selenium levels are not reported to have any anti-carcinogenic effects. However, supranutritional doses of selenium (>200 µg Se/day), corresponding to >0.4 ppm Se in diet, have been shown to lower the development and risk of dying from cancers of prostate, lung, colon [206], although the study was primarily set up to determine if selenium decreases the incidence of skin cancer. Meta-analysis from a number of studies has shown that selenium has the most consistent protective effect when comparison was made between levels of serum selenium, retinol, vitamin E, and β-carotene in relation to cancer risk [280]. But there is very limited knowledge regarding the chemopreventive effects of selenium supplementation and various forms of leukemias, which are gathering increasing evidence of being stem cell disorders.

Cyclopentenone prostaglandins (CyPGs) such as the AA-COX-HPGDS product, 15d-PGJ_2, have been reported to have pro-apoptotic effects on a variety of tumor models including leukemic cells [123, 151, 180, 183]. Bioinformatic approaches have speculated a potential role of 15d-PGJ_2 as a means of inducing apoptosis in leukemia stem cells (LSCs) [261]. Along these lines, a recent report from our laboratory demonstrated that 25 nM 15d-PGJ_2 is enough to completely eradicate LSC populations *ex vivo* [120]. Having already shown in Chapter 2 that selenium supplementation can modulate the AA-COX-HPGDS metabolism in favor of increased

endogenous production of 15d-PGJ_2, we investigated if dietary selenium supplementation has the ability to kill LSCs and prevent the development of disease using murine models of leukemia. We found that 0.4 ppm Se in the diet prevents development of leukemia in mice by selectively ablating LSCs through mechanisms involving a favorable modulation of the AA-COX pathway, and receptor-mediated downstream signaling that leads to the activation of tumor suppression gene, p53.

Spleen is not just a major lymphoid organ, but also an important site for extramedullary hematopoiesis [281]. Splenomegaly is thus a common clinical feature of infiltrative malignancies including myeloid leukemias. Hence, assessing the spleen size has been used as one of the critical end-points to investigate the effects of dietary selenium after induction of leukemia in mice. The normal size of spleens seen in Se-supplemented mice as compared to the 10-fold increase in spleen size in Se-deficient mice upon FV-infection is a dramatic finding, and although the FV-infection model cannot be reproduced in humans, the result signifies that supranutritional levels of selenium are affecting the polyclonal expansion of infected cells in the spleen and bone marrow, preventing the development of mutations that lead to LSC formation. The inability to distinguish splenic red pulp from white pulp demonstrates the grossly disrupted histo-architecture of the FV-infected spleen in Se-deficient mice. The splenomegaly in the BCR-ABL model, though not as pronounced as in the FV-model, indicates infiltration nevertheless. It is particularly significant because splenomegaly is considered as one of the most important prognostic factors for patients with CML [282, 283]. Importantly, the WBC counts reflect the spleen sizes confirming the anti-leukemic effect of Se-supplementation across three different models. Additionally, the absence of LSCs from the spleen and bone marrow in Se-supplemented mice in all the models demonstrates that selenium indeed targets LSCs, unlike conventional chemotherapeutic agents. Most importantly, the eradication of LSCs from spleens

by selenium underlines the relevance of selenium as a chemopreventive agent in CML, given the fact that leukemic spleen cells have been shown to be more potent than bone-marrow -derived cells in their contribution to the development of CML as well as resistance to imatinib [281].

It has long been recognized that just like HSCs have the ability to reconstitute bone marrow of irradiated recipients, LSCs can be defined functionally by their ability to transfer malignancy on xenotransplantation into an immunodeficient recipient animal, usually non-obese diabetic - severe combined immunodeficiency (NOD-SCID) mice [194, 230]. Leukemic blasts or bulk tumor cells, which are more differentiated ($CD34^{+}CD38^{+}$) as compared to LSCs ($CD34^{+}CD38^{-}$), do not retain the ability to engraft upon transplantation in susceptible recipients [255]. Results from our secondary and tertiary transplantation experiments in the BCR-ABL model provide further support for the role of supplementation of selenium in diet to prevent leukemogenesis. Instead of the NOD-SCID mice, we used lethally-irradiated recipients for LSC engraftment. Transplantation of splenocytes as well as bone marrow cells from BCR-ABL -transplanted Se-supplemented mice into susceptible Se-adequate mice failed to produce disease. These tertiary transplants did not show any signs of elevated WBCs or splenomegaly, reflected by the complete absence of primary donor-derived CD45.1 LSCs, unlike the mice receiving transplants from Se-adequate mice, which succumbed to leukemia. This experiment also exemplifies 'transmissibility' as a valid indicator of effectiveness of any chemopreventive or therapeutic regimen in a cancer stem cell model. Another major factor to be considered here is the ability to prevent relapse of disease. Although the tyrosine-kinase inhibitor, imatinib, is effective in reducing the bulk tumor and inducing quick remission in CML patients, the inability of this chemotherapeutic agent to target LSCs contributes towards relapse of the disease [200]. The results from imatinib treatment for one week indicate that selenium supplementation is highly effective in preventing the relapse of disease. However, future experiments with a prolonged

duration of imatinib treatment or the use of newer tyrosine kinase inhibitors such as dasatinib or nilotinib should provide better insight into the use of selenium as an adjunctive therapy with these inhibitors.

Having demonstrated the anti-leukemic chemopreventive effect of supplemental levels of dietary selenium, we tested whether the modulation of the AA-COX pathway mediates the effects of Se-supplementation. Indomethacin, being a non-selective inhibitor of COX (COX-1 and COX-2), suppresses the exclusive production of AA-derived prostaglandins (PGs) in the animal. The *in vivo* studies in both, FV- and BCR-ABL-, models involving treatment with indomethacin reveal that enzymatic inhibition of the AA-COX metabolism results in revoking the anti-LSC effects through one or more of the PGs. The CyPG, 15d-PGJ_2, emerges as a likely candidate given its pro-apoptotic properties [123, 151, 180, 181]. In fact, our studies with daily injections of 15d-PGJ_2 that rescues the mice treated with indomethacin, depict the importance of fatty acid metabolic pathway in cell-fate decisions. Further implications of these studies are discussed in Chapter 5. The PGE_2 analog is used as a negative control to show that the most likely AA-COX metabolite that induces apoptosis is a PGD_2 product, and not PGE_2, which is reported to contribute to the pathology of inflammation and cancer [284]. With respect to hematopoiesis and stem cells, the PGE_2 analog has been shown to increase survival, promote self-renewal, and enhance engraftment of HSCs by increased β-catenin signaling [285]. Additional evidence supporting the role of 15d-PGJ_2 is provided by the *ex vivo* experiment involving the use of H-PGDS inhibitor, HQL-79, pre-treatment of which blocks anti-LSC effects of selenite. It will be interesting to investigate the effects of HQL-79 administration *in vivo*, as well as the use of H-PGDS knock-out mice to study effects of Se-supplementation.

An interesting observation from the *ex vivo* model is the specificity of the effects of Se-supplementation on LSCs, based on the fact that selenite selectively kills BCR-ABL GFP^+ cells and spares the MSCV GFP^+ HSCs (controls). This implies that the selenium-dependent production of 15d-PGJ_2 targets the LSCs, but not the normal HSCs. This can be explained by either (i) an autocrine effect of 15d-PGJ_2, where LSCs generate 15d-PGJ_2 under Se-supplemented conditions, to undergo apoptosis, or (ii) a paracrine effect of 15d-PGJ_2, where other cells in the vicinity of LSCs, such as the monocytes-macrophages or T-cells in spleen and/or bone marrow, produce 15d-PGJ_2 in Se-supplemented conditions, and this 15d-PGJ_2 acts on LSCs specifically to induce apoptosis. Real-time gene expression analysis for H-PGDS demonstrates that sorted LSCs do not express H-PGDS (data not shown), which is essential for generating 15d-PGJ_2, and thus make the possibility of the autocrine effect less likely. On the other hand, it has been shown recently that monocytes constitute 94% of the total cells in the sub-capsular red pulp of spleen, which acts as a huge reservoir of undifferentiated extramedullary monocytes capable of accommodating demands of rapid-onset inflammation [286]. Furthermore, the argument that 15d-PGJ_2 has paracrine effects on LSCs is supported by the fact that Se-supplemented macrophages produce increased quantities of 15d-PGJ_2, which has also been detected in the serum [107]. This creates the possibility of a scenario in which there is increased production of 15d-PGJ_2 from the macrophages in spleen under a Se-supplemented state of nutrition, and this 15d-PGJ_2 exerts pro-apoptotic effects specifically on LSCs in a paracrine fashion. Studies have reported that 15d-PGJ_2 is detectable in nanomolar concentrations *in vivo* [107, 262].

Given the ability of CyPGs to target LSCs at concentration in the nanomolar range, the possible involvement of cell-surface DP receptors, DP1 and DP2, was considered. MK0524 and CAY10471, which are antagonists for DP1 and DP2 receptors, respectively, completely blocked the anti-LSCs effects of (i) selenium as well as (ii) DP agonists (PGD_2 methyl ester and ZK119192), upon pre-

treatment of unsorted splenocytes and sorted LSCs, respectively, at 10 nM concentration. In an effort to further delineate the mechanism that ensues downstream upon receptor activation, the role of protein kinases, PKA and PKC, were considered. Pharmacologic inhibition of PKA and PKC also prevented 15d-PGJ_2-dependent apoptosis of sorted LSCs, suggesting that DP receptors may play a role in mediating the paracrine effects of 15d-PGJ_2 on LSCs under Se-supplemented conditions.

Activation of the tumor suppressor gene p53 as the final step in initiating the apoptotic cascade in Se-supplemented mice, and the blocking effect of ATM-kinase inhibitor on Se-mediated eradication of LSCs imply the possibility of a strong link between the activation of DP receptors and the subsequent activation of p53-apoptotic pathway. Studies are currently underway to examine DP-mediated changes in cellular Ca^{++} and cAMP levels, which can modulate the phosphorylation status of ATM-kinase and/or p53 [287, 288]. Transcriptional repressor, BCL-6, is also being considered as one of these missing links, given its role in regulating p53 in CML [289]. Inhibition of NF-κB has been thought to be one of the strategies in the eradication of LSCs as described in Chapter 1; 15d-PGJ_2, being a potent endogenous regulator of NF-κB activation, makes a valid argument to investigate NF-κB – related mechanisms. However, recent studies from our laboratory indicate that the expression and nuclear translocation of NF-κB in BCR-ABL LSCs is very low [120], suggesting that the CyPG-mediated pro-apoptotic effects does not depend on NF-κB activation in LSCs. Finally, there is a possibility that 15d-PGJ_2 might exert pro-apoptotic effects through the redox regulation of cellular proteins, independent of its receptor-mediated effects [130]. But such a mechanism is less likely at low concentrations where CyPGs mainly act through cell surface DP receptors.

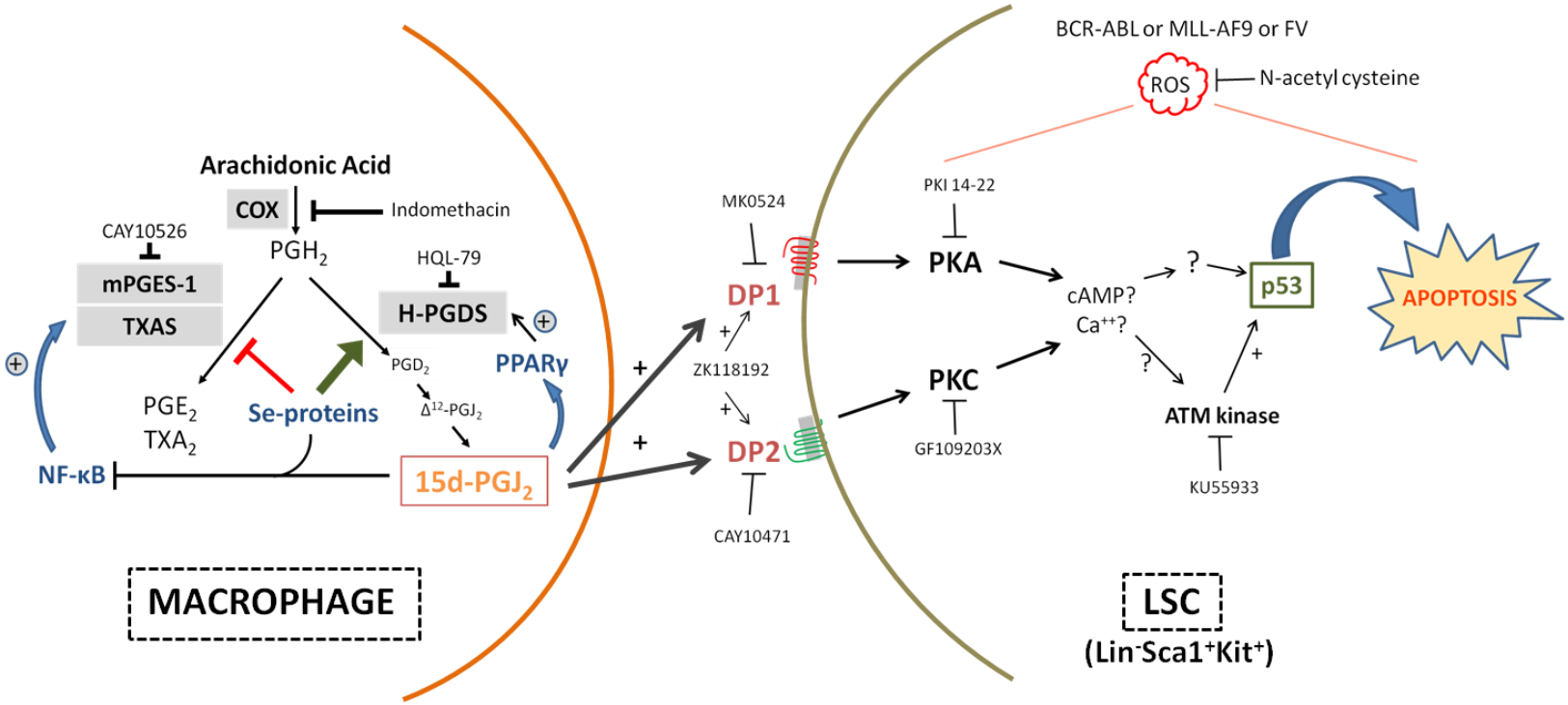

Fig. 29 Schematic showing proposed mechanisms involved in the Se-mediated apoptosis of LSCs. 15d-PGJ_2 produced in macrophages under selenium supplemented conditions acts in a paracrine manner on LSCs through DP receptors, in hematopoietic tissues like spleen and bone marrow. Elevated ROS in LSCs (unlike HSCs) makes the cell susceptible to apoptosis through DP-mediated downstream activation of p53. Activators and inhibitors that affect the 15d-PGJ_2-dependent apoptosis of LSCs are shown.

In summary, the data presented here strongly support the ability of dietary supplementation of selenium to prevent the development of leukemia in three different murine models by effectively eradicating the LSC populations. Pharmacologic inhibition at various levels of the AA-COX-HPGDS pathway suggests a critical role for CyPGs including 15d-PGJ_2 in mediating the effects of supplemental selenium levels (see Fig. 29). Further studies to elucidate the precise molecular mechanisms involved in 15d-PGJ_2-mediated apoptosis of LSCs where DP receptor ligation - followed activation of diverse signal transduction networks leading to p53 activation should provide novel insights with high potential for developing anti-leukemic therapies.

Chapter 4

Gambogic acid covalently modifies IκB-kinase-β subunit to mediate suppression of lipopolysaccharide-induced activation of NF-κB in macrophages

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Abstract

Gambogic acid (GA) is a polyprenylated xanthone abundant in the resin of *Garcinia morella* and *G. hanburyi* with a long history of use as a complementary and alternative medicine. The anti-tumor activity of GA has been well demonstrated and is thought to arise partly from the associated anti-inflammatory activity. Recent studies have indicated that the anti-tumor activity of GA is mediated by its ligation of the transferrin receptor TfR1. Since the cellular expression of TfR1 is down-regulated by lipopolysaccharide (LPS), we hypothesized that an alternative pathway exists in immune cells, such as macrophages, where GA could mitigate the expression of pro-inflammatory genes. Here we demonstrate that GA inhibits the LPS-dependent expression of nuclear factor-κB (NF-κB) target pro-inflammatory genes in macrophages. Western immunoblot, NF-κB luciferase reporter, and gel shift analyses revealed that GA strongly blocked the activation of NF-κB induced by LPS; while 9,10-dihydroGA that lacks the reactive α,β-unsaturated carbonyl group was ineffective. Moreover, GA was able to decrease nuclear p65 levels in RAW264.7 macrophages, where the expression of TfR1 was down-regulated by RNA interference. *In-vitro* kinase assays coupled with interaction studies using biotinylated GA as well as proteomic analysis demonstrated that IKKβ, a key kinase of the NF-κB signaling axis, was covalently modified by GA at Cys179 causing significant inhibition of its kinase activity. Taken together, these data demonstrate the potent anti-inflammatory activity of GA.

Introduction

Gambogic acid (GA) is a polyprenylated xanthone isolated from the resin of *G. hanburyi*, commonly used in food preparations in many Asian countries [290]. Recent literature has demonstrated its efficacy as a potent anti-tumor drug in rodent and canine models [291, 292]. Most importantly, GA treatment induced apoptosis of human gastric carcinoma cells [293] and human hepatoma cells in mice at 2-8 mg/kg body weight [294]. Kasibhatla *et al.* have reported that the mechanism of GA-induced apoptosis is mediated through the activation of caspases via the transferrin receptor (TfR1) [295]. Recently, Pandey *et al.* also demonstrated that GA inhibited NF-κB signaling pathway through its interaction with TfR1 [296]. However, the scenario may be different in non-cancerous cells, such as macrophages, where the expression of TfR1 is down-regulated by LPS-treatment [297]. We, therefore, reasoned that GA could possibly inhibit NF-κB activation through an alternate pathway. Given that Michael acceptors like 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 and 4-hydroxynonenal (4-HNE) can interact with proteins of the NF-κB signaling axis [298, 299], we hypothesized that GA could interact similarly and modulate their function. In fact, rearrangement of the olefinic bond at C_{10} (Fig. 30) to a saturated bond in GA resulted in significantly decreased anti-proliferative activities, suggesting that the α,β-unsaturated group in GA was essential for biological activity [290, 295].

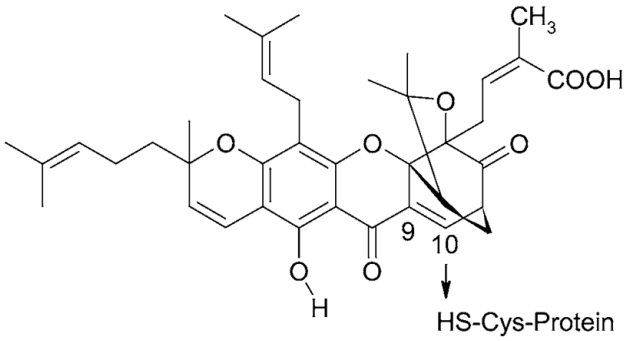

Fig. 30 Structure of GA. The putative site of addition to Cys thiol is shown. Reduction of the unsaturation (C9-C10) converts GA to DGA.

The NF-κB represents a family of transcription factors that participate in the regulation of diverse biological processes, including immune, inflammatory and apoptotic responses [300-303][304]. Given its ability to regulate expression of inflammatory enzymes, cytokines, chemokines, immunoreceptors, and cell adhesion molecules, NF-κB has often been termed a "central mediator of the immune response" [301, 305]. Macrophages are critical cellular participants in immune regulation and are activated by diverse stimuli (like LPS), to synthesize and secrete cytokines, which initiate and control inflammatory and immune functions [306]. In LPS-treated macrophages, the canonical (classical) pathway of NF-κB is mainly mediated by the IKKβ subunit; [307]. Under normal conditions, the signals mediating NF-κB activation are transient, which are instrumental for programmed cell proliferation and survival. However, activation of NF-κB exacerbates pro-inflammatory gene expression, inhibits apoptosis, and thus, contributes to inflammation.

Bioactive natural compounds such as sulforaphanes from broccoli [308], curcumin from turmeric [309], caffeic acid phenethylether from the propolis of honeybee [310], zerumbone from ginger [311] and many others cause transcriptional down-regulation of pro-inflammatory genes by inhibiting the pathway of NF-κB activation [312]. In addition, endogenous Michael acceptors with α,β -unsaturated carbonyl moiety such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 and 4-HNE [299] inhibit pro-inflammatory gene expression by targeting the NF-κB-dependent transcription via covalent interaction with the IKKβ subunit [169], Cys62 in p50 [313], Cys38 in p65 [314], in addition to its interaction with Cys in c-Jun to promote homodimerization [174]. It was hypothesized that GA could inhibit the NF-κB pathway through the α,β-unsaturated carbonyl group to impart potent anti-inflammatory activity. The findings suggest that the anti-inflammatory effect of GA occurs via the inhibition of IKKβ activity by covalent modification

leading to the consequent inhibition of NF-κB-dependent transcription of pro-inflammatory genes.

Methods and Materials

Cell Culture: The mouse RAW 264.7 macrophage cell line, human embryonic kidney (HEK293) cells, and human monocytic U937 cells, obtained from American Type Culture Collection (Manassas, VA), were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (Hyclone), 2 mM L-Glutamine (Invitrogen) and 10 μg/ml ciprofloxacin (Sigma) at 37 ^{o}C in a humidified atmosphere of 5% CO_2. The cells were sub-cultured either in 6-well or 12-well tissue culture plates.

Flow Cytometric Analysis of TfR1 Expression: The expression of TfR1 (CD71) in RAW264.7 macrophages before and after LPS treatment (1 μg/ml; 12 h) was quantitated by flow cytometry. The cells were incubated with blocking antibody (CD16/CD32; BD Biosciences) for 2 h followed by incubation with anti-CD71-FITC antibody (BD Biosciences). Both anti-CD16/CD32 and anti-CD71-FITC were kindly provided by Dr. Robert Paulson, Penn State University. Results were analyzed using FlowJo® software program (Tree Star, Inc.).

Preparation of Total Cell Lysates and Nuclear Extracts: Gambogic acid and its inactive dihydro-derivative, 9,10-dihydrogambogic acid (DGA), were purchased from Gaia Chemical Corporation, Gaylordsville, CT. To study the effect of GA on the expression of pro-inflammatory genes, RAW264.7 and U937 cells were grown to 80 % confluence and treated with various concentration of GA for 30 min. After pretreatment with GA, cells were stimulated with LPS (1 μg/ml) for 2 h. Cells were washed with PBS and lysed in mammalian protein extraction reagent (M-PER, Pierce) at 4 ^{o}C for 20 min. Lysates were centrifuged at 16,000 x *g* for 10 min and the resulting supernatants were frozen until further analyses. Protein concentrations in the supernatants were determined by BCA protein assay kit (Pierce). Nuclear extracts were isolated using NE-PER kit according to the manufacturer's instructions (Pierce).

Western Blot Analysis: Nuclear and cytosolic proteins (~10 μg) were resolved by electrophoresis on an SDS-PAGE gel (T = 10 %) and subjected to Western immunoblotting as described previously from our laboratory [298]. Primary antibodies for COX-2 (Cayman Chemicals), iNOS (Cayman Chemicals), p65 (Santa Cruz), pIκBα (Cell Signaling), TfR1 (Cell Signaling), IKKβ (Imgenex) were used. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and RNA polymerase II (Santa Cruz) were used as loading controls. In pull-down experiments, neutravidin-agarose (Pierce) or Anti-HA agarose beads (Santa Cruz) were employed. The bands were visualized by enhanced chemiluminescence (ECL) assay kit (Pierce). The membranes were also stained with Ponceau Red to verify equal loading and uniform transfer of proteins.

TNF-α Secretion Assay: To determine the effect of GA on TNF-α secretion, RAW 264.7 cells were treated with 1 μM GA for 30 min and stimulated with LPS (1 μg/ml) for different time points. The culture supernatants were isolated and the accumulation of TNF-α in cultured medium was determined using a commercially available TNF-α ELISA kit (Diaclone Research).

Quantitation of Nitrite Production: Nitric oxide production in culture media supernatant was assayed by measuring the stable degradation product of NO, nitrite, using the Griess reagent (Sigma). RAW 264.7 cells were grown in 12-well plates and incubated with different concentrations of GA (0.1, 0.5, 1.0, and 1.5 μM) for 30 min. The cells, were then stimulated with LPS (1 μg/ml) for 12 h. After 12 h of LPS stimulation, the culture media supernatants were isolated and mixed with an equal volume of Griess reagent and incubated at room temperature for 15 min. The absorbance was measured at 550 nm in a microplate reader. Sodium nitrite (10 μM-100 μM) was used to create a standard calibration curve.

Immunoprecipitation of p65: The nuclear translocation of p65 was examined by immunoprecipitation. Nuclear proteins (50 μg) were immunoprecipitated with 0.5 μg of anti-p65

polyclonal agarose conjugate (Santa Cruz Biotechnology) overnight at 4 °C. The agarose beads were extensively washed with PBS (4 x 400 µl) and subjected to SDS-PAGE (10%) followed by Western blot analysis as described earlier.

Transient Transfection Assays: The effect of GA on NF-□B-dependent reporter gene transcription induced with LPS was analyzed by luciferase assay. Briefly, RAW264.7 cells were seeded at a concentration of $2x10^5$ cells/well in six-well plates. After overnight culture, the cells in each well were transfected with 1 µg of DNA (0.75 µg of COX-2, COX-2 double mutant or 5X multimerized κB-luciferase reporter plasmid (Stratagene) and 0.25 µg pRLTK renilla luciferase control plasmid, Promega) along with 6 µl of lipofectamine-2000 (Invitrogen Life Technologies) in serum-free DMEM media. After a 6 h exposure to the transfection mixture, complete medium with 5 % FBS was added to the cells and incubated at 37 ^oC for an additional 16 h. The transfected cells were treated with various concentrations of GA (0.1-1.5 µM) for 30 min and the cells were stimulated with LPS (1 µg/ml) for 6 h. The cells were harvested and luciferase activity was measured by dual luciferase assay (Promega). Renilla luciferase activity was used to normalize transfection efficiency.

RNA Interference: RAW264.7 cells were plated in six-well plates and allowed to adhere overnight. Such cells were transfected with TfR1 siRNA (1 µg/well) or si-control (Dharmacon) using the Mirus SiQuest TransIT® reagent (Mirus Bio Corporation). After 48 hours, cells were treated with 1 µM GA for 30 min at 37 ^oC followed by stimulation with 1 µg/ml LPS for 2 h. Cytoplasmic and nuclear fractions were separated from cells using the protocol described earlier. The cytoplasmic and nuclear fractions were used in Western blot analyses using specific antibodies. GAPDH and SP1 were used as controls for cytoplasmic and nuclear fractions, respectively.

Preparation of Nuclear Extracts, and Electrophoretic Mobility Shift Assay: Cytoplasmic fraction was separated from nuclei by lysing the RAW264.7 cells or U937 cells in a buffer containing 10 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 % NP-40, 0.1 mM DTT, 1 mM PMSF, 5 µg/mL aprotinin, 5 µg/mL leupeptin and 1 µg/mL pepstatin, followed by centrifugation at 10000 x g for 10 min at 4 ^{o}C. The nuclear pellet was then lysed with a buffer containing 20 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA and protease inhibitors (see mentioned above). The lysates were sonicated (3 pulses) at 60% power on ice and centrifuged at 14000 x g for 10 min at 4 ^{o}C. Concentrations of proteins in this nuclear extract were determined using BCA reagent (Pierce). The DNA sequence for the sense strand of the NF-κB oligonucleotide was 5'-GATCCAGTTGAGGGGACTTTCCCAGGC-3' (Qiagen). Complementary strands were annealed and 4pmol/µl of the resultant double-stranded oligonucleotide was labeled with [γ-^{32}P] ATP (3000 Ci/mol at 10 mCi/ml) using T4-polynucleotide kinase (New England Biolabs), incubating it at 37 ^{o}C for 30 min. To determine the percent incorporation, labeled oligonucleotides were separated using Biogel 6 spin columns and counts were taken in the scintillation counter. For the binding reaction, 10 µg of nuclear extracts were incubated for 10 min at room temperature with 5x gel-shift binding buffer (5 mM MgCl2, 2.5 mM DTT, 2.5 mM EDTA, 250 mM NaCl, 50 mM Tris-HCl pH 7.5), 50 µg/ml poly (dI-dC) and ddH2O to bring up an equal volume. ^{32}P-labeled NF-κB oligonucleotide (40,000 CPM) was added to the reaction, and after an incubation of 15 min at room temperature, 5x loading dye was added. The samples were loaded on pre-cast 4% acrylamide gel (Biorad) for electrophoresis at 120 V for 45-50 min in TBE buffer. The gel was dried, exposed to X-ray film overnight at -80 ^{o}C and subsequently developed. To confirm specificity of NF-κB binding, unlabeled oligonucleotide (3.5 pmoles) was used as a specific competitor. For super-shift experiments, the nuclear extract was incubated

with 2 μg of anti-p50 (Santa Cruz Biotechnology, CA) followed by ^{32}P-labeled oligonucleotides as described above.

Preparation of Biotinylated GA: The carboxyl group of GA was modified by amidation with EZ-link (5-biotinamido)pentylamine (Pierce). Briefly, GA (~2 mg) and EZ-link (5-biotinamido)pentylamine (5 mg) were dissolved in DMSO (300 μl) and were allowed to react with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) in 100 mM MES, pH 5.5, at 37 ^{o}C for 3 h. The reaction was stopped by extracting the product with ethylacetate and MES buffer (1:1; v/v). The extracted biotinylated GA was purified using reverse phase HPLC with a linear gradient of acetonitrile (10-100%). The biotinylated GA was dried under nitrogen and dissolved in acetonitrile for further use.

Interaction of Biotinylated GA with IKKβ in HEK Cells: To determine the interaction of biotinylated GA with IKKβ, HEK293 cells were transfected with 5 μg HA-tagged IKKβ wild type mammalian expression construct along with 20 μl of lipofectamine for 16 h. After transfection, the cells were washed with PBS, harvested, and lysed in M-PER. Cell lysates containing 50 μg of protein were treated with 1mM DTT and/or 1.5 μM of biotinylated GA for 2 h at 37 °C and immunoprecipitated with anti-HA agarose beads (Santa Cruz, CA) overnight at 4 °C with constant shaking. The beads were washed three times with TBST buffer by centrifugation at 16,000 x *g* for 10 min. The proteins were eluted by boiling the beads in SDS sample buffer for 5 min and analyzed by SDS-PAGE. The biotinylated proteins were electro-transferred and immuno-detected using the North2South chemiluminescent detection kit (Pierce). The cell lysates, where IKKβ-HA was over-expressed, were incubated with biotinylated-GA mixed with increasing amount of glutathione (GSH; 0.1- 3 mM) to test the effect of GSH on the interaction with IKKβ. To test if Cys179 was being modified by GA, we used the HA-tagged human IKKβ wild-

type and IKKβ ^{179}Cys→Ala mutant to compare the reactivity of GA-biotin. The expression constructs were kindly provided by Dr. Michael Karin at University of California at San Diego, CA. The human HA-tagged IKKβ wild type or Cys179→Ala mutant expression constructs were transfected into RAW264.7 macrophages and cell lysates were treated with biotinylated GA as described above.

Mass Spectrometric Analysis of Post-Translational Modification of IKKβ: To confirm the modification of IKKβ by GA, murine IKKβ peptide-(173-186), LDQGSLCTSFVGTL (synthesized at the Peptide Synthesis Facility, Penn State College of Medicine, Hershey, PA) was incubated with Me_2SO_4 and purified GA (mol/mol) for 30 min at 37 ^{o}C in PBS. The samples were analyzed by M@LDI-TOF-MS for modification at the Mass Spectrometry Facility, Penn State University, University Park, PA.

Inhibition of IKKβ Activity by GA in vitro: To determine whether GA inhibits IKK activation, RAW264.7 cells were transfected with HA-tagged IKKβ wild-type expression vector for 24 h as described earlier. The cells were lysed and the clarified lysates were then incubated with anti-HA prebound to agarose at 4 ^{o}C overnight. The beads were washed with TBST containing phosphatase inhibitors and treated with GA (1.0 μM) for 2 h at 37 ^{o}C. The kinase reaction was performed on the immunoprecipitates in kinase buffer with GST-IκBα as the substrate for 2 h at 30 ^{o}C. The reaction was stopped by adding 10 μl of 1X SDS sample buffer and the reaction mixtures were subjected to SDS-PAGE followed by Western blot analysis. The membrane was probed with monoclonal anti-pIκBα antibodies and reprobed with anti-IKKβ after treating the membrane with the stripping reagent (Pierce).

Cell Viability Assay: Cell viability was determined using CCK-8 kit to count number of living cells (Dojindo Molecular Technologies, Inc., Gaithersburg, MD). CCK-8 utilizes WST-8 (2-(2-methoxy-

4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), which produces a water-soluble formazan dye upon bio-reduction by cellular dehydrogenases to an orange formazan product that is soluble in the tissue culture medium. The amount of formazan produced is directly proportional to the number of living cells. RAW264.7 cells were plated at a concentration of 5,000 cells in 100 μl of complete medium per well in 96-well plates for 24 h prior to treatment. The cells were treated with the indicated concentrations of GA for different time points. Thereafter, 10 μl of CCK-8 reagent was added to each well and incubated for an additional 3 h. The absorbance was read in a Packard microplate reader at 450 nm and the cell viability was calculated. In addition, RAW264.7 cells were treated with four-log orders of GA (0.01-10 μM) for 24 h and the cell viability was assessed using trypan blue exclusion method. Ratio of live to total cells was calculated using a hemocytometer.

Statistical Analysis: When necessary, data are expressed as mean ± S.D., and the Student's *t* test was used in statistical analysis for comparison. $P<0.05$ was used as the criterion for statistical significance.

Results

GA downregulates LPS-induced expression of COX-2, TNF-α and iNOS

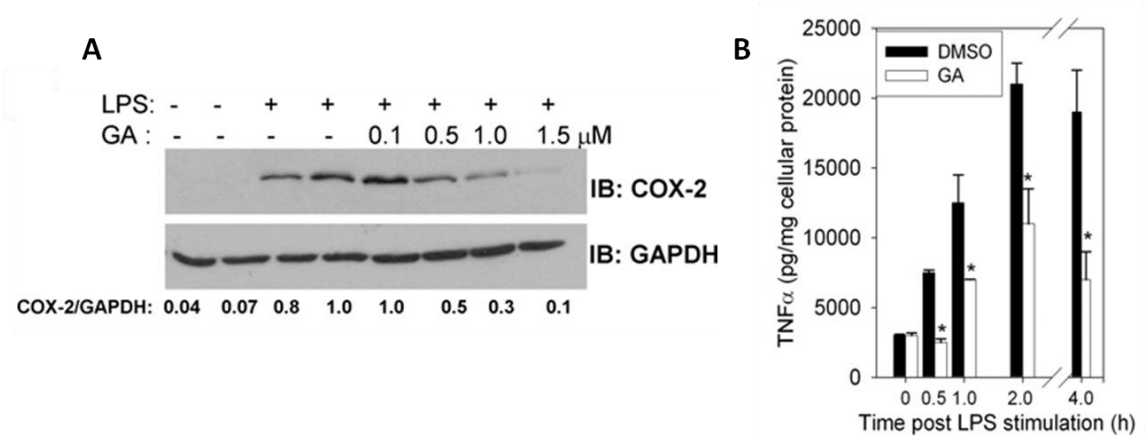

Fig. 31 Effect of GA on expression of COX-2 and TNF-α in LPS activated RAW 264.7 cells. Panel A: The cells were incubated with various concentrations of GA (0.1-1.5 μM) for 30 min followed by stimulation with LPS (1 μg/ml) for 2-4 h at 37 °C. The culture media supernatants and corresponding cell lysates were used for the analyses of COX-2 expression. Panel B shows time-dependent inhibition of the production of TNF-α by GA (1 μM) in LPS-treated RAW264.7 macrophages. The results are expressed as the mean ± S.D. of three independent experiments. Representative Western blots of n= 3 are shown.

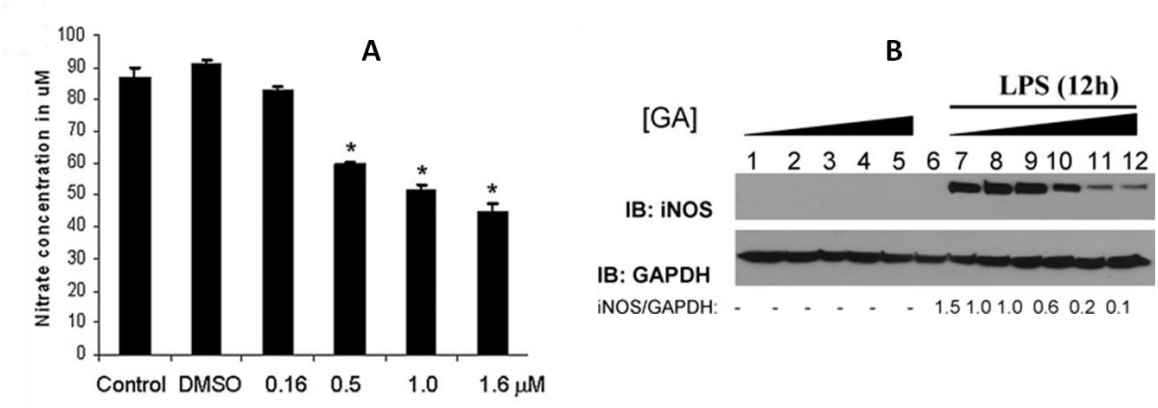

Fig. 32 Effect of GA on nitrite production and iNOS expression in LPS-induced RAW 264.7 macrophages. Panel A: Cells were pretreated with GA (0.1- 1.5 μM) for 30 min before incubation with 1 μg/ml of LPS for 12 h. Nitrite levels in the cell culture medium are expressed as the mean ± S.D. of three independent experiments performed in triplicate.

*p<0.05. Panel B: whole cell extracts were analyzed for iNOS expression by Western blot analysis. Representative of n= 3 shown.

To investigate the anti-inflammatory effects of GA, the expression of three prototypical inflammatory markers, COX-2, TNF-α, and iNOS, was analyzed in RAW macrophages. As shown in Fig. 31A, GA pretreatment markedly reduced the expression of COX-2 in LPS-treated macrophages in a concentration-dependent manner. The EC_{50} was found to be ~ 0.5 μM. At 1 μM, there was complete inhibition of COX-2 expression. Along the same lines, the production of TNF-α in the extracellular media was analyzed in RAW264.7 macrophages. As shown in Fig. 31B, the production of TNF-α was inhibited in a time- dependent manner in GA pretreated RAW macrophages. The inhibitory effect was apparent as early as 30 min post LPS stimulation; while at later time points (2-4 h), the inhibition in TNFα was even more conspicuous. We also analyzed the expression of iNOS in RAW 264.7 macrophages before and after treatment with GA. As shown in Fig. 32A, pretreatment of RAW 264.7 cells with increasing concentrations of GA promoted a dose-dependent inhibition in the amount of nitrite produced by these cells in response to LPS. The EC_{50} was calculated to be ~ 0.5 μM of GA. Western blot analysis of the corresponding cell extracts showed that GA pretreatment inhibited the LPS induced stimulation of iNOS expression (Fig. 32B). The EC_{50} in this case was calculated to be ~0.5 μM of GA, which corroborates well with those obtained with activity. The fact that the expression of all three pro-inflammatory genes was inhibited by GA suggested that the effect of GA was likely mediated at the level of transcription.

Modulation of p65 expression by GA is independent of Transferrin receptor (TfR1)

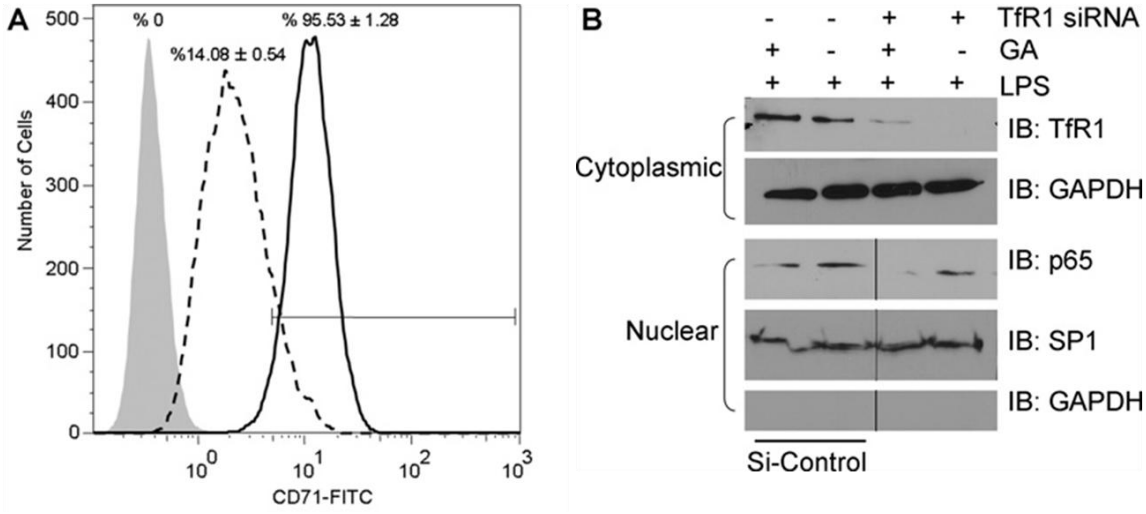

Fig. 33 TfR1-independent modulation of p65 in GA pretreated macrophages. Panel shows flow cytometry histograms of RAW264.7 cells treated with or without LPS (1 μg/ml) for 12 h. Cells were blocked with FcR blocking antibody (CD16/CD32), stained for CD71 (TfR1)-FITC and analyzed by flow cytometry. The histograms represent unstained (gray shaded), treated with LPS (dotted line), and untreated with LPS (solid line), respectively. Cell numbers described above are averages of triplicate experiments ± S.D. Panel B: RAW264.7 cells were plated in 6-well plates and were transfected with TfR1 siRNA or si-control at 1 μg/well. After 48 h, cells were treated with 1 μM GA or vehicle (DMSO) for 30 min at 37 ^{o}C followed by treatment with 1 μg/ml LPS for 2 h. Cytoplasmic and nuclear extracts were prepared from these cells. Representative Western blot of n = 2 shown.

Kasibhatla *et al.* [295] and Pandey *et al.* [296] have demonstrated that GA binds to TfR1 to negatively affect transcription of pro-inflammatory genes in cancer cells. However, flow-cytometric studies indicated a >85 % down regulation of TfR1 (also called CD71) in LPS-treated RAW264.7 cells (Fig. 33A). To address the fact that GA targeted the NF-κB pathway independently of TfR1, we examined the LPS-dependent nuclear translocation of p65 in TfR1 knockdown cells. Results shown in Fig. 33B clearly indicate that even in the absence of TfR1, GA caused a significant decrease in the nuclear levels of p65 in cells treated with LPS. Thus, given

the possibility of the existence of alternative cellular targets other than TfR1, we examined the effect of GA on the activation of the NF-κB pathway.

GA blocks LPS-induced Nuclear Translocation of p65 Subunit of NF-κB

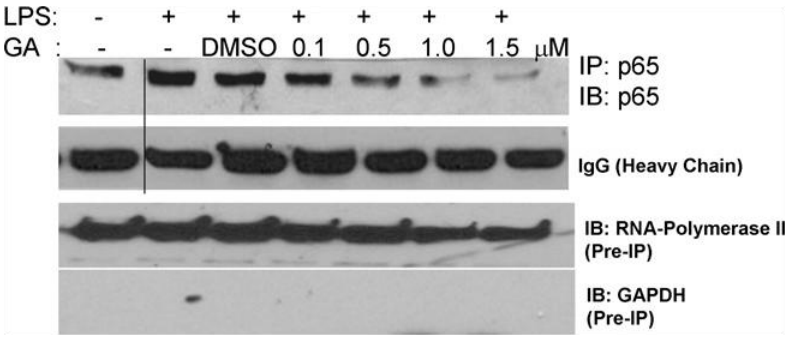

Fig. 34 GA inhibits LPS-induced nuclear translocation of p65. RAW264.7 cells were pretreated with 0.1-1.5 μM GA for 30 min and then stimulated with LPS (1 μg/ml) for 2 h. Nuclear proteins were analyzed for the presence of p65 by immunoprecipitation followed by Western blot analysis. IgG-heavy chain was used to confirm near equal loading of the immunoprecipitate. Cell lysates (pre-IP) were analyzed for Western blotting for GAPDH and RNAP-II as markers of cytosolic and nuclear fractions, respectively. Representative Western blot of n = 4.

We determined the effect of GA on the LPS-induced nuclear translocation of the p65 subunit of NF-κB by immunoprecipitation of nuclear protein extracts. LPS treatment elicited a rapid nuclear accumulation of p65 in control and DMSO treated cells. Pre-incubation with GA abolished the LPS-stimulated p65 translocation to the nucleus in RAW264.7 macrophages (Fig. 34). RAW264.7 cells showed enhanced sensitivity towards GA in that the inhibition of p65 translocation was robust at concentrations as low as 0.5 μM; while at higher concentrations (1.0 and 1.5 μM), there was complete inhibition of nuclear translocation of p65. Taken together, these results indicate that GA blocks the nuclear translocation of p65 subunit of NF-κB.

GA decreases DNA-binding of NF-κB

To determine if GA inhibited LPS-induced NF-κB transcriptional activity, transient transfections with a multimerized NF-κB driven luciferase vector, murine COX-2 wild-type (COX-2 WT), and COX-2ΔκB promoter luciferase vectors were carried out (Fig. 35A, B). We tested the modulation of murine COX-2 promoter reporter construct by GA, which has two NF-κB sites that are pivotal to LPS induced transcriptional activation [106, 315]. Pre-incubation of such COX-2 WT transfected cells with various concentrations of GA clearly indicated that that the LPS-induced NF-κB activation was significantly decreased at ≥0.5 μM of GA and at concentration ≥ 1 μM, there was hardly any activity observed (Fig. 35A). The NF-κB double mutant COX-2 reporter was inactive and exhibited no effect upon LPS and/or GA treatment (Fig. 35A). Furthermore, we studied the effect of GA on the LPS-dependent activation of the multimerized NF-κB luciferase reporter, $(κB)_5$-Luc (Fig. 35B). The results of the inhibition of LPS-dependent luciferase activity by GA corroborate well with those obtained earlier with the COX-2 reporter assays, with the EC_{50} in the range of 0.3-0.5 μM (Fig. 35B). DGA was unable to repress the NF-κB activity at 1 μM.

In support of the reporter studies, we analyzed nuclear extracts from LPS stimulated RAW264.7 macrophages (Fig. 35C) and human U937 monocytic cells (Fig. 35D) before or after GA and DGA treatment for binding to NF-κB oligonucleotide in gel shift assays. LPS at a concentration of 1 μg/ml strongly activated NF-κB activity in both the macrophage cell types. Pretreatment with GA markedly inhibited LPS-dependent NF-κB activation in both cell types. As seen earlier, pretreatment with DGA was ineffective in inhibiting the activation of NF-κB. Taken together, these studies unequivocally indicate that GA inhibits the activation of NF-κB and that the α,β-unsaturated moiety is essential for the inhibitory activity.

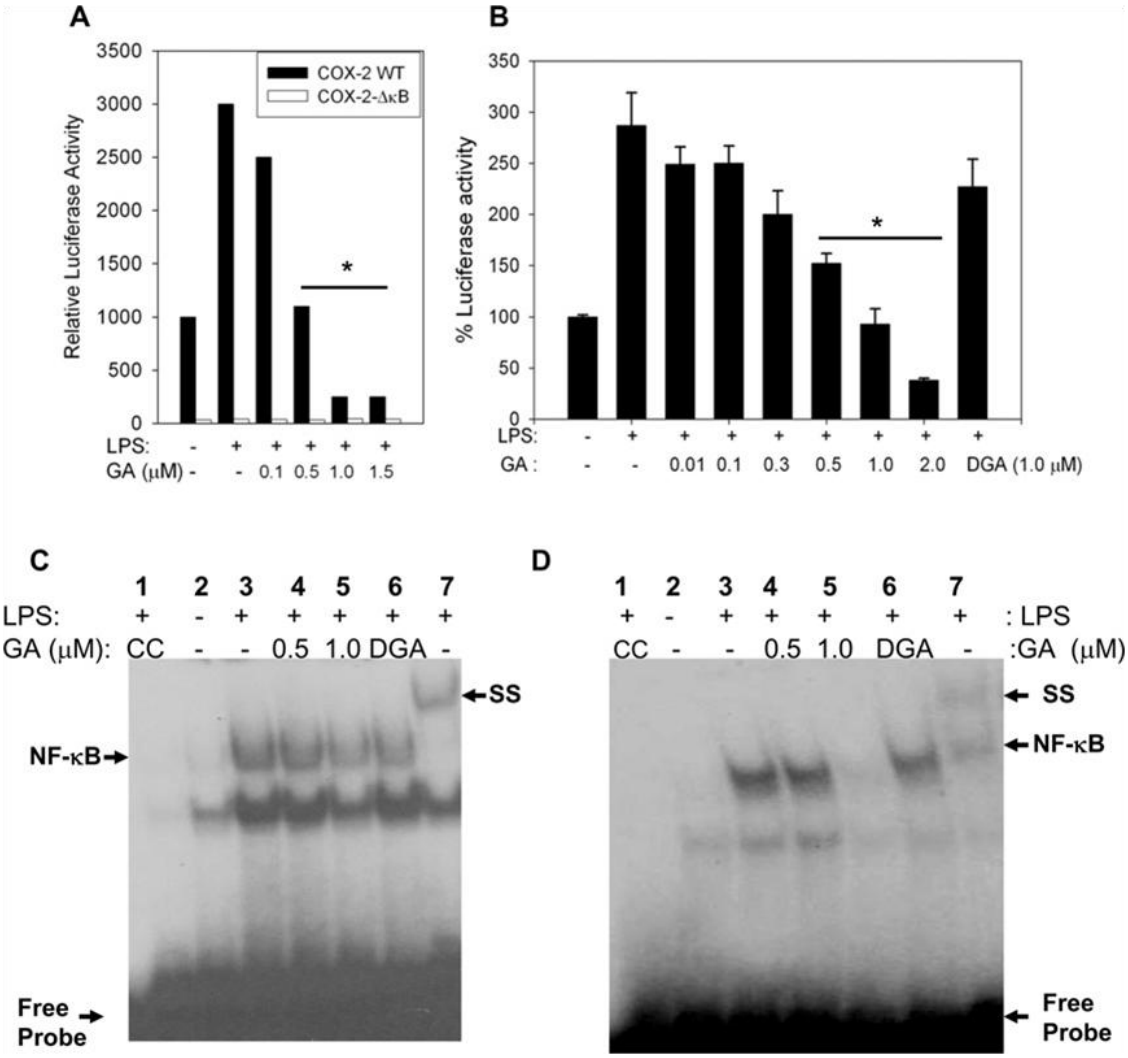

Fig. 35 GA represses NF-κB dependent reporter gene expression induced by LPS. *A*, Inhibition of NF-κB dependent COX-2 reporter activity using murine COX-2 wild-type (COX-2WT) and ΔκB-mutant (COX-2-ΔκB) promoter-luciferase reporter constructs by GA (0.1-1.5 μM). *B*, inhibition of NF-κB-luciferase activity by GA. Cells transfected with multimerized NF-κB-luciferase and pRL-TK vectors were treated with GA (0.1-1.5 μM) followed by stimulation with LPS for 6 h. Data shown are averages ± S.D. of four independent experiments. *C and D*, EMSA for the binding of NF-κB in the nuclear extracts isolated from RAW264.7 and U937 cells, respectively. Cells were treated with indicated concentrations of GA followed by stimulation with LPS for 2 h. CC corresponds to cold competitive oligonucleotide control. SS represents super-shift with anti-p50 antibody incubated with the LPS-treated nuclear extracts followed by ^{32}P-labeled NF-κB double-strand oligonucleotides. DGA was used at 1 μM in all the experiments as a control.

GA inhibits IKKβ enzymatic activity

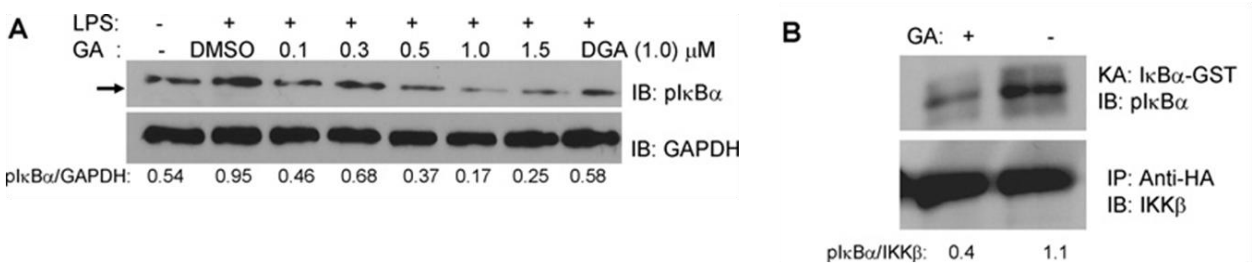

Fig.36 Effect of GA on LPS-induced IKK activity. A, RAW264.7 cell lysates from RAW264.7 cells treated with DMSO or GA (0.1-1.5 μM) followed by stimulation with LPS for 2 h. B, IKKβ-HA tagged protein was expressed in RAW264.7 cells and the immunoprecipitate (IKKβ: anti-HA complex) was incubated with DMSO or GA (1 μM) for 30 min followed by in-vitro kinase activity assay (KA) using the GST-IκBα (1-55) protein and ATP. In all experiments, the blots were reprobed for GAPDH or IKKβ to normalize for equal protein loading. Representative Western blots of n= 3 shown.

Based on our previous experiments with 15d-PGJ_2 [298], bioactive compounds with an α,β -unsaturated moiety have the potential to interact covalently with a key thiol in IKKβ. Therefore, we first examined if IKK activity was affected by GA. To determine the effect of GA on LPS induced IKK activation, we analyzed the cellular levels of pIκBα before and after LPS treatment. GA-pretreatment significantly decreased pIκBα in a dose dependent manner in RAW264.7 cells (Fig. 36A) at ≤0.5 μM of GA. To further investigate if GA targeted IKKβ, IKKβ-HA was over-expressed in RAW264.7 cells and the immunoprecipitates were examined for the effect of GA (1 μM) on kinase activity. As shown in Fig 36B, GA significantly inhibited the IKK activity suggesting the possibility of covalent interaction with IKKβ to inhibit the enzymatic activity.

GA binds covalently with IKKβ

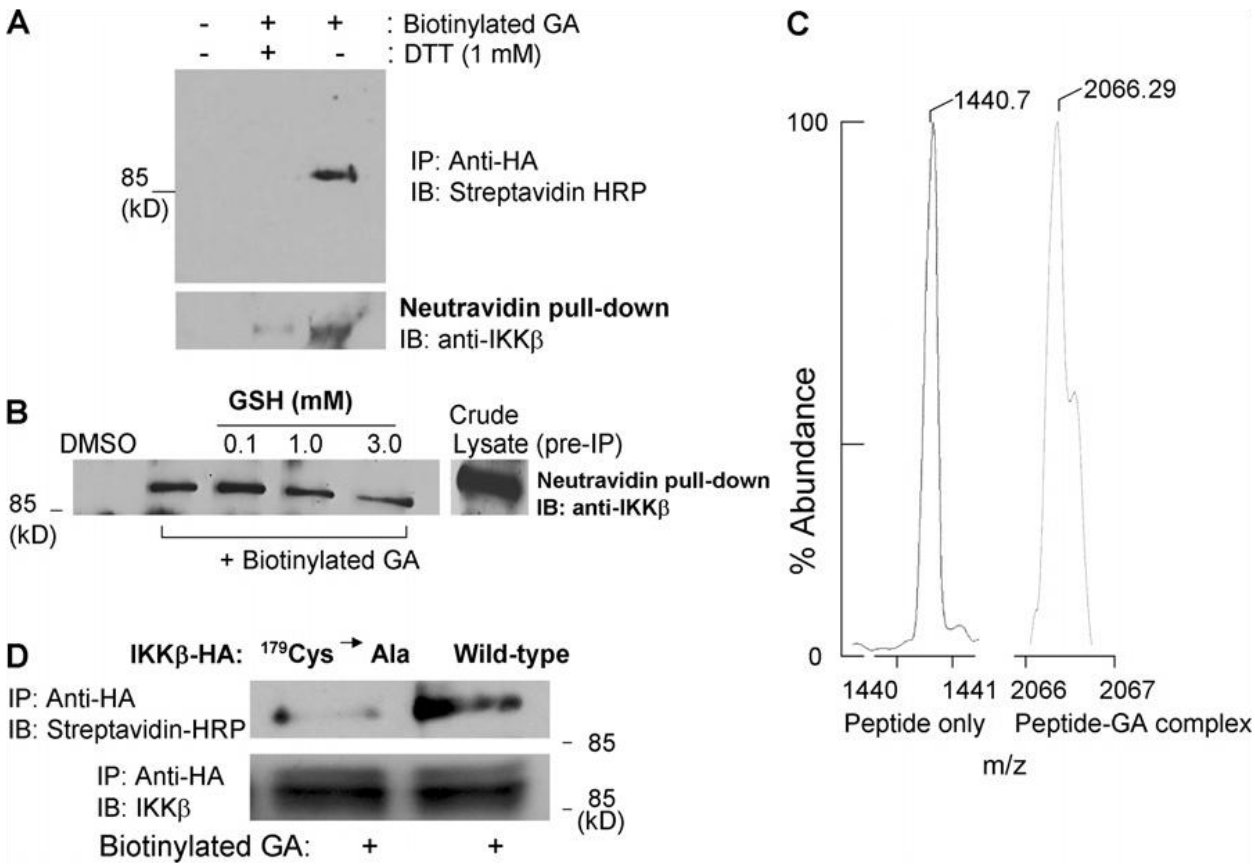

Fig. 37 Interaction of GA and IKKβ. Panel A: Total cell lysates (50 µg) from HEK293 cells transfected with IKKβ-HA plasmid were incubated with biotinylated- GA (1.5 µM) in the presence (lane 2) or absence (lane 3) of DTT. The lysates were subjected to immunoprecipitation with anti-HA followed by Western immunoblot with streptavidin-HRP (upper panel) or, a neutravidin-pull down followed by anti-IKKβ Western immunoblot (lower panel). Panel B displays the effect of GSH on the interaction of GA with IKKβ. GSH (0.1, 1, and 3 mM) was incubated with biotinylated GA (1 µM) for 2 h at 37 ^{o}C. The complex was added to IKKβ over-expressing HEK293 cell lysates (100 µg) for 1 h on ice. Neutravidin affinity chromatography was used to analyze biotinylated GA-modified IKKβ as described above. Panel C shows mass spectrometric analysis of the interaction of IKKβ peptide with DMSO and GA. Panel D shows interaction of GA with IKKβ-HA wild-type and 179 Cys→Ala mutant proteins expressed in RAW264.7 cells. Total cell lysates (50 µg) from these cells were incubated with biotinylated-GA (1 µM) for 30 min followed by immunoprecipitation by anti-HA and Western immunoblot using streptavidin-HRP. The blot was reprobed with anti-IKKβ to confirm equal protein expression and loading. Western blots shown are representative of n= 3.

To determine whether GA covalently reacted with IKKβ, a biotinylated derivative of GA was added to the lysates from HEK293 cells that were transfected with a pCMV-IKKβ-HA expression construct. The GA-IKKβ adduct was pulled down with anti-HA agarose beads and the biotinylation of IKKβ was examined. It appeared that GA reacted with IKKβ and treatment with 1 mM DTT disrupted the binding of GA with IKKβ (Fig. 37A, upper panel). Alternatively, the biotinylated proteins from the cell lysates were pulled-down with neutravidin-agarose beads. The affinity pull-downs indicated the presence of IKKβ as one of the proteins that interacted with GA (Fig. 37A, lower panel). In addition, we examined if the interaction between IKKβ and GA was modulated by physiological cellular thiols such as GSH. Although, increasing GSH concentration from 0.1 mM to 1 mM and 3 mM decreased the ability to IKKβ to bind GA, we were still able to detect interaction even at 3 mM (Fig. 37B). Both these experiments clearly indicated that GA covalently interacted with IKKβ and that the interaction withstood reducing and denaturing conditions during electrophoresis except when DTT or GSH was included during reaction. To further confirm the reaction, GA was incubated with the IKKβ (173-186) peptide and subjected to qualitative M@LDI-TOF-MS analysis. The results clearly demonstrated an increase in the mass of the peptide from 1440.7 to 2066.2, a difference of 625.5 *m/z* units, indicating that the peptide was covalently modified by GA (Fig. 37C). To address the importance of ^{179}Cys in the interaction with GA, an IKKβ (Cys/Ala) mutant expression construct was transfected into RAW264.7 cells. The cell lysates were incubated with GA as described above. Immunoprecipitation with anti-HA followed by Western immunoblot with streptavidin-HRP clearly showed lack of binding of GA to IKKβ (^{179}Cys/Ala) mutant compared to the wild-type IKKβ (Fig. 37D). Taken together, these studies indicate that GA interacts with the ^{179}Cys residue of IKKβ.

GA is not cytotoxic to RAW264.7 cells

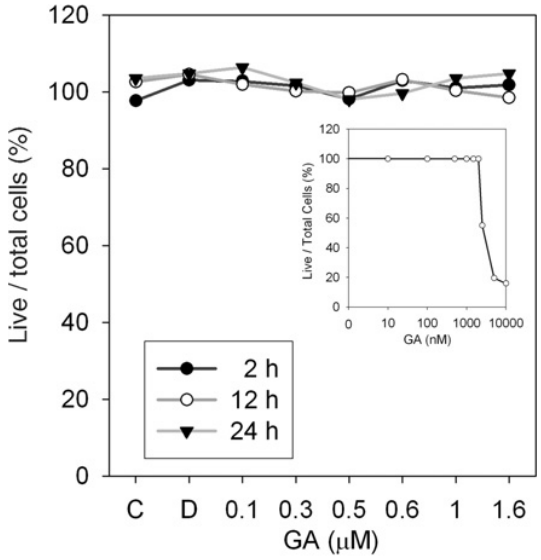

Fig. 38 Cytotoxic effects of GA. RAW264.7 cells were treated with GA (0.1 μM-1.6 μM) for indicated time points. Thereafter, 10 μl of CCK-8 reagent was added to each well and incubated for an additional 3 h. Cell viability was read in a plate reader at 450 nm. *Inset:* RAW264.7 cells were treated with GA (0.01-10 μM) for 24 h followed by trypan blue staining.

To further test if GA exhibited any effect on viability of RAW264.7 cells used in this investigation, cytotoxic assays were performed. Results shown in Fig. 38 indicate that in RAW 264.7 cells, no cytotoxic effect was observed even at 24 h of incubation. Trypan blue exclusion method also showed that GA did not cause apoptosis RAW264.7 macrophages toxic effects of GA to about 2 μM, after which there was increase in cell death (Fig. 38, inset).

Discussion

Many signal transduction pathways converge at the multi-subunit IKK complex to mediate activation of NF-κB-dependent gene expression, which is critical to metastatogenesis, tumor promotion and inflammation [316]. Thus, the control of NF-κB activation has significant therapeutic implications. Here, we demonstrate that GA abrogates LPS-activated NF-κB pathway through the inhibition of IKKβ. However, GA has been shown to bind TfR1 that is significantly overexpressed in different types of cancers [295]. Although GA interacted with TfR1 non-covalently; GAO was found to be inactive [295], which suggests that the importance of the α,β-unsaturated group in GA. The use of the dihydro-analog of GA, DGA, in our studies further confirms the requirement of the enone moiety in GA for its NF-κB inhibitory activity. Furthermore, Pandey *et al.* [296] demonstrated that the binding of GA to TfR1 triggered apoptosis in human myeloid leukemia cells via the potentiation of TNFα-induced apoptosis through modulation of the NF-κB pathway. Flow cytometric analysis of TfR1 expression in macrophages indicated a >85 % down-regulation of expression upon LPS-treatment, which agree with the results of Wardrop and Richardson [297]. Studies with siRNA-mediated knockdown of TfR1 confirmed the existence of intracellular targets such as IKKβ to be functional. Therefore, given the down-regulated expression of TfR1 in "inflammatory" macrophages, it is clear that the anti-inflammatory activity of GA arises in part from its interaction with intracellular proteins, particularly components of the NF-κB pathway such as IKKβ.

Based on the results from M@LDI-TOF-MS and site-directed mutagenesis experiments, we strongly believe that ^{179}Cys in IKKβ is an intracellular target. The interaction of GA with IKKβ was inhibited to a significant extent by increasing levels of GSH, a cellular thiol antioxidant. It could be predicted that imbalances in the [GSH]:[GSSG] ratio towards the oxidizing state, as in many

inflammatory disease pathologies that activates NF-κB [317], may in fact allow GA to impart its anti-inflammatory activity. Thus, glutathionylation represents a key cellular metabolic event in the regulation of the anti-inflammatory activity of GA.

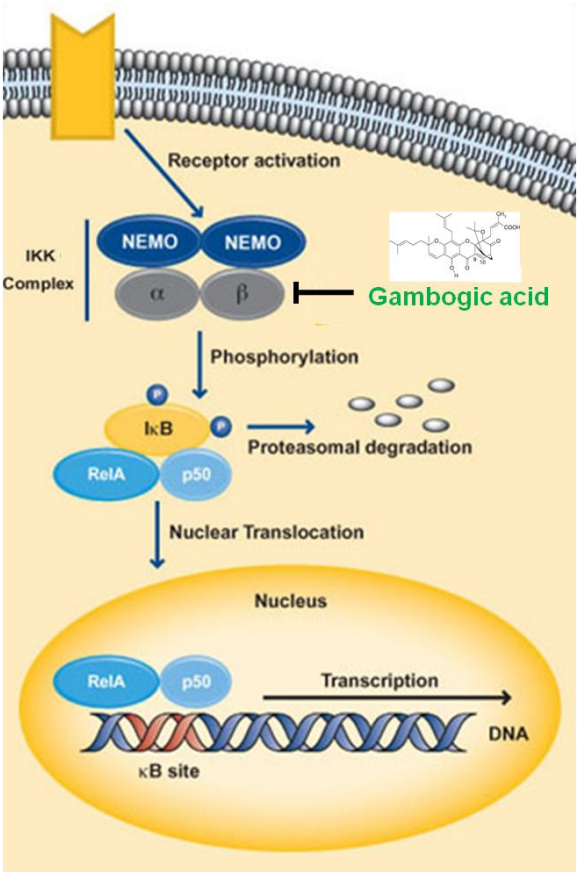

Fig. 39 Schematic showing the effect of GA on NF-κB pathway. (Adapted and modified from www.abcam.com)

It is known that electrophilic α,β-unsaturated carbonyl group in bioactive compounds selectively react with protein thiols. Electrophiles such as 15-deoxy-$\Delta^{12,14}$-PGJ_2 [169] [298] and 4-HNE [299] interact with IKKβ leading to complete abrogation of its kinase activity. To test if the interactome of GA and 15-deoxy-$\Delta^{12,14}$-PGJ_2 with cellular proteins overlapped, we analyzed the ability of GA to modify thioredoxin and p50 that are already established as key protein targets of 15-deoxy-$\Delta^{12,14}$-PGJ_2 [132, 313]. Studies showed that GA interacted with both these proteins

(data not shown). Molecular modeling studies have provided some insight into the interaction of 15-deoxy-$\Delta^{12,14}$-PGJ_2 with Cys thiols in the above proteins to be controlled by molecular recognition by fit of shape and complementarity in addition to accessibility and environment of the Cys thiol [318]. Thus, it is likely that a subset of cellular proteins may meet these requirements to interact with hydrophobic electrophiles such as 15-deoxy-$\Delta^{12,14}$-PGJ_2 and GA. Needless to say, the specificity of covalent interaction of GA with Cys thiols in target proteins will need to be further characterized using proteomics, which might provide clues regarding the environment of the Cys thiol and the structural features that are necessary for interaction. Based on these studies, we believe that the spectrum of activities exhibited by GA could be partly attributed to interaction with proteins covalently, as shown here, in addition to the ability to interact non-covalently with TfR1 to effect downstream gene expression.

In summary, the results indicate the existence of an alternate pathway of inhibition of NF-κB activation by GA via specifically modifying ^{179}Cys of IKKβ leading to the down-regulation of expression of COX-2, iNOS, and TNF-α, which are implicated in inflammation.

Chapter 5

Summary, Implications, and Future Directions

Selenium is an essential micronutrient for living organisms just like iron or calcium. However, the ability of selenium to exert a major part of its biological actions by incorporation into a set of proteins known as selenoproteins in the form of the 21st amino acid, selenocysteine (Sec), makes it particularly unique. This peculiarity also conveys the significance of nature maintaining a constant pressure on the genome throughout evolution to not only conserve but also expand the machinery necessary for Sec biosynthesis and its insertion into selenoproteins. Today, the health benefits of selenium are known in relation to oxidative stress, inflammatory diseases, infections, and cancers. One of the seminal molecular findings explaining the role of selenium in a broad range of diseases is the ability of selenium to inhibit the activation of (NF-κB), a family of transcription factors, known as the "central mediator of the immune system" because of its critical role in mediating inflammation and carcinogenesis in numerous pathological models.

Prostaglandins play crucial roles in physiological processes in form of 'short-acting, local hormones' as well as in the pathological events of inflammation and cancer. AA is a 20-carbon fatty acid that is part of the phospholipid cell membrane bilayer, and it is acted upon by COX - 1 and 2 as the first step in the synthesis of different PGs. 15d-PGJ_2 is a widely studied prostaglandin on account of its anti-inflammatory and anti-carcinogenic properties. While there are reports on apoptosis of cancer cell lines by CyPGs at high micromolar concentrations [182-185, 187], there are no studies that describe similar effects on CSCs, especially at concentrations that are orders of magnitude lower that are more relevant physiologically. The CSC model proposed over a decade ago brought a paradigm shift in the way pharmacological strategies have been studied for the treatment of various cancers [188]. The CSC hypothesis was the outcome of research that went into understanding the origin of myeloid leukemias, where it was found out that the clonogenic and leukemogenic activity resides in a minor, more primitive fraction of leukemia cell population, later termed as LSCs [194, 255]. These LSCs are responsible

for relapse of leukemia following conventional chemotherapy, and hence, the eradication of LSCs is required for curing the disease permanently. Alternatively, there has been an ever-increasing interest in studying the biological properties of natural botanicals and their active ingredients. GA is one such natural phytochemical derived from the plant *Garcinia hanburyi* that has been extensively studied for its anti-cancer properties [291-294]. Not much is known about the anti-inflammatory potential of GA, though.

Macrophages undergo respiratory oxidative burst characterized by transient increase in ROS during states of activation such as phagocytosis. During such 'classical' (M1) activation, macrophages also produce inflammatory mediators like IL-1β, IL-6, TNFα, PGE_2, PGD_2, and TXA_2. Tight regulation of macrophage activation is crucial to prevent an acute physiological response turning into a chronic destructive pathological state. Hence, it is important to understand the mechanisms that govern macrophage activation, and the onset and resolution of inflammation. It is known that PGE_2 is the pro-inflammatory mediator responsible for the early stages of an acute inflammatory response to a variety of 'injuries', and PGD_2 metabolites are the anti-inflammatory eicosanoids that are released at later stages and function to clear the inflammation, or in other words, to bring a about a timely resolution [74, 240].

Selenoproteins form a major component of the antioxidant defense system that maintains the redox tone required for optimal functioning of cellular processes. Studies described in Chapter 2 demonstrate that the presence of selenoproteins in macrophages is essential to increase the levels of PGD_2 and its downstream product, 15d-PGJ_2, while decreasing those of PGE_2 and TXA_2. This modulation of AA-COX metabolites is achieved at the level of expression of various PG synthases including H-PGDS, mPGES-1, and TXAS. The importance of selenoproteins is underlined by the observation that only certain forms of selenium (selenite, methylseleninic

acid), which make selenium easily bioavailable for synthesis of Sec, can actually regulate the expression of different PG synthases. This implies that the biochemical form and metabolic fate of selenium should be given utmost attention when selenium is considered for use in research studies and clinical trials. For example, L-selenomethionine (SeMet), an organic form of selenium that exists predominantly in cereal grains and meat, is incorporated in a non-specific manner instead of methionine (Met) into proteins outside the selenoproteome. Although this allows SeMet to serve as a reserve selenium store and be available for the expression of selenoproteins eventually as a function of protein turn over. Another aspect to note is the possibility of subtle changes in the structure of proteins upon incorporation of SeMet, instead of Met, that could lead to perturbation of the function of proteins. Taken together, such non-specific incorporation of selenium might explain, in part, the failure of the highly anticipated SELECT trial, carried out to test the effect of two antioxidants, selenium (in form of SeMet) and vitamin E (α-tocopherol), on the risk of prostate cancer [319]. In comparison, γ-glutamyl-Se-methylselenocysteine (GMSeCys) and Se-methyl-selenocysteine (MSeCys), which are characteristic of selenium accumulator plant species such as brassica family (broccoli, radish), onions, and garlic, can give rise to methylselenol (CH_3SeOH) by the action of β-lyases, and thus rapidly contribute to selenoprotein synthesis [320]. However, no human data is available on the bioavailability or retention of plant compounds like GMSeCys and MSeCys. Adequate knowledge on the speciation of selenium is important to draw conclusions on selenium requirement for human health and disease [320], thus forming a potential focus for future studies.

Another aspect from Chapter 2 that needs to be explored is determining which selenoprotein(s) is responsible for regulating the gene expression of PG synthases. Using knockout mice it has been shown that the deficiency of GPx-1, and accompanying oxidative stress enhances inflammation and the development of atherosclerosis by promoting TNF-α stimulated-ROS-

dependent signaling cascades [321]. Similar reports, along with the fact that enzymes, 5-lipoxygenase (LOX) and 15-LOX, of the AA-LOX pathway are known to be regulated by GPx-1 [322, 323], make GPx-1, a highly abundant selenoprotein, as one of the prime candidates to be studied for a specific role in regulation of AA-COX pathway and PG synthesis. However, it needs to be clarified that the reason for showing the protein expression of GPx-1 in some of our experiments is solely to provide an idea of the selenium status of the macrophages, given the sensitivity of GPx-1 expression to changes in selenium status, and not to establish any causal association with the expression of various PG synthases. The recently discovered selenoprotein H (SelH) is a redox-sensing DNA-binding protein that can activate transcription of genes involved in antioxidant defense and phase II detoxification [16], making it a worthy candidate for studying PG regulation.

Studies to elucidate the regulatory mechanisms of the AA-COX pathway by selenium suggested the activation of murine *Hpgds* gene to be mediated by PPARγ binding to PPREs in the *Hpgds* promoter. The fact that 15d-PGJ_2, a downstream metabolite generated by the action of H-PGDS, is also a potent endogenous ligand for PPARγ activation, serves as a positive feedback loop (H-PGDS → 15d-PGJ_2 → PPARγ → H-PGDS) that is activated in the presence of selenoproteins, to the wide repertoire of anti-inflammatory molecular properties of the lipid-sensing nuclear receptor, PPARγ. It is interesting to note that PPARγ is required for the maturation of 'alternatively' (M2) activated macrophages, which have a pro-resolution phenotype and a beneficial role in nutrient homeostasis [324]. The increased activity of arginase-1, a PPARγ-driven prototypic M2 marker, by selenium [114], and our finding that this increase in arginase-1 is blocked by the pharmacological inhibition of H-PGDS by HQL-79 [266], suggest that selenium may also play a role in the class switching of macrophage phenotypes via the shunting of the AA-COX pathway towards 15d-PGJ_2. Further studies using animal models are needed to

demonstrate the translational relevance of such a class switching. Helminth infections such as *Schistosomia mansoni*, *Fasciola hepatica*, or *Nippostrongylus brassilensis* trigger Th2-type host immune response in form of M2 activation [325], and thus provide useful models to validate the effects of differential selenium status. Such studies are currently being carried out in our laboratory.

Numerous studies have reported mechanisms involved in the inhibition of NF-κB by 15d-PGJ_2, including the direct covalent modification of critical thiols of key proteins in the NF-κB activation pathway, and indirectly through the activation of PPARγ (see Chapter 1). The enzymes mPGES-1 and TXAS, which catalyze the formation of pro-inflammatory prostaglandins, PGE_2 and TXA_2, respectively, are NF-κB -dependent genes. We show that the inhibition of NF-κB or mPGES-1 leads to increased H-PGDS levels. Taken together with the fact that 15d-PGJ_2 inhibits NF-κB and the subsequent mPGES-1 expression, our results depict a well-connected scheme involving selenoproteins, AA-COX metabolites with varying functions, and two key transcription factors, PPARγ and NF-κB (see Fig. 17). Thus, we relate the differential regulation of PG synthases in macrophages by selenoproteins to the inhibition of NF-κB axis, where the PPARγ-activating CyPG, 15d-PGJ_2, plays a central role.

The presence of an α,β-unsaturated carbonyl group in the structure of 15d-PGJ_2 makes it one of the few known endogenously produced Michael acceptors, which are highly electrophilic and hence are capable of reacting with specific cysteine thiols, resulting in covalent modification and changes in protein function [130]. A considerable number of biologically active, plant-derived compounds that act as Michael acceptors have been identified. Gambogic acid (GA), a polyprenylated xanthone derived from the resin of the plant, *Garcinia hanburyi*, is one such botanical that possesses a reactive α,β-unsaturated carbonyl group. GA has been used

historically as a folk medicine, and well-studied for its anti-carcinogenic properties over the past two decades [326]. Taking a clue from the structure-related mechanisms involved in the inhibition of NF-κB by 15d-PGJ_2, we have explored the anti-inflammatory potential of GA using murine macrophage cell line models. Our results show that GA inhibited lipopolysaccharide (LPS) -induced activation of NF-κB by the covalent thiol modification of Cys^{179} in the active site of IKKβ, a rate-limiting enzyme in the canonical NF-κB activation pathway. Nuclear translocation and subsequent DNA-binding activity of the p65 subunit of NF-κB was decreased by GA in macrophages, but not with 9,10-dihydro GA (DGA), confirming the crucial role of the α,β-unsaturated carbonyl group in the observed effects of GA. However, it is necessary to validate these promising results using *in vivo* models of inflammation. More knowledge is needed about the pharmacokinetic properties of GA in order to pursue *in vivo* studies.

While NF-κB is a critical player in inflammation, the roles of this family of transcription factors in cellular proliferation and cell survival are also well known [327, 328]. Thus, the ability of endogenous CyPGs to trigger pathways of apoptosis also seems important to study. Besides a role in curbing inflammation and favoring a resolution response in the tissue, 15d-PGJ_2 is well known for its anti-proliferative properties. The mechanisms by which 15d-PGJ_2 induces apoptosis of tumor cells or cell lines include p53-dependent or -independent pathways. However, there has always been a debate in literature over the endogenous production of 15d-PGJ_2 and whether the *in vivo* concentrations are enough to exert the effects seen in cell culture models. An earlier report from our laboratory has shown that 15d-PGJ_2, at low nanomolar concentrations, induces apoptosis *ex vivo* in cultured LSCs from spleens from the Friend-virus (FV) leukemia model ($M34^+Kit^+Sca^+$) and the BCR-ABL model ($GFP^+Kit^+Sca^+$) [120]. This result is well supported by a computational study, in which 15d-PGJ_2 was identified as one of the four compounds from a high-throughput screen to have a highly similar public gene expression

signature as the “query” (template) compound, parthenolide (PTN), in eradicating AML LSCs [261]. This strengthened our hypothesis that selenium supplementation has the ability to generate sufficient endogenous levels of 15d-PGJ_2 by a favorable modulation of the AA-COX pathway in such a way that LSC populations can be targeted to apoptosis *in vivo*.

The two main models of leukemia we have used for this study are: FV-infection erythroleukemia model, and BCR-ABL -transduced HSC transplantation model. Although the mechanisms of leukemogenesis are different in these two models, they represent useful experimental murine models for studying the role of chemopreventive or chemotherapeutic strategies directed towards LSCs. The presence of LSCs was confirmed in both of these models using flow cytometry analysis for appropriate cell markers as well as by traditional colony-forming assays in culture. A third model, MLL-$AF9^+$ HSC transplantation model, which closely resembles a subtype of human AML, has been used for a few major experiments. To investigate the effects of selenium in these models, mice were maintained on Se-deficient (0.01 ppm selenite), Se-adequate (0.1 ppm selenite), or Se-supplemented (0.4 ppm selenite; or 3 ppm methylseleninic acid) diets until the desired selenium levels were achieved in the body. This was followed by induction of leukemia by either FV-infection or BCR-ABL^+ HSC transplantation. Our results indicate that while mice on both, Se-deficient and Se-adequate diets succumbed to leukemia within two-three weeks, mice on Se-supplemented diets survived. WBC counts and spleen pathology were used as end points to assess the disease. Interestingly, selenium supplementation led to an eradication of the LSC populations from these mice, unlike the mice on Se-deficient or Se-adequate diets. The results from serial transplantation experiments testify the absolute ‘wiping-out’ effect of Se-supplementation on LSCs, reaffirming serial transplantation as a very powerful tool to gauge the efficiency of LSC-eradication strategies in the future. The results also indicate that adequate levels of selenium are not enough for anti-carcinogenic effects, which is in agreement with

previously reported studies where levels close to 0.1 ppm of selenite in the diet are sufficient for the selenoproteins to perform physiological functions, but not assuming chemopreventive roles [252]. Knowing that levels of selenium in the range of 8-10 ppm are considered toxic [329, 330], it is prudent to investigate the health benefits of selenium at levels all across a range of ~0.4 ppm to ~2-3 ppm in various cancer models. However, as discussed earlier, a careful analysis on the various forms of selenium used with regards to their pharmacokinetic properties is absolutely necessary.

Another interesting aspect to consider for future studies is to look at the chemotherapeutic potential of supplemental selenium in leukemias. It is surprising that there has been very limited research conducted to look at the chemotherapeutic potential of selenium as opposed to its chemopreventive benefits; this dearth of knowledge applies to not only leukemias but cancers in general. In fact, a report from 1956 shows that when selenocysteine was given orally to four leukemic patients (two with acute leukemia and two with chronic leukemia), there was a dramatic improvement in WBC counts and their spleens returned to unpalpable sizes [331]. Surprisingly, there has not been much research done in this field since this promising report. One of our results shows that the BCR-ABL - transplanted mice on Se-adequate diets show relapse after stopping imatinib treatment, while those on Se-supplemented diets display no signs of relapse. It will be interesting to examine if switching the Se-adequate mice onto a Se-supplemented diet upon transplantation affects the outcome of the disease. Additionally, effects of combining selenium supplementation and lower doses of chemotherapy should be studied in leukemia treatment, as this may lead to a decrease in the incidence of adverse effects of chemotherapeutic drugs.

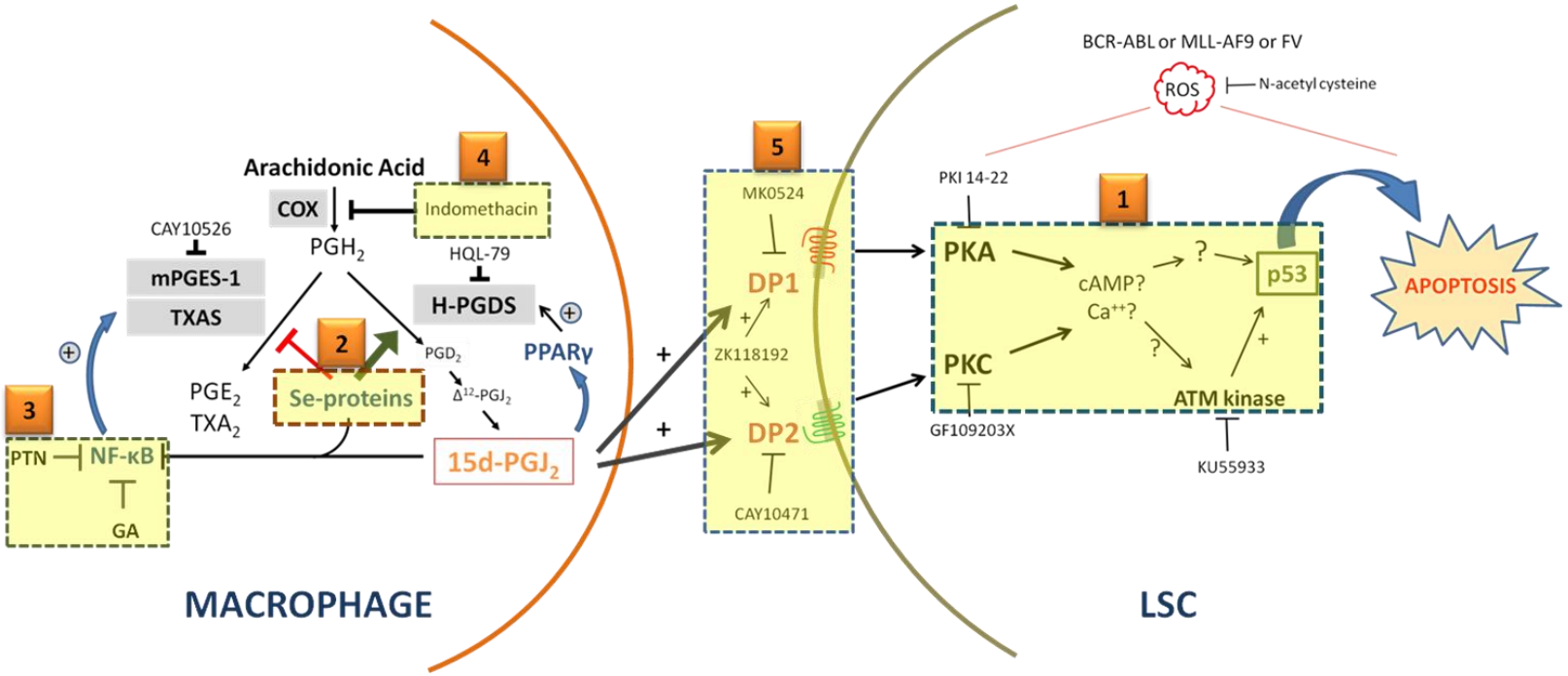

Fig. 40 Schematic showing some of the potential future directions for this project. Box 1 – Delineate exact downstream signaling involved in the Se - 15d-PGJ_2 – mediated apoptosis of LSCs; Box 2 – Determine which specific selenoprotein(s) is involved in the regulation of PG synthases; Box 3 – Investigate the anti-leukemic potential of natural NF-κB inhibitors like GA; Box 4 – Re-evaluate the use of NSAIDs in leukemic patients; Box 5 – Re-consider the use of DP antagonists (e.g. Ramatroban) as anti-asthma therapy.

In order to connect our striking results with AA-COX pathway metabolites, we have used indomethacin, a non-steroidal anti-inflammatory drug (NSAID), which inhibits COX-1 and COX-2 activity, and therefore the production of all classes of AA-derived PGs. *In vivo* administration of indomethacin blocks the chemoprotective effects of supplemental selenium in both models of leukemia in mice, suggesting an essential role of PGs. Furthermore, HQL-79, an inhibitor of H-PGDS activity, also prevents the anti-LSC effects of selenite *ex vivo*, suggesting that a block in the AA-COX pathway is most probably at the level of the production of PGD_2 or its downstream metabolite, 15d-PGJ_2. This is confirmed when administration of 15d-PGJ_2 for one week during the second week post-FV infection rescues the indomethacin-treated Se-supplemented mice. Effects of HQL-79 need to be looked at using *in vivo* models, though. An important implication to arise out of this result is regarding the use of indomethacin or any other NSAID in leukemic patients. A recently published study states that long-term use of acetaminophen is associated with almost two-fold increase in the incidence of hematological malignances [332]. In this era of poly-pharmacy, such results make a compelling case for retrospective studies to investigate the incidence of leukemias in patients on selenium supplements who have also been consuming aspirin or non-aspirin NSAIDs.

The studies involving pre-treatment of LSCs with DP receptor antagonists suggest that 15d-PGJ_2 likely acts through these receptors to activate downstream apoptotic pathways. Inhibition of PKA or PKC also blocks the pro-apoptotic effects of 15d-PGJ_2, giving further insight about the signaling mechanisms involved. The DP receptors are known to mediate effects of PGD_2 on mast cells in allergic conditions where PGD_2 contributes to increased airway-responsiveness and inflammation related to allergic asthma [333]. In fact, ramatroban, a DP_2 receptor antagonist, is a drug being considered for use in patients with bronchial asthma. Our results indicate the need to conduct studies to investigate the role of ramatroban or other similar DP antagonists as

potential confounders in patients with leukemia on Se-supplementation. Such provocative associations have to be addressed in epidemiological studies followed by mechanistic studies to examine the remarkable selectivity of selenium and/or CyPGs towards LSCs. The expression of certain fusion oncoproteins specifically in LSCs may explain their susceptibility to CyPGs, which needs to be explored to get a better understanding of the role of supplemental selenium-induced increase in 15d-PGJ_2 in the eradication of LSCs. Models for the blast-crisis (BC) phase of CML such as those based on cooperation between BCR-ABL and NUP98/HOXA9 oncoproteins [334] also provides basis for investigating the beneficial effects selenium and/or CyPGs on more aggressive and terminal stages of leukemia. Given the compelling preliminary data and the availability of various different models to perform such studies, time seems propitious to conduct extensive studies to delineate mechanisms and initiate clinical trials.

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