Vitamin K3 suppressed inflammatory and immune responses in a redox-dependent manner

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Abstract
Recent investigations suggest that cellular redox status may play a key role in the regulation of several immune functions. Treatment of lymphocytes with vitamin K3 (menadione) resulted in a significant decrease in cellular GSH/GSSG ratio and concomitant increase in the ROS levels. It also suppressed Concanavalin A (Con A)-induced proliferation and cytokine production in lymphocytes and CD4+ T cells in vitro. Immunosuppressive effects of menadione were abrogated only by thiol containing antioxidants. Mass spectrometric analysis showed that menadione directly interacted with thiol antioxidant GSH. Menadione completely suppressed Con A-induced activation of ERK, JNK and NF-κB in lymphocytes. It also significantly decreased the homeostasis driven proliferation of syngeneic CD4+T cells. Further, menadione significantly delayed graft-vs-host disease morbidity and mortality in mice. Menadione suppressed phytohemagglutinin-induced cytokine production in human peripheral blood mononuclear cells. These results reveal that cellular redox perturbation by menadione is responsible for significant suppression of lymphocyte responses.

Keywords: NF-κB, cytokines, lymphocytes, GVHD, GSH

Introduction
Vitamin K is a group of essential fat-soluble vitamins that act as co-factors of a number of plasma proteins mostly required for blood coagulation, such as prothrombin and factors VII, IX and X [1]. Vitamin K3 (2-methyl-1,4-naphthoquinone), also known as menadione, is a synthetic precursor (provitamin) to various other types of vitamin K required by the body. Since it is easily synthesized chemically it has been considered as a suitable and inexpensive source of vitamin K. Menadione has been shown to have potent anti-cancer effects against various types of carcinoma, including that of hepatic, oral, pharyngeal, mammary and bladder as well as leukemia cell lines in vitro [2]. Mechanisms like the mitochondria-related apoptotic pathway [3] and the disruption of the microtubules network by binding to tubulin in human carcinoma cells [4] have been proposed to explain the growth inhibitory and cytotoxic effects of menadione in tumour cells. Other naphthoquinones like juglone, plumbagin and 1,4-naphthoquinone, which are structurally related to menadione, have also been shown to exert anti-proliferative effects. Their mechanism of action was through suppression of constitutive and inducible NF-κB expression [5]. NF-κB is a central transcription factor that regulates the expression of various cytokines, growth factors and effector enzymes in response to ligation of many receptors involved in immunity, including T-cell receptors (TCRs) and B-cell receptors (BCRs), TNF receptor, CD40, B-cell activating factor receptor, lymphotoxin β receptor and the Toll/IL-1R family [6–8]. Historically, the main effect of menadione is believed to be due to oxidative stress via redox cycling of the quinone to produce...
reactive oxygen species [9,10]. Among the most susceptible oxidant-sensitive targets are protein thiol groups, which can be reversibly oxidized to sulphenic acid (-SOH) [11]. Reversible oxidation is believed to not only protect proteins from irreversible oxidation but may also modulate protein function. Further, there are several proteins that are involved in regulation of immune responses like IKKB, IkB-α and AP1 which are known to have critical cysteine residues. These residues are easily oxidized with change in cellular redox that can alter the function of the protein [12–14]. Earlier reports have also demonstrated that sustained oxidative stress inhibits NF-κB activation partially, by inactivating the proteasome [15].

The ability of vitamin K3 to modulate immune responses remains largely unexplored. We investigated the effects of vitamin K3 on mitogen-induced immune responses in murine lymphocytes in vitro and in vivo. Further, the immunomodulatory effects of Vitamin K3 in human peripheral blood mononuclear cells were studied and are described herein.

Materials and methods

Chemicals

Menadione, RPMI-1640 medium, HEPES, ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), phenylmethylsulphonyl fluoride (PMSF), leupeptin, aprotonin, benzamidine, diithiothreitol (DTT), glutathione (GSH), N-acetyl cysteine (NAC), Mannitol, nodenet P-40, phytomelagglutinin (PHA) and dimethyl sulphoxide (DMSO) were purchased from Sigma Chemical Co. (St Louis, MO, USA). A 100 mM solution of menadione was prepared in dimethyl sulfoxide and stored as small aliquots at –20 °C. The guidelines issued by the Institutional Animal Ethics Committee of Bhabha Atomic Research Centre, Government of India, regarding the maintenance and dissections of small animals were strictly followed.

Animals

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

Treatment with menadione

A stock solution of 20 mM menadione was prepared in DMSO and small aliquots were stored at –20°C. The cells were pre-treated (incubated) with different doses (1–10 μM) of menadione for 4 h before the initiation of culture, in all the experiments conducted in vitro.

Proliferation assay

Splenocytes were obtained by squeezing the mouse spleen through a nylon mesh in a petri plate containing RPMI medium. RBC were lysed by brief hypotonic shock. Splenocytes were stained with CFSE (20 μM, 5 min, 37°C) and washed three times using ice-cold RPMI medium containing 10% FCS, 100 IU/ml penicillin and 100 mg/ml streptomycin. Two million splenocytes were treated with menadione (50 nM to 10 μM, 4 h) and were stimulated with Con A (5 μg/ml) for 72 h at 37°C in 2 ml RPMI with 10% FCS in a 95% air/5% CO2 atmosphere. Vehicle-treated cells served as control. Cell proliferation was measured by dye dilution in a flowcytometer (Partec CyFlow, Gotlitz, Germany) [16]. The percentage of daughter cells that showed a decrease in CFSE fluorescence intensity was calculated using Flomax software and expressed as daughter cells.

Estimation of apoptosis.

The percentage of apoptotic cells was estimated using a flowcytometer. One million splenocytes were treated with menadione (10 μM) for 24 h at 37°C in RPMI1640 medium supplemented with 10% foetal calf serum (FCS) in a 5% CO2 atmosphere. Vehicle-treated cells served as control. The cells were washed with PBS and incubated with 1 ml of staining solution containing 0.5 μg/ml propidium iodide, 0.1% sodium citrate and 0.1% Triton X-100 overnight. A total of 20 000 cells were acquired in a Partec CyFlow flowcytometer and analysed using Flomax® software. The pre-G1 population represented the apoptotic cells.

Measurement of cytokine secretion by mouse spleen cells

Mouse splenocytes were pre-treated with menadione (1–10 μM) for 4 h, before they were stimulated with Con A (5 μg/ml) or anti-CD3/CD28 mAb and cultured for 24 h at 37°C. The concentration of cytokines IL-2, IL-4, IL-6 and IFN-γ were estimated in the
culture supernatants from control unstimulated cells as well as stimulated cells using cytokine ELISA sets (BD Pharmingen, Franklin Lakes, NJ, USA) [17,18]. The supernatant obtained from Con A stimulated cells, in the absence of menadione, was used as positive control.

Similarly, the cytokine levels induced by LPS were estimated in the culture supernatant of splenic adherent macrophages. Spleen cells (5 x 10⁶ cells/well) were incubated in a 24-well cell culture plate for 3 h at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The non-adherent cells were removed by aspiration. The adherent cells (macrophages) were incubated with menadione (10 μM for 4 h) and then stimulated with LPS (50 μg/ml) and further cultured for 6 h or 24 h at 37°C. The concentration of cytokines IL-6 and TNF-α after 6 h and IL-1β after 24 h of LPS stimulation, were estimated using cytokine ELISA sets (BD Pharmingen).

Measurement of nitric oxide
The concentration of nitric oxide in the supernatant of splenic adherent macrophages pre-treated with menadione and cultured for 24 h at 37°C in the presence of LPS was measured by using Griess reagent as described earlier [19].

Human lymphocyte cytokine assay
Informed, signed consent was taken from all the registered healthy volunteers before undertaking these studies. The guidelines issued by the Medical Ethics Committee of Bhabha Atomic Research Centre, Government of India, were strictly followed during these studies. Peripheral venous blood (10 ml) was collected from the cubital vein of healthy volunteers under all aseptic precautions and transferred immediately into heparinized vacutainer tubes (BD Pharmingen). The blood was diluted 1:1 with sterile RPMI medium and gently layered on to 10 ml Histopaque 1077 in a 50 ml centrifuge tube. The tubes were centrifuged at 400 x g for 20 min and peripheral blood mononuclear cells (PBMC) were collected fromuffy coat. The PBMC were washed three times with RPMI medium and viable cells were counted using trypan blue dye exclusion. PBMC (2 x 10⁶/ml) were stimulated with PHA (10 μg/ml) in the presence or absence of menadione (1 and 5 μM) and cultured for 24 h at 37°C in 2 ml RPMI with 10% FCS in a 95% air/5% CO₂ atmosphere. Untreated cells served as control. The culture supernatants were collected and used for estimation of different cytokines. Concentrations of IL-2, IL-4, IL-5, IL-6 and TNF-α in the culture supernatants were measured using commercially available ELISA kits. The supernatants obtained from unstimulated cells and PHA stimulated cells were used as negative and positive control, respectively.

Determination of intracellular glutathione and glutathione disulphide levels. The ratio of GSH:GSSG was estimated using the enzymatic recycling method [20].

Pro-oxidant measurements
To detect intracellular ROS, lymphocytes were preincubated with 20 μM oxidation-sensitive dihydrodichlorofluorescein diacetate (H₂DCF-DA) for 25 min at 37°C before being treated with various concentrations of menadione. After 1 h of incubation, the increase in fluorescence resulting from oxidation of H₂DCF to DCF was measured using a spectrofluorimeter [17].

Mass spectrometry
Mass spectrometric analysis was performed to characterize the products formed during reaction of menadione with GSH using a Varian ProStar 410 AutoSampler in combination with Varian 1200 L LC–MS equipment triple quadrupole mass (QqQ) spectrometer with ESI source (Varian, Inc., Santa Clara, CA, USA). The ESI source operated in positive ionization mode.

Detection of intracellular proteins. Three million lymphocytes cells were cultured in the presence or absence of menadione for 4 h and then stimulated with Con A for 1 h at 37°C. Cultured cells were fixed with 4% paraformaldehyde for 10 min at room temp and excess of paraformaldehyde was removed by washing once with wash buffer (PBS containing 1% BSA). Before staining with monoclonal antibody against phosphor-ERK and phosphor-JNK, cells were permeabilized with PBST (PBS containing 0.02% Tween-20) three times for 5 min each at room temp followed by two washes with wash buffer and then incubated with the indicated mAbs for 30 min at room temp, washed twice and analysed on a Partec Cyflow flowcytometer.

Electrophoretic mobility shift assay
Splenocytes were treated with menadione (10 μM, 4 h) and were stimulated with Con A (5 μg/ml) for 1 h at 37°C and nuclear extracts were prepared [21]. The nuclear pellets were resuspended in 25 μl of ice cold nuclear extraction buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2.0 pg/ml leupeptin, 2.0 μg/ml aprotinin and 0.5 mg/ml benzamidine) and the tubes were incubated on ice for 15 min with intermittent agitation. This nuclear extract was microcentrifuged for 5 min at 12 000 rpm and the supernatant was collected in fresh tubes and frozen at −70°C. EMSA was performed by incubating 10 μg of nuclear proteins with 16 fmol of 32P-end-labelled, 45-mer double stranded NF-κB binding oligonucleotides from the
human immunodeficiency virus long terminal repeat (5′-TTGTTACAAGGGACCTTCCGCTGGGG-
GACCTTCCAGGGAGGCCTGG-3′; boldface indicates NF-κB binding sites) in the presence of 0.5 μg of poly (2′-deoxyinosinic-2′-deoxycytidylic acid) (poly (dI–dC)) in binding buffer (25 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.5 mM dithiothreitol, 1% Nonidet P 40, 5% glycerol and 50 mM NaCl) for 30 min at 37°C. The DNA–protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels using buffer containing 50 mM Tris, 200 mM glycerine and 1 mM EDTA, pH 8.5. The dried gel was exposed to a phosphorimaging plate and the radioactive bands were visualized using a phosphorimage plate scanner (Amersham Biosciences, Piscataway, NJ, USA).

Lymphopenia induction

Animals were placed in ventilated perspex boxes and exposed to 600 cGy whole body γ-radiation at a dose of 420 cGy/min in a Gamma cell 220 irradiator (AECL, Mississauga, Ontario, Canada). Lymphopenia induction was confirmed by counting the number of total splenic lymphocytes 96 h after irradiation [16].

Adoptive transfer and homeostasis driven proliferation in vivo

CD4+ T cells were purified from splenic lymphocytes using anti CD4 antibody coated magnetic nano-particles (BD Pharmingen) in a MACS cell sorter. CD4 + T cells were stained with CFSE and cultured in the presence of menadione for 4 h at 37°C in a 5% CO2 incubator. Untreated cells served as control. The unbound drug was removed by washing the cells once with RPMI medium. In each group 1.5 million purified CD4 + T cells were injected i.v. into the tail vein of lymphopenic mice. At least four mice were used in each group. The reconstituted mice were sacrificed 96 h after injection and spleens were recovered. Fifty thousand lymphocytes in each group were analysed by flow-cytometry. These cells were used to enumerate frequency of donor cells and to quantify the cell proliferation by CFSE dye dilution [16].

Induction of graft vs host disease (GVHD)

Balb/c mice were exposed to 600 cGy whole body gamma-radiation (WBI) (Gamma Cell 220, AECL, Mississauga, Ontario, Canada). To induce GVHD in immunocompromised Balb/c mice, 8 × 106 splenocytes from C57BL/6 donors were injected i.v. 48 h after irradiation [22]. Each mouse in the control group received vehicle treated splenocytes, whereas each mouse in the experimental group received splenocytes treated with 10 μM menadione for 4 h. The recipient mice were monitored daily to assess the signs of GVHD. In total, 10 recipient mice in the control group and 10 recipient mice in the menadione-treated group were evaluated. GVHD became evident from rapid and sustained weight loss as well as from features such as hunchback, diarrhoea, hair loss and death.

Results

Menadione inhibited Con A induced T-cell proliferation

The effect of menadione on Con A induced lymphocyte proliferation as assessed by CFSE dye dilution is shown in Figures 1A and B. Menadione completely inhibited the Con A induced proliferation of lymphocytes. The majority of the menadione-treated cells failed to enter the cell division cycle, as shown by lack of decrease in the CFSE fluorescence intensity in flowcytometric histograms (Figure 1A). The percentage of daughter cells was obtained after 72 h. The inhibition of T-cell proliferation by menadione was not due to cytotoxicity because lymphocytes incubated with menadione (10 μM) for 24 h did not show any increase in apoptosis (pre-G1 peak) as compared to that in control cells as estimated by PI staining (Figure 1C). Further, cytotoxic effects of menadione studied by DNA ladder assay, live and dead assay and acridine orange/ethidium bromide staining methods show that menadione did not induce any cytotoxicity in unstimulated lymphocytes (data not shown).

Menadione inhibited Con A induced cytokine production

Figure 1D shows the levels of IL-2, IL-6 and IFN-γ cytokines in culture supernatant of menadione pre-treated (1–10 μM) or untreated cells stimulated with Con A (5 μg/ml). Con A activated cells showed significantly higher secretion of IL-2, IL-6 as well as IFN-γ as compared to that in control vehicle treated cells. Pre-treatment of cells with menadione significantly inhibited Con A induced levels of IL-2, IL-6 and IFN-γ cytokines (Figure 1D).

Menadione inhibited anti-CD3/CD28 mAb and LPS induced activation of CD4 + T-cells and macrophages, respectively

Figure 2A shows the levels of various cytokines secreted by purified CD4 + T cells in response to stimulation by anti-CD3/CD28 mAb stimulation with or without menadione pre-treatment. CD4 + T cells stimulated with anti-CD3/CD28 mAb produced significantly higher levels of IL-2, IL-4, IL-6 and IFN-γ cytokines (Figure 2A). Whereas, prior treatment of cells with menadione (10 μM for 4 h) completely inhibited anti-CD3/CD28 mAb induced secretion of IL-2, IL-4, IL-6 and IFN-γ (Figure 2A). Further, it was also observed that treatment of splenic adherent macrophages with
Menadione prior to stimulation with LPS completely inhibited the secretion of nitric oxide, IL-6, IL-1β and TNF-α (Figures 2B and C).

Menadione inhibited cytokine secretion by PHA activated human PBMCs

To study the effect of menadione on human PBMC, the pattern of cytokine secretion was assessed in PHA stimulated cells in the presence or absence of menadione in vitro. We observed that menadione (5 μM) was effective in inhibiting PHA induced cytokine secretion by human PBMC in vitro (Figure 2D).

Menadione modulates cellular ROS levels and GSH/GSSG ratio

Figure 3A shows the levels of ROS in control and menadione-treated lymphocytes. Menadione induced an increase in the basal ROS levels in lymphocytes which was further augmented in the presence of diethylmaleate, a GSH-depleting agent (Figure 3A). To estimate the effect of menadione on intracellular GSH/GSSG ratio, lymphocytes were incubated with menadione for 4 h. Menadione treated cells showed a significant decrease in the ratio of GSH/GSSG as compared to the untreated cells (Figure 3B).

Immunosuppressive effects of menadione were abrogated by thiol antioxidants

Figure 3C shows the effect of thiol and non-thiol antioxidants on the immunosuppressive effects of menadione. Thiol-containing antioxidant (GSH) abrogated the immunosuppressive effect of menadione (Figure 3C). However, antioxidants that do not contain a thiol group (trolox, MnTBAP and manniitol) did not have any effect on the immunosuppressive action of menadione (Figure 3C). Interestingly,
The immunosuppressive effects of well known drugs like rapamycin and Ly294002 (PI3kinase inhibitor) were not abrogated by thiol (GSH, NAC) or non-thiol (MnTBAP) antioxidants (Figure 3D).

**Interaction of menadione with GSH**

Since the immunosuppressive effects of menadione were sensitive to the presence of thiol antioxidants, experiments were carried out to determine whether menadione physically interacted with thiol groups. Menadione was incubated with GSH and subjected to mass-spectrometric analysis. Molecular mass analysis revealed that the major peak corresponded to menadione–GSH adduct (m/z: 478.1) (Figure 3E). There were other minor peaks appearing at different molecular masses, which could not be characterized.

**Menadione suppressed Con A induced ERK, JNK and NF-κB activation in lymphocytes**

Figure 4 shows the effect of menadione on Con A induced ERK, JNK and NF-κB activation in lymphocytes. Treatment of lymphocytes with Con A led to an increase in the levels of pERK and pJNK, whereas in cells pre-treated with menadione there was a decrease in their levels (Figures 4A and B). Interestingly, menadione alone induced phosphorylation of ERK but not JNK. Lymphocytes stimulated with Con A showed NF-κB activation in the nuclear fraction as compared to that in vehicle treated control cells. However, cells treated with menadione prior to stimulation with Con A did not show NF-κB activation (Figure 4C).

**Menadione inhibited HDP of CD4+ T cells**

Figure 5A shows the effect of menadione treatment (10 μM, 4 h in vitro) on HDP of CD4+ T cells in autologous lymphopenic hosts. The dot-plots show donor derived CFSE+CD4+ T cells in the host spleen 96 h after reconstitution (Figure 5A). The frequency of menadione treated donor CD4+ T cells was lower as compared to that of untreated CD4+ T cells injected into the lymphopenic mice (Figure 5A). Thus, menadione-treated cells failed to survive or divide under lymphopenic conditions.
Menadione treatment of lymphocytes prior to allogenic transplantation delayed the induction of GVHD in mice

To test immunosuppressive activity of menadione in a model of GVHD, splenocytes from C57BL/6 mice were incubated with menadione (10 μM, 4 h) and transferred to WBI treated immunocompromised Balb/c mice. The recipient Balb/c mice of vehicle-treated C57BL/6 cells developed GVHD that led to 100% death within 8 days (Figure 3B), demonstrating typical symptoms of GVHD, including alopecia, hunched posture, diarrhoea and progressive weight loss. However, in the recipients of menadione-treated cells the development of GVHD was delayed and 20% of the mice survived and showed better health for more than 30 days (Figure 5B). Furthermore, the mice injected with cells that were treated with menadione experienced insignificant weight loss as compared to the control group (Figure 5B).

Discussion

Menadione or Vitamin K3 has been recently shown to possess potent anti-proliferative effects in various tumour cells [2]. These anti-tumour properties of vitamin K3 have been attributed to its ability to generate reactive oxygen species (ROS) including H$_2$O$_2$, O$_2$•$^-$ and OH, during its metabolism inside the cell. Since many critical signalling mediators of immune response are known to be sensitive to alterations in the cellular redox status the present study focused on immunomodulatory effects of menadione. Our data clearly shows that menadione was able to completely inhibit Con A induced T-cell proliferation and cytokine production (Figure 1). This inhibition of lymphocyte proliferation and cytokine secretion was not due to cell death, as menadione up to 10 μM did not induce cell death in lymphocytes. Menadione inhibited anti-CD3/CD28 mAb induced cytokine secretion by
CD4⁺ T cells in vitro (Figure 2A). Interestingly, menadione also mitigated LPS induced secretion of nitric oxide, IL-1β, TNF-α and IL-6 by macrophages (Figures 3B–D). These results suggest that menadione may act on several different cell types and also exhibit anti-inflammatory activity. Anti-tumour effects of menadione have been attributed to its ability to modulate cellular redox status. This was also confirmed in lymphocytes treated with menadione which showed an increase in the cellular ROS levels and a decrease in cellular GSH/GSSG ratio (Figures 3A and B). However, the subsequent and the most intriguing finding of this study was that the immunomodulatory action of menadione was associated with its ability to modulate cellular thiols and not on its ROS generating capability. This crucial role of cellular thiols and a redundant function of cellular ROS in vitamin K3 mediated immunomodulation could be inferred from the observation that only thiol-based antioxidant (GSH) was able to abrogate the immunosuppressive effects of vitamin K3, whereas non-thiol antioxidants like Trolox, MnTBAP and mannitol had no effect on the same (Figure 3C). Further, we observed that the anti-inflammatory action of well-known immunosuppressants rapamycin and Ly294002 could not be abrogated by thiol or non-thiol anti-oxidants (Figure 3D).
These results clearly demonstrate a novel mechanism of action of menadione as compared to the existing immunomodulatory agents.

It has previously been demonstrated using ESR studies that menadione reacts with GSH [23] and a similar reaction was observed using mass spectrometric analysis in this study (Figure 3E). It was possible that menadione might also be reacting with free thiol groups on cellular proteins and in the process modulating the activity of sensitive signalling mediators involved in mitogen-induced immune responses.

Activation of MAPK and NF-κB upon TCR cross-linking is critical for T-cell survival and activation [6]. Menadione suppressed Con A mediated activation of ERK and JNK in T-cells (Figures 4A and B). Inhibition of phosphorylation of ERK and JNK by menadione may result in suppression of immunologically important transcription factor AP-1. In unstimulated T-cells, NF-κB is sequestered in the cytoplasm by IκB-α. T-cell activation results in phosphorylation and degradation of IκB-α, leading to translocation of NF-κB to the nucleus [24]. Phosphorylation of IκB-α is mediated by the IκB kinase (IKK) complex, which contains two catalytic sub-units, IKK-α and IKK-β, and one regulatory sub-unit, IKK-γ. NF-κB activation is required for complete T-cell activation, survival and proliferation [25]. We observed that menadione was able to completely suppress Con A induced activation and nuclear translocation of NF-κB in normal lymphocytes (Figure 4C). Our results clearly demonstrate that menadione has multiple molecular targets in exhibiting immunosuppressive activity.

Several reports suggest that production of IL-2, IL-4, IL-6 and IFN-γ cytokines is transcriptionally regulated by NF-κB [26,27]. We propose that the observed suppression of T-cell proliferation and cytokine production by menadione may be due to its ability to inhibit MAPK and NF-κB activation. The inhibition of DNA binding ability of NF-κB by menadione may be through modulating critical cysteine residues present on P65 and P50 sub-units of NF-κB.
The *in vivo* anti-proliferative and anti-inflammatory effects of menadione were studied using murine models of homeostasis driven proliferation (HDP) and graft-vs-host disease (GVHD), respectively. Lymphocytes have been shown to proliferate *in vivo* in response to lymphopenia by a process known as HDP [28]. Treatment of CD4⁺ lymphocytes with menadione severely hampers the ability of these cells to proliferate as is evident from the diminished frequency of menadione-treated CFSE labelled CD4⁺ donor T-cells in the spleen of reconstituted lymphopenic mice (Figure 5A). This inhibition of homeostatic proliferation and survival of donor CD4⁺ T-cells following treatment with menadione clearly demonstrates that modulation of cellular redox can also alter the proliferative responses of lymphocytes *in vivo*. We had earlier demonstrated that NF-κB plays a crucial role in the HDP of lymphocytes in lymphopenic mice and that inhibition of NF-κB can prevent HDP of lymphocytes [29]. Thus, the observed NF-κB suppressive property of menadione may also be contributing to the observed inhibition of HDP.

Development of GVHD mediated activation of lymphocytes of donor origin has over time proved to be a major hurdle in successful transplantation. Lymphocyte activation and cytokine secretion is one of the initial events during the development of GVHD and modulation of lymphocyte activation leading to suppression of alloreactive T-cells may have potential application in the treatment of GVHD. Also, modulation of NF-κB activation pathway which leads to suppression of alloreactive T-cells has been shown to have therapeutic potential in the prevention of graft rejection [30]. Treatment of donor lymphocytes with menadione delayed the development of acute GVHD in mice receiving allogenic donor lymphocytes with menadione disrupted the microtubule networks by binding to tubulin: a novel mechanism of its antiproliferative activity. Biochemistry 2009;48:6963–6974.

In conclusion, the present report describes the immunomodulatory effects of menadione in murine lymphocytes and human PBMC. We also demonstrate that these anti-inflammatory effects of menadione are mediated via modulation of cellular redox status and inhibition of activation of NF-κB in lymphocytes. These properties and the ability of menadione to delay the induction of acute GVHD may find application in treatment of human inflammatory disorders.

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**Declaration of Interest**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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