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ORIGINAL ARTICLE

Premature T cell senescence in Ovx mice is inhibited by repletion of estrogen and medicarpin: a possible mechanism for alleviating bone loss

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Abstract
Summary Presently the relationship between CD28, biological marker of senescence, and ovariectomy is not well understood. We show that ovariectomy leads to CD28 loss on T cells and estrogen (E2) repletion and medicarpin (Med) inhibits this effect. We thus propose that Med/E2 prevents bone loss by delaying premature T cell senescence.
Introduction Estrogen deficiency triggers reproductive aging by accelerating the amplification of TNF-α-producing T cells, thereby leading to bone loss. To date, no study has been carried out to explain the relationship between $CD4^+CD28$null T cells and ovariectomy or osteoporosis. We aim to determine the effect of Ovx on CD28 expression on T cells and effects of E2 and medicarpin (a pterocarpan phytoalexin) with proven osteoprotective effect on altered T cell responses.
Methods Adult, female Balb/c mice were taken for the study. The groups were: sham, Ovx, Ovx+Med or E2. Treatments were given daily by oral gavage. At autopsy bone marrow and spleen were flushed out and cells labelled with antibodies for FACS analysis. Serum was collected for ELISA.
Results In Ovx mice, Med/E2 at their respective osteoprotective doses resulted in thymus involution and lowered Ovx-induced increase in serum TNF-α level and its mRNA levels in the BM T cells. Med/E2 reduced BM and spleen $CD4^+$ T cell proliferation and prevented CD28 loss on $CD4^+$ T cells. Further, Med abrogated TNF-α-induced loss of CD28 expression in the BM T cells.
Conclusions To our knowledge this is the first report to determine the mechanism of CD28 loss on T cells as a result of ovariectomy. Our study demonstrates that Ovx leads to the generation of premature senescent $CD4^+CD28$null T cells, an effect inhibited by E2 and Med. We propose that one of the mechanisms by which Med/E2 alleviates Ovx-induced bone loss is by delaying T cell senescence and enhancing CD28 expression.

Keywords Immunological senescence · Menopause · Osteoporosis · TNF-α

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Introduction

Osteoporosis is an asymptomatic systemic disease of the skeleton characterized by decreased bone mass and loss of microarchitectural integrity. There is increasing evidence that the bone resorption and probably bone formation are modified by immune system through a complex interaction involving T and B lymphocytes, dendritic cells, cytokines, and cell–cell interactions, which are in turn further modified by circulating

hormones [1]. The main risk factors for pathogenesis of osteoporosis are aging and estrogen deficiency. Estrogen (E2) deprivation leads to bone resorption by affecting inflammatory responses including cytokine and growth factor levels, T cell activation, and free radical production to heightened levels [2–4]. Among the inflammatory cytokines interleukin-1 and TNF-α are the most potent osteoclastogenic stimulators under E2 deprivation [4, 5]. Ovariectomy (Ovx) induced estrogen estrogen deficiency causes an expansion of the T cell pool in the bone marrow (BM) by increasing T cell activation, a phenomenon that results in increased T cell proliferation and lifespan [2, 6]. Ovx is also known to upregulate TNF-α production from T cells by increasing the number of TNF-α-producing cells. Immunophenotypical analyses of peripheral blood lymphocyte reveal that several subsets of T lymphocytes ($CD3^+$, $CD4^+$, and $CD8^+$) are increased in osteoporotic patients [7–9].

Studies have been carried out where phenotypic T cell profiles of elderly individuals indicated increase in $CD4^+$ T cells deficient in the expression of CD28, a membrane glycoprotein typically found on T cells that provides the requisite costimulatory signal for T cell activation [10]. Loss of CD28 is the most evident phenotypic change in cellular senescence [11, 12]. Although CD28 is constitutively expressed on all T cells, CD28null T cells are typically found in the aging immune system, in both $CD4^+$ and $CD8^+$ subsets [11, 13–16]. Also, in many chronic inflammatory conditions like rheumatoid arthritis (RA), persistent immune activation accelerates the premature senescence of T lymphocytes and thus increase in CD28 null T cells [11]. Additionally, there are reports that CD28 downregulation on T cells is mediated by TNF-α. TNF-α induces a quantitative decrease in CD28 expression at the protein and mRNA levels, represses the CD28 gene promoter, and induces the downregulation of site α- and β-binding proteins [17, 18].

To date, no study has been carried out to explain the relationship between $CD4^+$CD28null T cells and ovariectomy or osteoporosis in mice or humans. If recurrent or persistent inflammation contributes to osteoporosis, then $CD4^+$CD28null T cells may predict not only the severity of inflammation but also osteoporosis. We hypothesize that Ovx increases the production of $CD4^+$CD28null premature senescent cells, and these cells secrete inflammatory cytokines like TNF-α which in turn enhance bone resorption. Additionally, phytoestrogens confer substantial benefits to bone health without posing the risk of cancer associated with HRT [19, 20]. Recently, we have shown bone conserving effect of medicarpin (Med), a pterocarpan-type phytoalexin present in dietary legume, in Ovx mice, suggesting its E2-"like" effect on bone. Med was devoid of any uterotrophic effect [21]. In this study, we aim to determine the effect of estrogen deficiency on CD28 expression on T cells and the role of E2/Med in this phenomenon. We also evaluate the role of Med/E2 on proliferation of TNF-α-producing T cells and TNF-α-mediated CD28 downregulation on $CD4^+$ T cells.

Methods

Reagents and chemicals

Mouse lymphocytes from BM and spleen were cultured in complete RPMI-1640 medium (Wisent Inc., St-Bruno, QC, Canada) supplemented with 10% fetal bovine serum, penicillin (500 U/ml), and streptomycin (500 mg/ml). Trizol was purchased from GIBCO-BRL, Invitrogen Corp., Carlsbad, CA, and TNF-α and DCF-DA from Sigma-Aldrich (St. Louis, MO, USA). RPE-cy-7, PE-cy-5.5-conjugated anti-mouse CD4, PE-conjugated anti-mouse CD8, and FITC-conjugated anti-mouse CD28 antibodies were purchased from BD Biosciences (Mississauga, ON, CA). CD4 (L3T4), CD4 isolation kit, and CD28 microbeads were purchased from Miltenyi Biotech, Germany and TNF-α ELISA kit from Immunodiagnostic Systems Ltd., UK.

In vivo study

The study was conducted in accordance with current legislation on animal experiments (Institutional Animal Ethical Committee at Central Drug Research Institute). Adult Balb/c mice (9–10 weeks old) were taken for the study [22–25]. All mice were housed at 25°C, in 12-h light to 12-h dark cycles. Normal chow diet and water were provided ad libitum. Ten mice per group were taken for the study, and all mice in each group were assayed and included in the statistical analyses (n=10). The groups were as follows: sham-operated (ovary intact) mice, which served as the control group and were given vehicle (gum acacia in distilled water); Ovx+vehicle; Ovx+10.0 mg kg^{-1} day^{-1} Med [21]; and Ovx+0.01 mg kg^{-1} day^{-1} E2 [26, 27]. All treatments were given by oral gavage and continued for 6 weeks. At the completion of study, animals were autopsied. After autopsy, bones and spleens were collected in PBS. BM and spleen cells were flushed out and labelled with fluorescent antibodies (Abs) for analysis of $CD3^+$, $CD4^+$, and $CD4^+CD28^+$ cells. Total lymphocytes from the BM were isolated by using Hisep LSM 1084 (Himedia) by means of

density (1.084±0.0010 g/ml) gradient centrifugation technique which gave >90% pure lymphocyte [28, 29]. Pure $CD4^+$, $CD4^+CD28^+$, and $CD4^+$CD28null T cells were retrieved from the BM by positive and negative selection using microbeads-based isolation by MACS separator according to the manufacturer's protocol (EasySep Biotin Selection Kit; Stem Cell Technologies Inc., Vancouver, BC, Canada). These purified cells were then collected in Trizol for real-time PCR (qPCR) and ex vivo cultured for ROS generation study. Thymuses were collected for histological studies. Thymuses were weighed and were placed in 4% formaldehyde, dehydrated, sectioned, and stained with eosin and hematoxylin. The sections were coded and examined in a Leica microscope (Leica, Wetzlar, Germany) with the computer application Leica Qwin (Leica). Whole thymus, cortex, and medulla areas were measured, and the ratio between the cortex and total area (cortex area fraction) was calculated [30]. Serum was collected for ELISA. Serum TNF-α was measured in all the groups by using ELISA kit according to manufacturer's instructions.

Flow cytometry

Cells from BM and spleen were labelled with CD3, CD4, and CD28 antibodies (RPE-cy-7, PE-cy-5.5-conjugated anti-mouse CD4, APC-conjugated anti-mouse CD3, and FITC-conjugated anti-mouse CD28 Abs) to assess the number and percentage of $CD4^+$ and $CD4^+CD28^+$ cells. A parallel sample of cells was also incubated with Ig isotypic controls (BD Biosciences). Specificity of immunostaining was ascertained by the background fluorescence of cells incubated with Ig isotype controls. Fluorescence data from at least 10,000 cells were collected from each sample. Immunostaining was done as per manufacturer's instructions. In brief, single-cell suspension of the BM and spleen were prepared in PBS. Then cells were centrifuged at 500 rpm for 5 min, spun down, and pellet was suspended in 1 ml of PBS. Cells were counted using haemocytometer at a cell density of 10^6 cells/100μl PBS. Cells were immunostained with antibodies using standard protocols. After incubation cells were washed twice with PBS and transferred to FACS tubes for analysis. FACS Caliber and FACS Arya (BD Biosciences Mississauga, ON, Canada) were used to quantify the number and percentage of $CD4^+$ and $CD4^+CD28^+$ T cells in $CD3^+$ T cells in all the groups [18].

Total RNA isolation and quantitative real-time PCR

Total RNA was extracted from $CD4^+$, $CD4^+CD28^+$, and $CD4^+$CD28null cells harvested from bone marrow of mice from each group (n=3 mice/group) using Trizol (Invitrogen). Then cDNA was synthesized from 1 μg total RNA with the Revert AidTM H Minus first strand cDNA synthesis kit (Fermentas, USA). SYBR green chemistry was used for quantitative determination of the mRNAs for CD28, nucleolin, hnRNP-D0A, TNF-α, and a housekeeping gene, GAPDH, following an optimized protocol. The design of sense and antisense oligonucleotide primers was based on published cDNA sequences using the Universal ProbeLibrary (Roche Diagnostics, USA). For real-time PCR, cDNA was amplified with Light Cycler 480 (Roche Diagnostics Pvt. Ltd.).

The double-stranded DNA-specific dye SYBR Green I was incorporated into the PCR buffer provided in the Light Cycler 480 SYBER green I master (Roche Diagnostics Pvt. Ltd.) to allow for quantitative detection of the PCR product in a 20-μl reaction volume. The temperature profile of the reaction was 95°C for 5 min, 40 cycles of denaturation at 94°C for 2 min, annealing and extension at 62°C for 30 s and extension at 72°C for 30 s. GAPDH was used to normalize differences in RNA isolation, RNA degradation, and the efficiencies of the reverse transcription. The primer pair for CD28 was 5′-CAG AAT CCT CTG GAA CTT GAG G-3′ (sense) and 5′-CTT CCA GAC ATT CGG AGA CC-3′ (antisense), nucleolin 5′-CATGGTGAAGCTCG CAAAG-3′ (sense) and 5′-TCACTATCCTCTTC CACCTCCTT-3′ (antisense), hnRNP-D0A 5′-CAAGATC GACGCCAGTAAGA-3′ (sense) and 5′-GTGTCGTGGGGAGGAGTTT (antisense), TNF-α 5′-TCTTCTCATTCCTGCTTGTGG-3′(sense) 5′-GGTCTGGGCCATAGAACTGA-3′ (antisense), and that of GAPDH was 5′-GTC CTC TCC CAA GTC CAC ACA-3′ (sense) and 5′-CTG GTC TCA AGT CAG TGT ACA GGT AA-3′ (antisense).

In vitro culture of total lymphocytes for TNF-α-induced CD28 loss

For this experiment, lymphocytes were isolated by using Hisep LSM 1084 (Himedia) according to manufacturer's instructions. After isolation cells were seeded overnight in 48-well plates at 5×10^5 cells/well in 10% RPMI 1640 media. Cells were incubated with10^{-10} M of Med and 10^{-8} M E2 followed by incubation with TNF-α (10 ng/ml) for 24 h at 37°C. Cells were then incubated with FITC-conjugated anti-CD28 and APC-conjugated anti-CD3 antibody for 30 min in dark. After incubation cells were pelleted down and the supernatant was discarded. Cells were washed in PBS. After washing cells were transferred in FACS tubes and total $CD28^+$ cells were analyzed in $CD3^+$ T cells by FACS [31].

For inhibitor studies cells were pre-treated with ICI-182780 (Tocris Bioscience, USA) at 10^{-9} M concentration 30 min prior to Med/E2 and TNF-α treatment.

Ex vivo and in vitro culture of total lymphocytes for ROS generation study

For ex vivo studies, animals were autopsied and total lymphocytes were isolated from bone marrow of long bone (femur) of animals by density gradient centrifugation. Pure $CD4^+$ cells were isolated by MACS using standard protocols according to manufacturer's instructions. Purified cells were seeded in 48-well plates at a density of 3×10^5 cells/well for 24 h in 1% RPMI-1640 medium. For ROS measurement, cells were incubated with 2′,7′-dichlorofluorescin diacetate DCF-DA (10 μg/ml concentration) for 30 min. After incubation cells were pelleted and supernatant was discarded. Cells were washed in PBS. After washing cells were transferred in FACS tubes and analyzed by FACS for ROS generation [31].

For in vitro ROS generation, lymphocytes were isolated by using Hisep LSM 1084 (Himedia) according to manufacturer's instructions. After isolation cells were seeded in 48-well plates at 3×10^5 cells/well in 10% RPMI 1640 media. After 24 h cells were incubated with10^{-10} M of Med in 1% FCS containing RPMI 1640. This was followed by incubation with TNF-α (10 ng/ml) for 24 h at 37°C. For ROS measurement, cells were incubated with 10 μg/ml concentration of DCF-DA for 30 min. After incubation cells were pelleted down and supernatant was discarded. Cells were washed in PBS. After washing cells were transferred in FACS tubes and analyzed by FACS for ROS generation [31].

Statistical analysis

Data are expressed as mean ± SEM. The data obtained in experiments with multiple treatments were subjected to one-way ANOVA followed by Newman–Keuls test of significance using Prism version 3.0 software. Student's *t* test was used to study statistical significance in experiments with only two treatments. Qualitative observations have been represented following assessments made by three individuals blinded to the experimental designs.

Results

Effect of E2/Med on Ovx-induced increase in thymus weight

Ovx mice exhibited deterioration of trabecular microarchitecture compared with sham, and Ovx mice treated with Med (10.0 mg kg^{-1} day^{-1}) or E2 (0.01 mg kg^{-1} day^{-1}) for 6 weeks protected against Ovx-induced loss of trabecular microarchitecture (supplemental Table 1). At these osteoprotective doses, Med/E2 were given to Ovx mice for 6 weeks and it was observed that treatment of Med/E2 results in thymus involution (supplementary Fig. 1a). In addition, Ovx mice displayed an increased cortex area fraction compared to those of sham controls, whereas Ovx mice treated with either Med or E2 had thymus cortex fraction area comparable to sham group (supplementary Fig. 1b).

Effect of E2/Med on Ovx-induced ROS production, TNF-α expression in BM T cells, and circulating TNF-α levels

Ovariectomy causes an accumulation of reactive oxygen species in the BM T cells, which leads to increased production of TNF-α by activated T cells through upregulation of the costimulatory molecule CD80 on dendritic cells [32]. Our data show that Ovx led to significant increase in ROS production compared to sham group (Fig. 1a). However, treatment with Med and E2 led to significant reduction in ROS production (Fig. 1a). E2 deficiency is known to increase circulating TNF-α levels [33]. Our data show that Ovx mice had significantly higher circulating levels of TNF-α compared with sham group. Ovx mice treated with E2 or Med had reduced TNF-α levels that were comparable to that of sham group (Fig. 1b). Further, mice Ovx for 6 weeks had ~2.0-fold higher TNF-α mRNA levels in BM T ($CD4^+$) cells compared with sham (Fig. 1c). E2 or Med treatment of Ovx mice significantly reduced TNF-α mRNA levels compared to Ovx control group (Fig. 1c).

Effects of E2/Med on BM and spleen T cell subsets

Ovx is known to increase the proliferation of proinflammatory T helper cells including $CD4^+$ and $CD8^+$ T cells [33]. Mice Ovx for 6 weeks have higher frequency of $CD4^+$ T cells in BM compared with sham group (Fig. 2a). Treatment of Ovx mice with either E2 or Med resulted in ~50% reduction in the frequency of $CD4^+$ T cells in BM compared with Ovx group (Fig. 2a). Effect of Ovx on the secondary lymphoid organ (spleen) was also studied, and we found that Med/E2 decrease the Ovx-induced expansion of $CD4^+$ cells in spleen (Fig. 2b).

Effect of E2/Med on Ovx-induced alteration of CD28 expression in BM and spleen T cells

CD28 is the surface glycoprotein constitutively expressed on all $CD4^+$ T cells and loss of CD28 expression is an indicator of T cell senescence [11, 13]. $CD4^+CD28^+$ T cell numbers in BM of sham group mice were significantly higher than Ovx

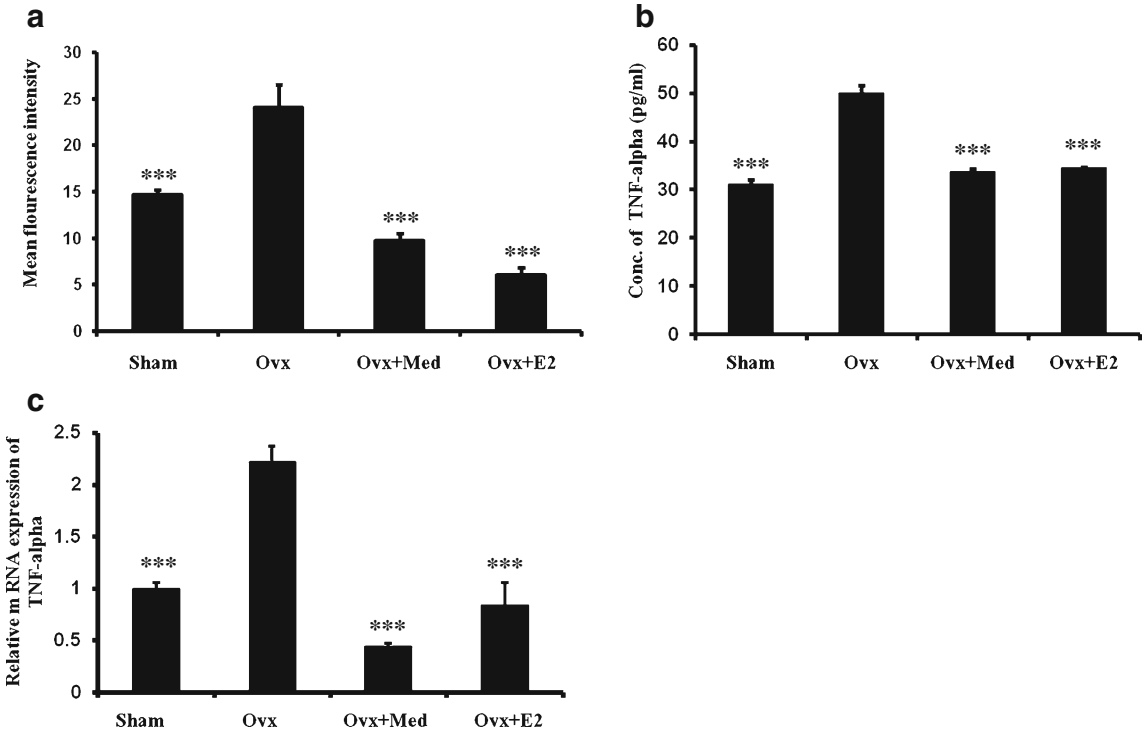

Fig. 1 Med or E2 treatment inhibits Ovx-induced oxidative stress and T cell TNF production. **a** Cellular ROS measurement was performed by incubating T cells with DCF-DA followed by FACS analysis. **b** Circulating TNF-α levels were measured in various groups by ELISA. **c** TNF-α mRNA levels in the BM $CD4^+$ T cells were measured in various groups by qPCR. $N=10$ mice/group; data are presented as mean ± SEM; $^{***}P<0.001$ compared with Ovx+vehicle group

group (11.57%±0.23 in sham vs. 1.5%±0.08 in Ovx, $P<0.001$) (Fig. 3a). Ovx mice treated with either Med or E2 had significantly higher frequency of $CD4^+CD28^+$ T cells in BM compared with Ovx group (6.5%±0.07 in Ovx+Med group, $P<0.001$; 5.8%±0.39 in Ovx+E2 group, $P<0.01$; Fig. 3a). The same is denoted in absolute numbers in Fig. 3b.

A similar pattern was observed with $CD4^+CD28^+$ T cells in spleen, as the number of $CD4^+CD28^+$ T cells in spleen were significantly reduced in Ovx group (135±18 compared to sham 2,670±128, $P<0.001$; Fig. 3c). Supplementation of Med or E2 treatment to Ovx mice increased the frequency of $CD4^+CD28^+$ T cells compared with vehicle-treated Ovx group (2,020±190, $P<0.001$ in Ovx+Med group and 1,850 ±143, $P<0.001$ in Ovx+E2 group; Fig. 3c).

As we had shown above that $CD4^+$ T cells isolated from Ovx mice produced more TNF-α and Ovx mice exhibited

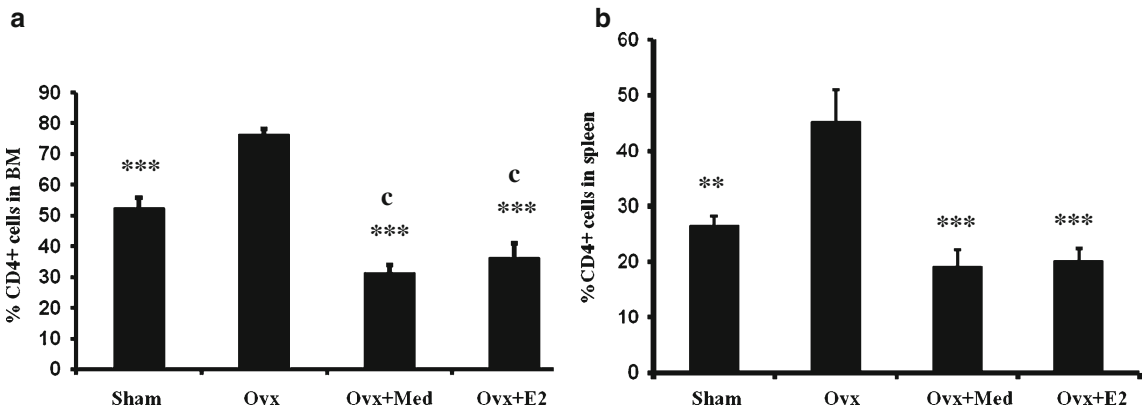

Fig. 2 Med or E2 treatment significantly decreased Ovx-induced increases in effector T cell subsets in the BM and spleen. **a** $CD4^+$ T cells in BM and **b** $CD4^+$ T cells in spleen were quantified by flow cytometry as described in "Methods." $N=10$ mice/group; data are presented as mean ± SEM; $^{**}P<0.01$, $^{***}P<0.001$ compared with Ovx+vehicle group and $^{c}P<0.001$ compared with sham group

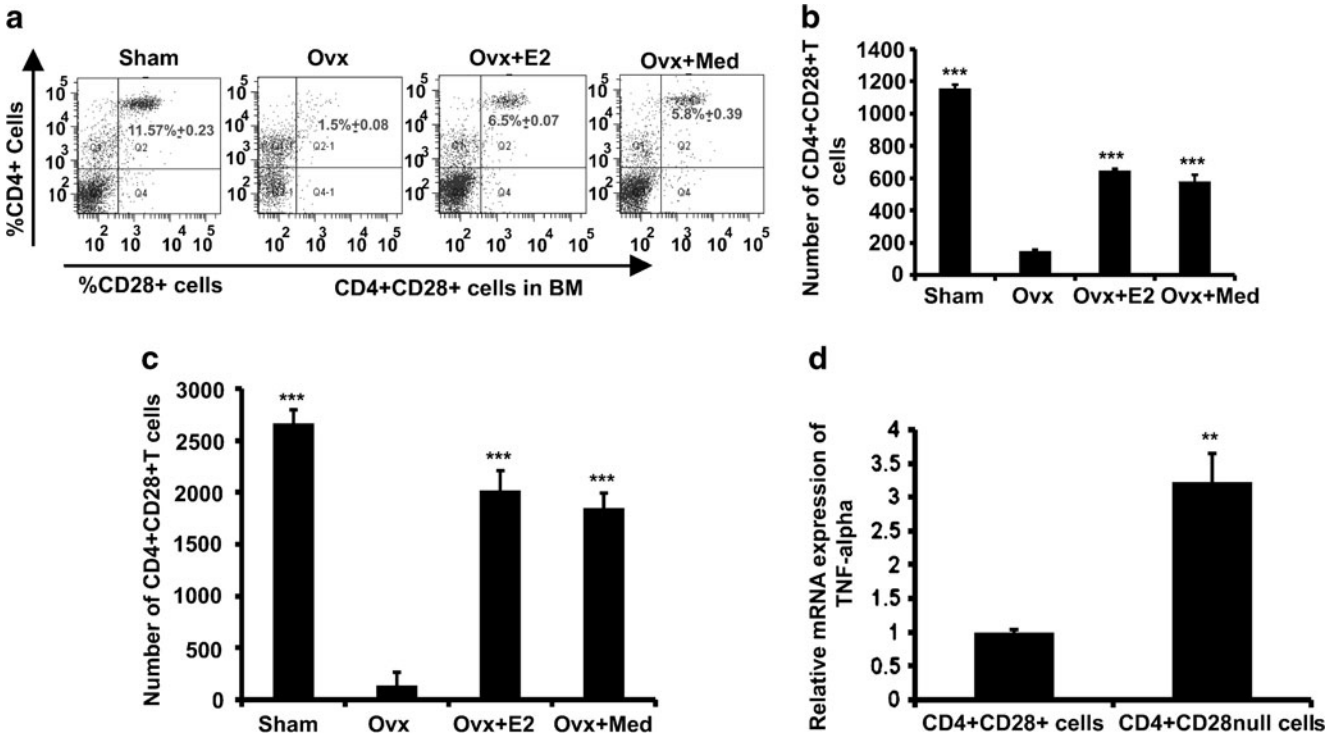

Fig. 3 Med or E2 treatment prevented Ovx-induced loss of CD28 on the BM as well as in spleen $CD4^+$ T cells. **a** Representative image **b** $CD4^+CD28^+$ T cells in BM, and **c** $CD4^+CD28^+$ T cells in the spleen of sham, Ovx, Ovx+Med, and Ovx+E2 were measured by flow cytometry as described in "Methods." **d** mRNA levels of TNF-α was measured by qPCR in $CD4^+CD28^+$ and $CD4^+CD28$null T cells. $N=10$ mice/group; data are presented as mean ± SEM; $^{**}P<0.01$ and $^{***}P<0.001$ compared with Ovx+vehicle group

greater loss of CD28 expression on $CD4^+$ T cells, it was necessary to study if increased TNF-α production is mainly contributed by $CD4^+CD28$null T cells. It was observed that TNF-α mRNA levels were several folds higher in $CD4^+CD28$null T cells compared to $CD4^+CD28^+$ T cells isolated from BM (Fig. 3d).

Effect of E2/Med in the expression of nucleolin and hnRNP-D0A in BM T cells

Nucleolin and hnRNP-D0A are proteins responsible for CD28 expression on T cells [13, 14]. Our data show that $CD4^+$ T cells in BM of Ovx mice had significantly lower mRNA levels of CD28, nucleolin, and hnRNP-D0A compared with sham group (Fig. 4a–c). Med/E2 treatment of Ovx mice resulted in significantly increased expression of CD28, nucleolin, and hnRNP-D0A mRNA levels comparable to that of sham (Fig. 4a–c).

Effects of E2/Med on TNF-α-mediated CD28 downregulation in BM T cells in vitro

We next studied the effect of E2/Med on TNF-α-induced suppression of CD28 levels in BM T cells, and whether Med acts via estrogen receptors. T cells were exposed to various treatments as shown in (Fig. 5a) and $CD28^+$ T cells were quantified by flow cytometry. Exogenous TNF-α significantly reduced the frequency of $CD28^+$ T cells, while either E2 or Med attenuated TNF-α-induced reduction of $CD28^+$ T cells (Fig. 5a). Presence of an antiestrogen, ICI-182,780, blunted the ability of Med to attenuate TNF-α-induced reduction of $CD28^+$ T cells.

Increased oxidative stress caused by increased ROS generation is known to induce cellular senescence. We next studied whether E2/Med treatment inhibited TNF-α-induced ROS generation in BM T cells and if treatment with estrogen receptor antagonist ICI 182,780 inhibits TNF-α-induced ROS generation. Our data show that TNF-α treatment resulted in significantly higher ROS accumulation [assessed by DCF-DA-positive lymphocytes using FACS in BM T cells compared with sham group (Fig. 5b)]. Treatment of BM T cells with either E2 or Med prior to TNF-α significantly reduced ROS levels compared with only TNF-α-treated cells (Fig. 5b). Presence of ICI 182, 780 blunted the ability of Med and E2 to inhibit TNF-α-induced ROS generation (Fig. 5b).

Discussion

There is now strong evidence to suggest that production of proinflammatory cytokines in response to estrogen with-

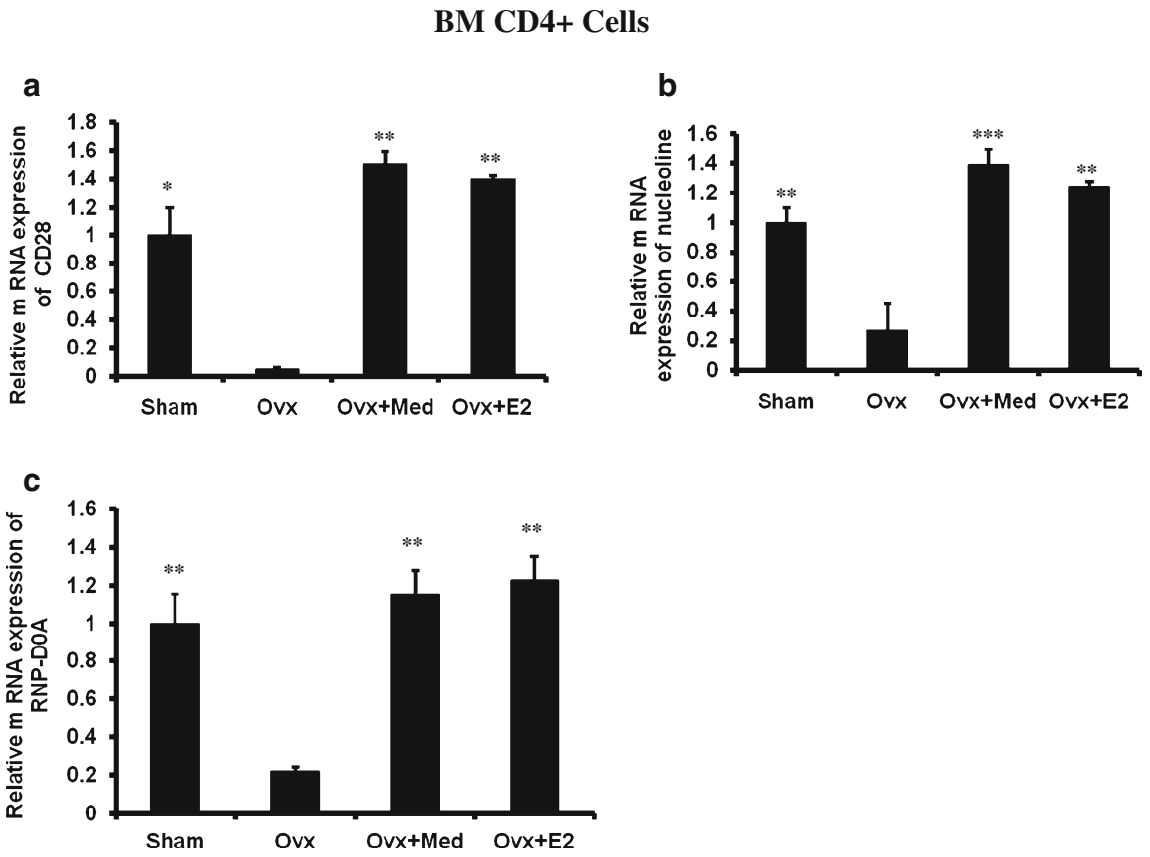

Fig. 4 Med or E2 treatment increased CD28, nucleolin, and hnRNP-D0A mRNA levels assessed by qPCR in the BM T cells of Ovx mice. mRNA levels of CD28, nucleolin, and hnRNP-D0A in $CD4^+$ T cells (**a**–**c**). All data are the mean ± SD of three independent experiments; $n=3$ mice/group; $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with Ovx+vehicle group

drawal at menopause is responsible for the characteristic loss of bone density through their effect on osteoclast activity [34]. Ovx results in an expansion of TNF-α-producing T cells, and TNF-α is well established for its role in bone resorption. Besides, TNF-α also mediates the downregulation of CD28, the biological marker of immunosenescence whose expression goes down in aging population and also in inflammatory conditions like rheumatoid arthritis. These observations led us to explore the relationship between Ovx-induced estrogen deficiency and CD28 loss on T cells as menopause onsets the reproductive aging and there is increased frequency of inflammatory diseases at menopause [1].

We have demonstrated that Med (10.0 mg kg^{-1} day^{-1} dose) or E2 (0.01 mg kg^{-1} day^{-1} dose) given for 6 weeks to Ovx mice protected against Ovx-induced loss of trabecular microarchitecture (supplemental Table 1). At their respective osteoprotective doses both Med and E2 decreased population of BM and spleen $CD4^+$ T cells and prevented the Ovx-induced loss of CD28 expression on BM and spleen $CD4^+$ T cells. A scheme summarizing the results of the present report and findings from earlier studies by others is provided in Fig. 6.

Ovx is known to increase thymic weight and cellularity, and E2 administration causes thymic atrophy [30]. Accordingly, Ovx increased thymic mass and cortex, resulting in increased positive selection of T cells [30, 35, 36] which enables them to mature and exit into the periphery. Thus, E2 deficiency contributes to increased thymic T cell output that could lead to bone loss. Our data demonstrate that Med, similar to E2, caused thymic involution and reduced cortical area in Ovx mice (supplementary Fig. 1a, b), suggesting that Med could reverse Ovx-induced impact of thymus on bone loss.

Reports have suggested that stimulation of TNF production by osteoclasts or BM cells is the mechanism by which ROS cause bone loss [37]. Studies by Grassi et al. [32] have shown that key effects of Ovx, the upregulation of Ag-dependent activation of T cells and the resulting T cell production of TNF, are mediated by ROS and abolished by treatment with antioxidants. Our data show that while Ovx led to significant increase in ROS production, treatment with Med and E2 inhibited Ovx-induced ROS generation (Fig. 1a). We further observed that the circulating levels of TNF-α were ~60% higher in Ovx mice compared with sham, which accords previous reports [38], and E2/Med

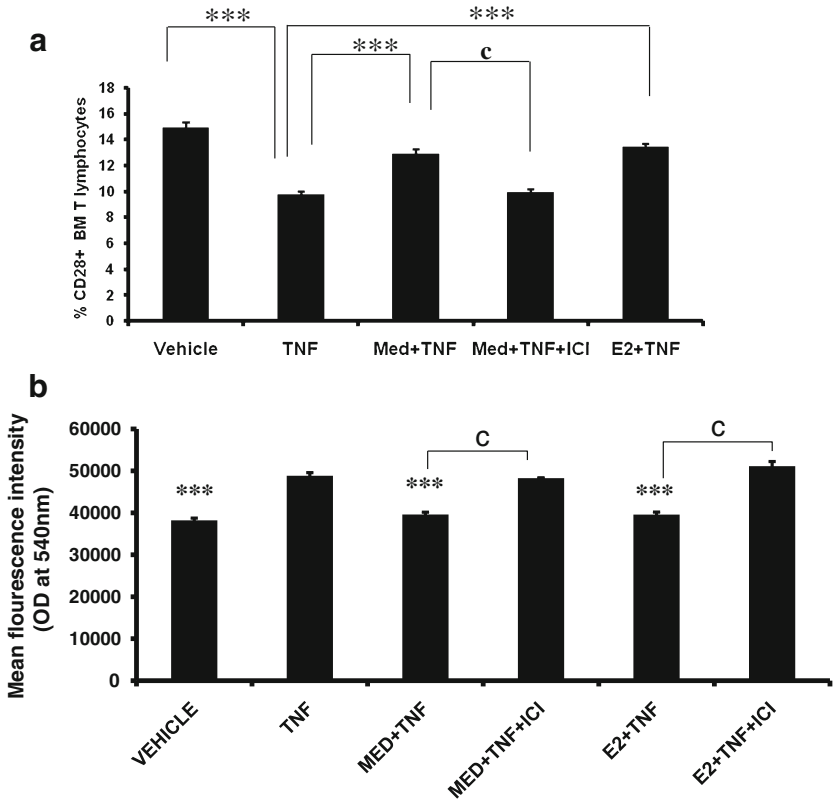

Fig. 5 Med or E2 abrogated TNF-α-mediated CD28 downregulation in the BM lymphocytes. **a** BM lymphocytes (5×10^5 cells/well) were seeded in 24-well plates. For ICI 182,780 (ICI) treatment group, the cells were pre-treated with ICI for 30 min. Various treatments as shown were given to BM cells for 24 h [E2—10^{-9} M, Med—10^{-10} M, TNF-α—10.0 ng/ml]. At the end of incubation, cells were stained with antibodies against CD3, CD4, and CD28 and subjected to flow cytometry as described in "Methods." Data are presented as mean ± SEM from three independent experiments; ***$P<0.001$ compared with TNF-α-treated cells and $^cP<0.001$ compared between Med+TNF and Med+TNF+ICI-treated cells. **b** Med or E2 abrogated TNF-α-induced ROS generation. BM lymphocytes (3×10^5 cells/well) were seeded in 24-well plates. Cellular ROS measurement was performed by incubating T cells with DCF-DA followed by FACS analysis after various treatments given for 30 min. Data are presented as mean ± SEM from three independent experiments; ***$P<0.001$ compared with vehicle-untreated cells and $^cP<0.001$ compared between Med+TNF vs. Med+TNF+ICI-treated cells; E2+TNF vs. E2+TNF+ICI-treated cells

treatment of Ovx mice totally obliterated Ovx-induced increase in circulating TNF-α levels (Fig. 1b). Moreover, TNF-α mRNA levels were elevated by >2.0-fold in BM $CD4^+$ T cells of Ovx mice compared with sham, and E2/Med treatment completely abolished Ovx-induced upregulation of TNF-α mRNA levels (Fig. 1c). This observation is different from that obtained by Roggia et al. [33] where they showed that there is an increase in TNF-producing T cells; however, there is no alteration in the amount of TNF-α alpha produced by each cell. We speculate that Ovx results in increased frequency of premature senescent T cells which may lead to increased production of TNF-α. Together, our data suggest that E2/Med alleviates systemic and local (via BM T cells) rises in TNF-α caused by E2 deprivation and in the process prevents bone wasting.

Similar to that in the thymus, Ovx causes an expansion of the T cell pool in BM (Fig. 2a) and spleen (Fig. 2b) by increasing T cell proliferation and lifespan [33, 39]. We observed that E2 as well as Med reduced the proportion of Ovx-induced increases in BM (Fig. 2a) and spleen $CD4^+$ T cells (Fig. 2b) to levels that were more than the sham (ovary intact) mice. It is possible that administered doses of E2 and Med used in our study exerted physiological effect on ERs of T cells.

According to inflammation theory of aging, T cells deficient in CD28 expression become prematurely senescent and acquire the ability to produce proinflammatory cytokines. In mammals, E2 deficiency is an aging process, signalling the end of reproductive life [40]. However, the role of E2 in CD28 expression is not known. A striking finding of this work is that Ovx resulted in loss of CD28 expression in BM and spleen $CD4^+$ T cells (Fig. 3). Further, supplementing Ovx mice with E2 completely restored the $CD28^+$ T cells to sham level which confirmed the role of E2 in the regulation of CD28 expression. However, Med was ~2.0-fold more effective than E2 in increasing BM $CD28^+$ T cells (Fig. 3a, b) where as Med is nearly equally effective in splenic T cells (Fig. 3c). These data suggest that Ovx triggers T cell

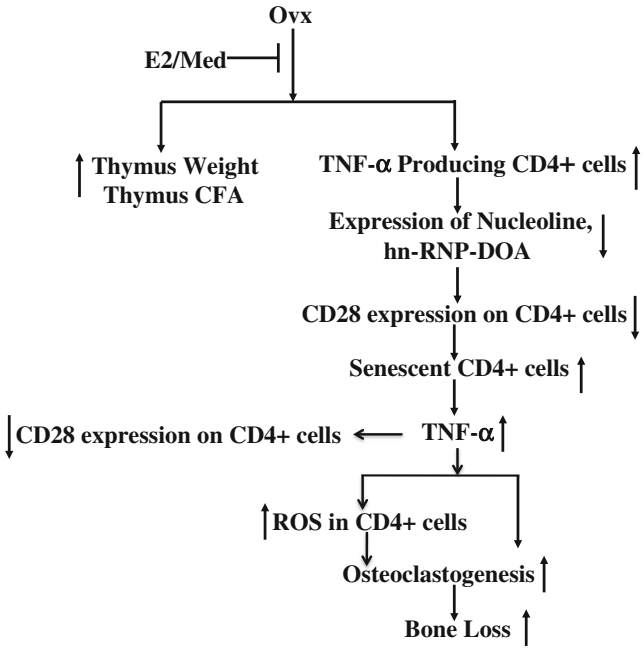

Fig. 6 Schematic diagram showing various Ovx-activated immunological alterations contributing to bone loss. (1) Ovx (E2 deficiency) increased thymus weight and cortical fraction area, (2) Ovx increased TNF-α-producing T cells in the BM, (3) Ovx reduced expression of nucleoline and hnRNP-D0A in the BM T cells, (4) Ovx downregulates the expression of CD28 on T cells, and (5) Ovx results in the production of more senescent T cell population that produce more TNF-α thus leading to decreased CD28 expression on $CD4^+$ T cells and enhanced osteoclastogenesis

senescence in the BM as well as in spleen by reducing CD28 expression, and E2 or Med counteract this loss. We found that both Med and E2 reversed the effects induced by Ovx in primary and secondary lymphoid organs. A similar observation was also made in $CD8^+$ T cells where CD28 expression was significantly low in BM and spleen of Ovx mice compared to sham. Supplementing Ovx mice with E2 or Med restored the percentage of $CD28^+$ T cells to sham levels (supplementary Fig. 2). Also, we show that increased TNF-α levels observed in $CD4^+$ T cells of the Ovx mice is mainly due to increased frequency of $CD4^+CD28$null T cells in the Ovx mice (Fig. 3d). To ascertain if increased TNF-α production by $CD4^+CD28$null T cells contributes to enhanced osteoclastogenesis, $CD4^+CD28$null T cells were co-cultured with bone marrow cells. We found that TRAP mRNA expression was significantly more in $CD4^+CD28$null T cells co-cultured with BM cells compared to control and treatment with Med and E2 decreased TRAP transcript levels in the co-culture (data not shown). Moreover, TNF-α levels when determined by ELISA in conditioned media of $CD4^+CD28$null T cells co-cultured with BM cells were higher and were decreased after Med and E2 treatment (data not shown). However, it is a preliminary observation and needs to be further validated.

We further demonstrated the mechanism by which Ovx caused reduction of CD28 expression in BM $CD4^+$ T cells and E2/Med enhanced CD28 expression. CD28 mRNA levels in BM T cells were significantly reduced in Ovx mice compared with sham. Med/E2 treatment of Ovx mice caused significantly greater increase in the levels of CD28 mRNA than Ovx group (Fig. 4a). Basal transcription of CD28 gene is regulated by nucleolin-hnRNP-D0A-site α-complex formation in the CD28 promoter [13, 14]. We found that mRNA levels of both nucleolin and hnRNP-D0A were significantly reduced in $CD4^+$ T cells in Ovx mice compared with sham group (Fig. 4b ,c). These data suggest that the loss of CD28 expression under E2 deficiency is caused by the reduction in the mRNA levels of nucleolin and hnRNP-D0A in $CD4^+$ T cells. Treatment of Ovx mice with Med/E2 significantly increased nucleolin and hnRNP-D0 mRNA levels in $CD4^+$ T cell subsets. However, Med-treated Ovx mice exhibited higher mRNA expression of nucleolin than Ovx +E2 group which correlates well with the FACS data, where Med is showing slightly greater effect on CD28 expression than E2 in Ovx mice (Fig. 4).

There is evidence that TNF-α downregulates CD28 expression on T cells leading to the emergence of senescent cell population [17]. Previous studies strongly indicate that among the consequences of chronic exposure of T cells to TNF-α is the repression of CD28 transcription that may lead to CD28null phenotype [17]. We show that treatment of exogenous TNF-α to BM T cells resulted in reduced numbers of $CD28^+$ T cells compared with vehicle-treated T cells, and the presence of either Med or E2 with TNF-α significantly reversed TNF-α-induced loss of CD28 on BM T cells. Further, presence of antiestrogen ICI-182,780 blocked the ability of Med to reverse TNF-α-induced loss of CD28 on T cells, suggesting that Med acts via ER in T cells (Fig. 5a). Thus, we hypothesize that E2/Med act via the ERs to abrogate TNF-α-induced loss of CD28 expression in T cells, a phenomenon that will contribute to preventing production in senescent population of T cells that in turn are responsible for increased TNF-α production under E2 deficiency.

Additionally, we demonstrate that Med, as effectively as E2, neutralized TNF-α-induced accumulation of ROS by BM T cells in vitro (Fig. 5b). Since increased ROS generation and oxidants contribute to the induction of T cell senescence by downregulating the expression of CD28 [41], our data suggest that E2/Med reversed Ovx-induced loss of CD28 in T cells and diminish ROS production (Fig. 5b). Studies by Somjen et al. [42] have shown that E2 and their agonists stimulate ROS production in human osteosarcoma cell line SaOS-2 and this effect is blocked in presence of antiestrogen. Thus, we studied if pretreatment with ICI 182,780 blunts the ability of E2 and Med to inhibit

TNF-α-induced ROS generation. We found that while Med and E2 inhibited TNF-α-induced ROS generation, pretreatment with ICI 182,780 blunts this effect. However, the involvement of ER-dependent mechanism may not be fully confirmed because oxidative stress, in turn, may result in the release of oxidizing fatty acids which might unfavorably affect overall T cells.

Conclusion

To our knowledge this is the first report to determine the mechanism of CD28 loss on T cells as a result of ovariectomy. Based on our findings, we propose that E2 or Med prevent premature T cell senescence and bone loss via (a) increasing mRNA levels of nucleolin, hnRNP-D0A, and CD28 in BM T cells; (b) antagonizing TNF-α-induced loss of CD28 expression in an estrogen dependent manner; and (c) abrogating TNF-α-induced ROS production. To conclude, one of the mechanisms by which Med/E2 may be alleviating Ovx-induced bone loss is by delaying T cell senescence and enhancing CD28 expression.

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Conflicts of interest None.

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