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Estrogen deficiency inducing shifted cytokines profile in bone marrow stromal cells inhibits Treg cells function in OVX mice

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Abstract

The study aims to reveal the effect of estrogen deficiency on Treg cells population in bone marrow in the development of osteoclastogenis with comparing the differences about Treg cells phenotypes and cytokines related with the homeostasis and functions maintenance of Treg cells in bone marrow in OVX mice and health control group. Wide-type C57BL/6 mice were operated OVX to mimic estrogen deficiency in PMO women. Treg cells population and their surface markers expressions were detected by flow cytometry. Cytokines profiles in bone marrow with examined by real-time PCR and ELISA analysis. Signal pathways and key modulators responsible to inflammatory cytokines expressions in bone marrow stromal cells were also detected with using western blotting. Estrogen deficiency in OVX mice decreased Treg cells and their functions, and cytokines profile in bone marrow were found shifted in bone marrow when compared with control group. Consistent to these observations, signal pathways in bone marrow stromal cells were reported altered by estrogen deficiency in our model. Estrogen deficiency effects Treg cells population and their functions in OVX mice with altering cytokines profile in bone marrow stromal cells.

Key words: Treg cells, bone marrow stromal cells, OVX mice, estrogen.

Introduction

Postmenopausal osteoporosis (PMO) is the most common metabolic bone disease in women after menopausal. Previous studies suggested the most frequency cause of bone lose in PMO women was decreased production of estrogen, the sex steroid that is required in the differentiation, apoptosis, and functions maintenance of osteoclast cells (OC) (1-3). Moreover, the regulatory of osteoclastogenic cytokines such as tumor necrosis factor alpha (TNF-alpha), IL-7, RANKL and M-CSF (4-8) expressions in bone marrow is also under the control of estrogen level. However, as research continues, the fundamental roles of T cells and their productions in the progression of PMO came into researchers' view. It was reported that T cells deficient nude mice was protected against bone loss in ovariectomized mice (OVX mice) model (9-11). And activated T cells population was confirmed to be the major source of TNF-alpha in the mechanism of osteoprorsis (10, 11). Furthermore, the over-activation of bone marrow stromal cells (SC) and disregulation of osteoblast (OB) and osteoclast cells formation were dependent on T cells receptor CD40l (12). Therefore, T cells were recognized as the key regulator in the interaction between bone metabolism and immune system.

Regulatory T cells (Treg) expressing transcription factor FoxP3 are dispensable in the cases of autoimmune and inflammatory diseases. In the etiology of PMO, the balance between osteoblast and osteoclast cells were regulated by Treg cells with producing anti-inflammatory cytokines IL-10 and TGF-beta (13, 14). Furthermore, Treg cells were capable to inhibit osteoclastogenic cytokines secretions in activated stromal cells via surface receptors such as CTLA-4 and PD-L (13, 15) and such suppressive functions of Treg cells were associated with estrogen level as *in vitro* data shown (16). However, little information is available regarding the intrinsic regulatory mechanisms of Treg cells in osteoclastogenesis *in vivo*. On the other hand, the cross-talk and feedback of activated T cells and bone marrow stromal cells were widely reported especially in the studies of TNF-alpha signals (10) and CD40-CD40L pathways (12), which suggested the functions of T cells lineages were strict regulation and control by local inflammatory cytokines.

To analyze the effects of estrogen on Treg population, we created the OVX mice model to mimic estrogen deficiency in PMO women and found that a significant reduction of Treg population. According to these, abundant TNF-alpha and decreased IL-6 expression were detect in bone marrow stromal cells. And further studies *in vitro* suggested shifted cytokines profile was associated with estrogen signal. Considering the roles of bone marrow stromal cells in osteoclastogenesis, we propose Treg cells population and their functions are regulated by bone marrow stromal cells, and such mechanisms are partial dependent on estrogen secretion.

Materials and methods

Mice and operation

Specific pathogen-free (SPF) wild-type C57BL/6 mie were purchased from Shanghai SLAC laboratory animal center (Shanghai) and operated according to institutional animal ethics guidelines. Sexually mature female mice were operated with either OVX or sham surgery at 12 wks of age and then killed 2 or 4 weeks after operation, and bone marrow cells were harvested for further analysis.

Reagents and antibodies

Phenol red–free RPMI 1640 was purchased from Gibco. Charcoal-stripped fetal calf serum (FCS) was purchased from Hyclone. Antimouse Allophycocyanin(APC)-conjugated CD3e, APC-conjugated Foxp3, phycoerythrin(PE)-conjugated CD11b, PE-conjugated ICOS, PE-conjugated PD-1 and PE-conjugated CTLA-4 were purchased from eBioscience. Fluorescein isothiocyanate (FITC)-conjugated B220 and FITC-conjugated CD3e was from biolegend. Mouse IL-6 and a tumor necrosis factor alpha (TNF-alpha-alpha) enzyme-linked immunosorbent assay (ELISA) kit was purchased from Dakewe. CpG1668 with the sequence 5-TCC-ATG–ACG-TTC-CTG-ATGCT-3 was synthesized by Invitrogen. Anti-mouse phospho-ikba, phospho-p38, phospho-erk, and GAPDH for Western blot analysis were purchased from Cell Signaling Technology. Transcriptional factor Foxp3 ICS staining kit was purchased from eBioscience.

Flow cytometric analysis

All the cells were washed twice in FACS medium phosphate buffered saline (PBS) containing 2% FCS and 0.1% NaN3. Then the cells were incubated for 30 minutes at 4°C with antibodies according to the standard procedure. An isotype control was used for each antibody. Fluorescence was measured using a FACSCalibur (Becton Dickinson), and data were analyzed using CellQuest software (Becton Dickinson).

Real-Time RT-PCR and Primers

The expression levels of mRNA of bone marrow stromal cells were quantified by realtime RT-PCR as previously described (10). All the primers used were designed by Primer Express Express Software v2.0 (PE Biosystems). Changes in relative gene expression between sham operated and ovx groups were calculated using the 2−ΔΔCT method with normalization to GAPDH expression levels.

Western blot analysis

Cells were lysed in lysis buffer with protease and phosphatase inhibitor. After centrifugation, cell lysates were subjected to 10% SDS-PAGE and transferred onto PVDF membranes (Roche). The membranes were blocked for 1 hour in TBST buffer containing 5% nonfat dry milk, and then the membranes were incubated with first antibodies at 4°C overnight and secondary antibodies for 30 minutes and then detected by Immobilon Western Chemiluminescent HRP Substrate (Millipore), and images were obtained using the FluorChem FC2 System (Alpha Innotech).

Statistical analysis

All results are expressed as mean±standard deviation (SD). All data were analyzed using the SPSS database and one-way ANOVA analysis, and P values less than 0.05 were considered to represent statistical significance.

Results

Estrogen deficiency suppresses bone marrow Treg cells functions in OVX mice

Ovariectomized (OVX) mice is a common animal model to mimic estrogen deficiency in the studies of bone loss in PMO women. In our work, wild-type mice with appropriate age were operated with OVX or sham operation and then euthanized 2 weeks after the operation to investigate the effects of estrogen on Treg

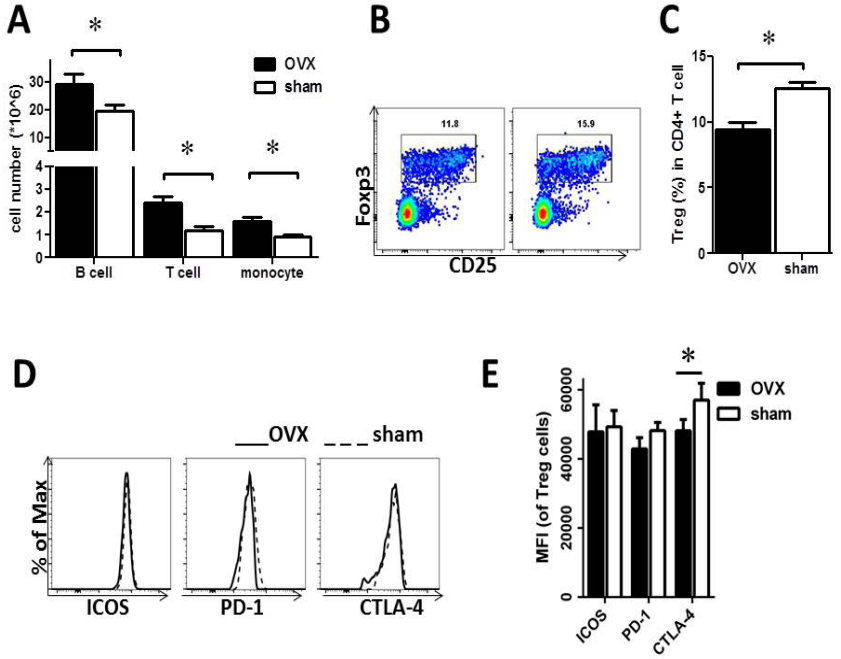

Figure 1. Estrogen deficiency in OVX mice suppresses Treg cells population and their functions. (A). lymphocytes count in bone marrow from OVX mice and sham group. B cells, T cells and monocytes were stained with either B220, CD3e or CD11b. (n=6). (B, C). Treg cell population in BM cells from OVX mice and sham group. Cells were gated on CD3e+ CD4+ population. (n=6, *, $P< 0.01$) (D, E) Median fluorescence intensities (MFI) of ICOS, PD-1 and CTLA-4 expression levels on Treg population in BM cells from OVX mice and sham group. Cells were gated on CD3e+ CD4+ Foxp3+ population (n=6, *, $P< 0.05$).

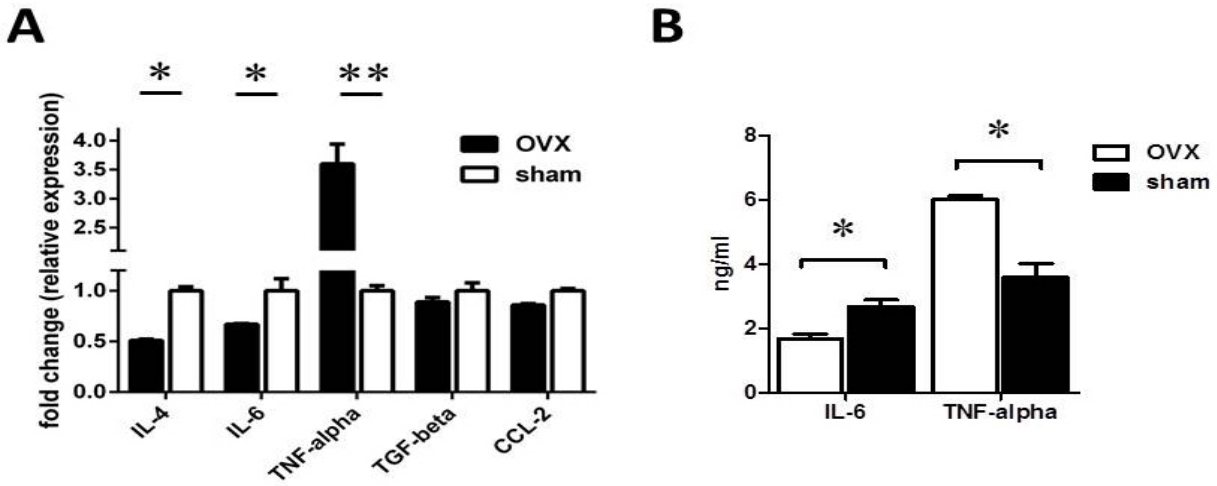

Figure 2. Estrogen deficiency in OVX mice alters cytokines profile in bone marrow. (A). QRT-PCR analysis of cytokines expression levels in bone marrow from OVX mice and sham group. (n=6) (B). ELISA analysis of inflammatory ctyokines IL-6 and TNF-alpha secretion levels in bone marrow (n=6, *, $P< 0.05$, **, $P< 0.01$).

cells population in bone marrow with using flow cytometry. As our data show, OVX operation increased the number of several immune cells groups such as CD3e+ T cells, B220+ B cells and CD11b+ myeloid cells by about 1.5~2 fold in OVX mice and sham group (Fig. 1a). However, the fraction of Treg cells who expressed transcriptional factor Foxp3 in bone marrow were found decreased in OVX mice (Fig. 1b,c). Notable, accumulation of TNF-alpha producing T cells in the bone marrow of OVX mice was widely reported and the proliferation of such activated T cells were contribute to the reduction of Treg cells population, we also examined Treg cells surface markers which responsible to their suppressive functions including programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and inducible T-cell co-stimulator (ICOS) in vivo. Treg cells in bone marrow were enhance with using magnetic-activated cell sorting (MACS) positive selection with CD3e and CD4 double positive and gated on Foxp3 expression with flow cytometry, and significant reductions of PD-1 and CTLA-4 expression levels but not ICOS were found in OVX mice compared with sham group (Fig. 1d.e). Thus, estrogen secretion maybe required in the maintenance of Treg cells functions in bone marrow in OVX mice model.

Estrogen deficiency shifts cytokines profile in bone marrow

Estrogen deficiency in OVX lead to a wide range of cellular responses including several signal transduction pathways involved in cytokines expression in bone marrow cells lineages. To investigate the alteration of bone marrow micro-environment, we collected bone marrow from OVX mice and the sham group, and determined the expression of cytokines which were either involved in the maintenance of Treg cells functions or related with bone loss such as TNF-alpha and IL-4. And we found a significant alterations of TNF-alpha and IL-4 expression as reported (7, 9) and down-regulated IL-6 expression *via* real-time PCR (Fig. 2a). Considering the key role of IL-6 in the differentiation of Treg cells both *in vitro* and *in vivo*, such unbalanced IL-6/TNF-alpha axis maybe partly contribute to the down-regulated functions in Treg cells population.

Estrogen deficiency alters cytokines expression in bone marrow stromal cells via promotes IRF family activation

The regulatory of cytokines expression in bone marrow is complex for the reasons that various bone marrow cells lineages including monocytes, memory immune cells and stromal cells secrete their specific cytokines and have a positive or negative feedback loop when they interact with other cell types. In order to identify the roles of estrogen in such a complex regulatory system, we purified bone marrow stromal cells with removing immune cells who expressed CD3e (T cells) or B220 (B cells) on their surface with MACS negative selection. These cells were treated with oligodeoxynucleotide CpG (a ligand to TLR9) to activate the TLR signal, and cytokine IL-6 and IL-12 was detected with ELISA 6 hour later to measure their ability on cytokines expression. As a result, we found these cells from OVX who lacked estrogen secretion in vivo produced less mount of IL-6 when compared with the control group (Fig. 3a).

It is known that the activation of TLR signal transduce a large variety of intercellular responses and lead to a wide range of gene expressions, and following 3 major signal pathways have been identified required in the regulation of inflammatory cytokines expressions: p38, extracellular signal-regulated kinases (erk) and NF-kappa b pathway. To further delineate the signal pathways involved in estrogen treatement, we then investigated all these pathways with western blotting (Fig. 3b) and found that estrogen deficiency in OVX mice inhibited the activation of both p38 and erk pathways, but had a slight effect on NF-kappa b pathway as our data show. These results indicated that estrogen deficiency affected the intercellular signal pathways which responsible to cytokines expression in bone marrow stromal cells.

Discussion

Osteoclastogenis in OVX mice in regulated by multiple factors including estrogen, inflammatory cytokines such as IL-4, IL,7, RANKL, TNF-alpha and activated T cells group as previous works reported(17), however, the roles of Treg cells in such process are poorly understood. In this study, we show that estrogen plays a major

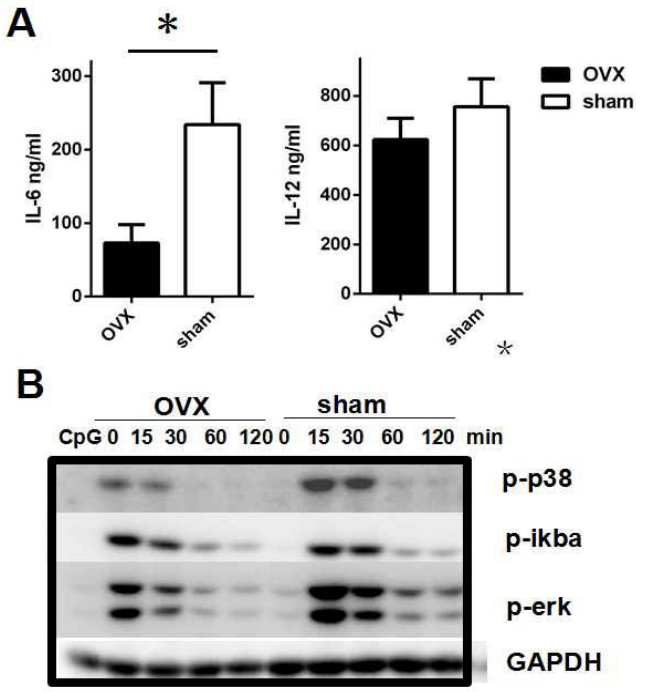

Figure 3. Estrogen deficiency affects signal pathways required for cytokines expression in BMSC. (A). ELISA analysis of pro-inflammatory cytokines IL-6 and IL-12 secretion levels in bone marrow stromal cells. BMSCs were purified from bone marrow with special depletion of lymphocytes *via* MACS negative selection and treated with CpG for 6 hours (n=6, *, $P< 0.05$). (B). Immunoblot of phosphorylated (p-) protein lysates of CpG treated BMSCs from OVX mice and sham group.

role in the regulation of Treg cells in OVX mice model. A significant reduction of Treg population was observed in bone marrow in estrogen deficiency OVX mice when compared with health control group. Moreover, surface markers which responsible to the suppressive ability of Treg cells such as CTLA-4 and PD-1 were down-regulated as well in our system. Considering the fact that estrogen was not required in the differentiation and functions maintenance of Treg cells(18), we proposed that estrogen altered cytokines profile in bone marrow, which enhanced local inflammatory and inhibited Treg functions, and finally our data about cytokines expressions in bone marrow cells support this hypothesis.

We then focused on the bone marrow stromal cells which was one of the major amount of inflammatory cytokines in bone marrow and found that these cells from OVX mice show higher sensitivity to Toll-like receptor (TLR) signal and produced more pro-inflammatory cytokines including TNF-alpha and type-1 interferon but less IL-6 when compared with untreated control cells. Consistent to these observations, molecular mechanisms studies suggested estrogen specifically enhanced TLR signals followed IRF family activation in bone marrow stromal cells. Comprehensive, we concluded that bone marrow stromal cells were the key regulator in estrogen inducing dis-function of Treg cells in OVX mice.

However, we noted that estrogen deficiency in OVX had various effects on bone marrow stromal cells. It was observed that estrogen enhanced the proliferation and differentiation *of BMSCs* in PMO women in clinical (19). And the mechanisms that estrogen promoted osteoclastogenic cytokines (20) expressions in bone marrow stromal cells via alterant p38 and NF-κB signals were well studied. Further studies focused on signal pathways show that estrogen treatment altered calcium signaling and affected followed PI3K/Akt/NF-κB activations in bone marrow stromal cells (21). Furthermore, studies on mesenchymal stem cells (22) reported estrogen deficiency in OVX mice resulted in Treg cells migration from bone marrow towards to peripheral tissues and led to apoptosis through MCP-1 secretion *in vitro*. And it was also reported that estrogen inhibited IL-6 secretion in bone marrow cells when treated with LPS *in vitro* (23), which was consistent with our observations of cytokines expression in bone marrow stromal cells.

In conclusion, our results demonstrate that estrogen deficiency effects Treg cells population and their functions in OVX mice with altering cytokines profile in bone marrow stromal cells and provide a novel approach to understand the progression of osteoclastogenis in OVX mice.

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