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Lack of estrogen down-regulates CXCR4 expression on Treg cells and reduces Treg cell population in bone marrow in OVX mice

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Abstract

Postmenopausal osteoporosis (PMO) is the most common metabolic bone disease in women after menopausal. Recent works focused on cross-talk between immune regulation and bone metabolism pathways and suggested Treg cells suppressed bone resorption and osteoclasts (OC) differentiation in bone marrow via cell-cell contact interaction and/or secreting of IL-10 and TGF-beta. In this study, we investigated the impact of estrogen on regulatory T cells (Treg cells) trafficking and staying in bone marrow and we found that a significant reduction of Treg cell population in bone marrow in estrogen deficiency ovariectomied (OVX) mice. We then studied the expressions of chemokines CXCL12/CXCR4 axes, which were critical to Treg cells migration and our data show the expression of CXCR4 on Treg cells was relative with oestrogen *in vivo*, however, the expression of CXCL12 was not. Furthermore, the loss of trafficking ability of Treg cells in OVX mice was recoverable in our system. These findings may mechanistically explain why Treg cells lose their suppressive functions on the regulation of OC cells and demonstrate a previously unappreciated role for estrogen, which may be critical to the novel therapy in clinical practice of PMO patients.

Key words: Postmenopausal osteoporosis, osteoclasts, Treg cells.

Introduction

Postmenopausal osteoporosis (PMO) is a widespread metabolic bone disease characterized by decreased bone mass and poor bone quality which occurs in women after menopausal and closed related with estrogen deficiency (1). Recent works suggested many factors were also involved in the pathogenesis of postmenopausal osteoporosis including lack of calcium and vitamin D and increased osteoporosis population in bones, etc. (2). Besides, estrogen followed cytokines expressions such as interleukin-1beta (IL-1beta), tumor necrosis factors-alpha (TNF-alpha), and interleukin-6 (IL-6) were also found disorder after menopausal (3) in clinical, which were critical to the functional maintenance of immune cells. Based on these observations, postmenopausal osteoporosis is recently recognized as a cytokines driven immune dys-regulation disease, however, with mechanisms still unclear.

It was well studied that bone resorption and formation in both human and mice were regulated *via* the balance of osteoclasts (OC) and osteoblasts (OB) (4). Loss of estrogen was reported to stimulate osteoclasts formation by increasing haematopoietic progenitors and promoting osteoclasts differentiation from progenitors in peripheral *via* IL-6 signal pathways based on recent reports (4, 5). And estrogen-estrogen receptor (ER) expression levels were also important for the differentiation, proliferation, functions maintenance and survival of osteoclast and osteoblast cells (6, 7). However, deficiency of estrogen only is not sufficient to induce bone loss in mostly animal models as published papers shown (8). It was reported that ovariectomy contrasted in T cells deficiency nude mice and T cells depletion mice did not result in osteoporosis (8, 9) although the functions and balance of osteoclasts and osteoblasts was changed after estrogen deficiency. Furthermore, the disorder secretions of pro-inflammatory cytokines such as TNF-alpha, TGF-beta and IL-1beta were reported regulated by activated T cells populations or even directly produced by T cells and was not dependent on estrogen levels (10, 11). Recently, researchers focused on the roles of regulatory T cells (Treg cells) in the PMO patients for the reasons that Treg cells were reported inhibit osteoclast differentiation from peripheral blood mononuclear cells (12, 13). In another work, Treg cells were shown able to increase bone density and resistance to ovariectomy-induced bone loss *via* inhibiting osteoclast differentiation (14). Moreover, Li et al. reported that the function of Treg cells in inhibiting osteoclast differentiation *in vitro* was dependent on estrogen (15).

However, little information of inhibitory mechanism of Treg cells in the case of osteoclastogenesis is available. Some groups accounted that TGF-beta and IL-4 produced by Treg cells were sufficient to inhibit osteoclast cells differentiation from peripheral blood mononuclear cells (PBMCs) (12). And other groups hold the view that cell to cell contact via CTLA-4 was necessary in Treg cells suppressing osteoclast cells formation (13). The goal of this work was to investigate the effect of estrogen on the migration ability of Treg cells in bone marrow, which was essential for both cell-cell contact dependent and independent manners.

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Materials and methods

Mice

CD90.1 mice was purchased form model animal research center from Nanjing University (Nanjing, China) and held in our animal room with a 12h light/dark cycle and given free access to food and water. 35 female mice aged 8 weeks old were performed with OVX operations, for details, the ovary, oviduct and top of the fallopian tubes were clamped and removed as previously reported (16, 17), and 20 age-matched mice were performed the sham operation as control group. Exogenous estrogen (17 β-estradiol) or placebo pellets were implanted in 10 OVX mice after operation to provided 0.72mg of estrogen for 4 weeks (28 days). with a anti-PE MACS beads

ELISA

Bone marrow fluid was collected from mice bone with PBS buffer with 0.5% BSA and was keep at -80 °C. CXCL 12 in bone marrow fluid was measured with Mouse CXCL12/SDF-1 alpha Quantikine ELISA Kit according to manufactures' instructions.

Cell isolation

Treg cells in bone marrow were purified with MACS sorter according to manufactures' instructions. Treg cells were labeled with anti-CD4 and anti-CD25 antibodies and selected with anti-PE MACS beads. In addition, Treg cell population in bone marrow was enriched with removing of B220+ CD11b+ CD11c+ non-T cells. The purity of CD4+ CD25hi Treg cells was more than 85%.

Antibodies, regents and kits

FITC/PE labeled anti-mouse CD4 antibody (GK1.5), PE labeled anti-mouse CD25 (ebio3C7) and APC labeled anti-mouse FoxP3 antibody (FJK-16s) were purchased from ebioscience. PE-Cy7 labeled anti-mouse CD184 (CXCR4) antibody (2B11), FITC-labeled anti-mouse CD90.1 antibody (G7) and PE-Cy7 labeled anti-mouse CD90.2 antibody (30-H12) were purchased from Becton Dickinson (BD). Intracellular Fixation & Permeabilization Buffer Set was purchased from ebioscience. Placebo pellets (17 β-estradiol) was purchased from Innovative Research of America. Data acquisition and analysis were performed on a FACSCalibur (BD).

Statistic analysis

Each experiment was repeated for at least three times. Results were presented as the mean ± standard deviation (SD). Statistical analysis was performed by using the Student's t test. Significance was defined at the $p<0.05$ (*) or $p<0.01$ (**) levels.

Results

Estrogen deficiency induced specific reduction of Treg cell population in bone marrow in OVX mice model

Treg cells are the major negative regulator in the processions of many diseases and recently reported involved in the development of PMO (12, 13). However, studies of cell fate and transcriptional factors regulation networks of Treg cells suggested that estrogen was not required in the differentiation and development of Treg cells both in vivo and in vitro (18). To investigate the influence of estrogen level on Treg cell numbers, we created the ovariectomied (OVX) mice model to mimic estrogen deficiency in postmenopausal patients. Four weeks after surgery, there was an decreasing expression of anti-inflammatory cytokines IL-10 and TGF-beta in the bone marrow in OVX mice (data not shown), which were required in Treg cells inhibiting OC development (8, 12, 13). This was consistent with that previously reported in OVX mice (19) and etrogen receptor (ER) deficient mice (20).

To affirm whether the reduction of cytokines was associated with Treg cells or not, we gated CD4+ CD25hi Treg cells from bone marrow in OVX mice and control group and examined the FoxP3 protein expression level via flow cytometry (fig. 1) for the reason that FoxP3 was critical to the following effector proteins expressions in Treg cells, and but no significant difference in MFI of FoxP3 expression was observed between OXV mice and the control, which hints that no change of Treg suppressor functions on single cell level. Moreover, percentages CD4+ CD25hi Foxp3+ Treg cell population in different organs, including peripheral blood (PB), spleen and bone marrow were also measured, and a significant reduction of Treg cell population in bone

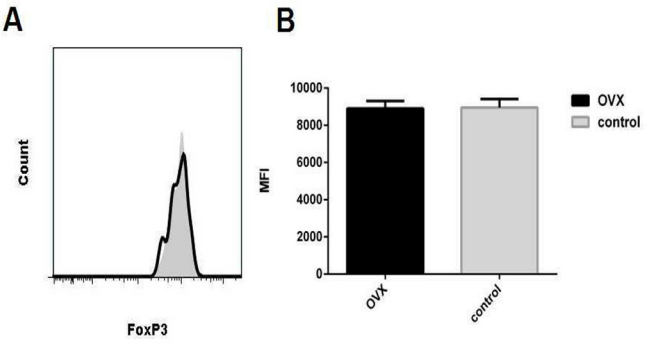

Figure 1. Foxp3 expression level in bone marrow Treg cell population. (a). Intercelluar Foxp3 exprssion levels in Treg cells from OVX mice or control group. Treg cells from bone marrow were stained with anti-CD3e, anti-CD4, antiCD25 and anti-Foxp3. Cells were gated on CD4+ CD25hi population. (B). MFI of Foxp3 expression in Treg cells from (A). (n=6 each group) *, $P < 0.05$; Data (mean ± SEM) are representative three independent experiments with similar results.

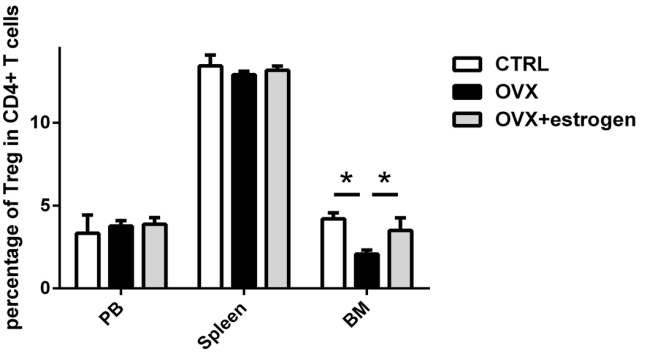

Figure 2. Treg cell population in different organs. Percentages of Treg cells in different organs. PB, peripheral blood; BM, bone marrow. Cells were stained as described in figure 1 and Treg cells were gated on CD4+ CD25hi Fopx3+ population. *, $P < 0.05$; Data (mean ± SEM) are representative three independent experiments with similar results.

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marrow was found in these OVX mice (fig. 2) compared with control group. We also reported that such reduction of Treg cell population was associated with estrogen level *in vivo* since that estrogen treatment in OVX mice recovered Treg cells reduction in bone marrow. Additional, these mice OVX mice did not exhibit high risk to infection and no autoimmunity disease development was observed in our model. Taken together, these observations suggested the percentage of Treg cell population was associated with estrogen expression level in *vivo*. However, the down-regulation of Treg cells functions in bone marrow seemed more likely to be related with the reduction of Treg cell population.

Estrogen deficiency down-regulated CXCR4 expression on Treg cells and impaired bone marrow homing

Functional studies about estrogen and estrogen receptors suggested that ER signal pathways linked responses were associated with the migration ability in many cancer cells such as breast cancer and thyroid cancer (21). Besides, estrogen/ER signals were involved in chemokines secretions such as CXCL13-CXCR5 axes (22) and inflammatory cytokines expression via NF-kappaB pathways (23) in clinical. Based on these conclusions, we hypothesized that estrogen had an impact on the trafficking ability of Treg cells, and deficiency of estrogen *in vivo* blocked Treg cells migrate towards bone marrow. Considering that CXCR4 and CXCL12 axes is the very important chemokine that was reported involved in Treg cells bone marrow trafficking (24), we then examined the surface expression levels of CXCR4 on Treg cells. As our data shown (fig. 3), percentage of CXCR4+ "trafficking " Treg cells in OVX was less than that in the control group, however, no significant difference was found in total CD4+ T cells group. Moreover, we also examined CXCL12, the ligands of CXCR4 in bone marrow with ELISA, and found similar CXCL12

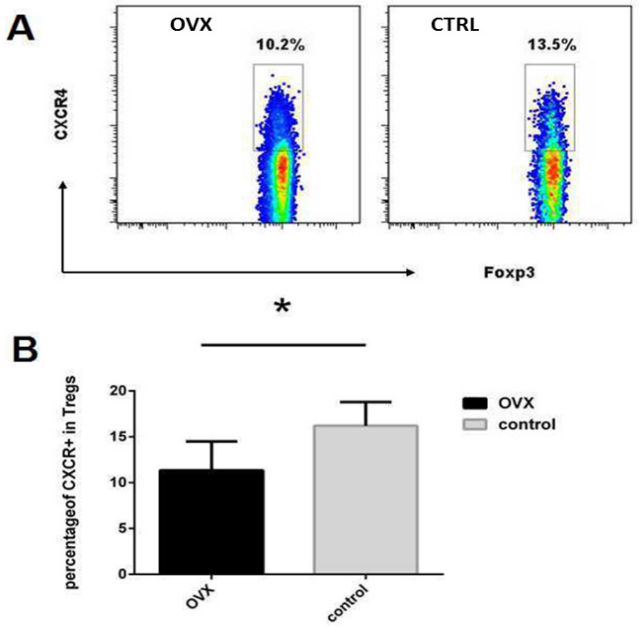

Figure 3. Impair migration ability of Treg cells in bone marrow. (a). Chemokine receptor CXCR4 expression level on the surface of Treg cells from bone marrow. Treg cells were gated on CD4+ CD-25hi Foxp3+ population. (B). Significant reduction (n=6, p=0.036) of CXCR4+ "trafficking" Treg cells were observed in bone marrow. Data (mean ± SEM) are representative three independent experiments with similar results.

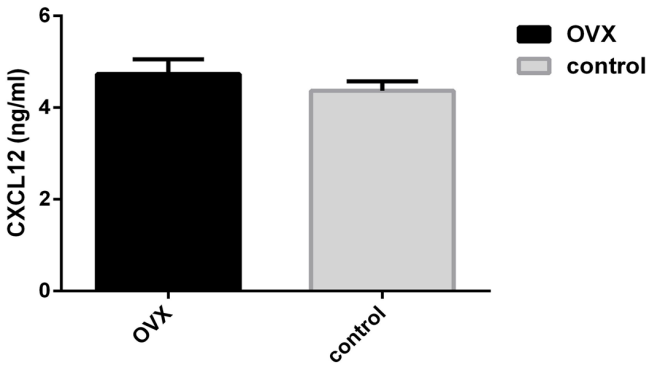

Figure 4. CXCL12 secretion levels in bone marrow. CXCL12 levels were detected with ELISA in bone marrow fluid from OVX mice and in control group. (n=6)

expression levels in OVX mice and the control group (fig. 4). Notable, changed CXCL12-CXCR4 axes was also observed in clinical cases reports of ovarian cancer who usually had an abnormal estrogen secretion *in vivo* (25), and also other cases like lung adenocarcinoma (26). Therefore, we held the view that estrogen associated up-regulation of CXCR4 on Treg cells impaired the homing ability of Treg cells in bone marrow.

Estrogen deficiency related down-regulation of trafficking ability in Treg cells was recoverable

Singal pathways studies in cancer cells threw light on the mechanisms that estrogen regulated CXCR4 expression in breast cancer cells (27), in which estrogen signals activated ER target genes such as SDF-1, and over-expression of SDF-1 promoted CXCR4 production. However, whether similar mechanism could be found in Treg cells or not was still unclear so far. We noted there was a positive feedback loop in the regulation of CXCR4 expression in *vivo*, which suggested CXCR4 expression was extremely sensitive to estrogen levels. Additionally, the CXCL12 secretion in bone marrow was not dependent on the estrogen deficiency as our data shown. Hence, we wondered whether estrogen deficiency resulting impairment of trafficking ability in Treg cell population was intrinsic or recoverable.

To answer this question, we sorted CD4+ CD25hi Treg cells from OVX mice bone marrow. Furthermore, we crossed CD90.2+ wildtype mice with CD90.1+ mice to gained CD90.1+CD90.2+ offspring, and then sorted functional bone marrow Treg cells from these mice. Notable, Treg cells from OVX mice expressed CD90.2 on their surface, however, the control cells expressed both CD90.1 and CD90.2. Treg cells from OVX or control mice were stained with CFSE and mixed at a ratio of 1:1 and transferred i.v. into CD90.1+ receiver with a dose of 1 million mixer cells per mice. After 3 days, Odds ratio of two Treg cell population was measured in bone marrow with FACS, and we found the trafficking ability of Treg cells from OVX was recovered (fig. 5) as the percentage of Treg cells from OVX mice occurred in bone marrow was almost equal to that of no treated control mice. Likewise, CFSE analysis also showed no significant difference (data not shown) in two groups of Treg cells, which excluded the possibility of Treg cells proliferation. These observations suggested the impairment of Treg cell migration in OVX was recoverable.

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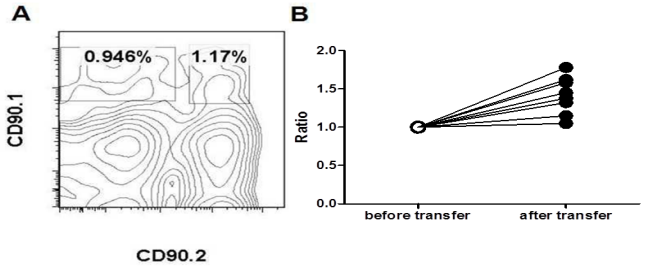

Figure 5. Comparison of migration ability in bone marrow Treg cell populations. (a). Both CD90.1+ Treg cells from OVX bone marrow and CD90.1+ CD90.2+ double positive Treg cells from control group bone marrow could be found in bone marrow of CD90.2+ receive mice. (b). No significant different were detected in odd ratio of two Treg cells in bone marrow. (n=12)

Discussion

CD4+ CD25hi Foxp3+ natural Treg cells is the major regulator of self-tolerance and immune homeostasis (28). In the course of PMO, the disorder of osteoclasts differentiation from CD11b+ precursors and over-activated functions of osteoclasts were common accepted related with dysfunctional Treg cell population in bone marrow. And as recent works shown, estrogen enhanced anti-inflammatory cytokines IL-10 and TGF-beta production in Treg cells in vitro, which suggested the primary role of estrogen in functions maintenance of Treg cells in vivo. However, the change of Treg cell number was not assessed in PMO patients although the decreasing of Treg cell population specific in bone marrow was observed in myelodysplastic syndromes (MDS) patients (29).

In our OVX mice model, which mimicked estrogen loss in PMO patients, a significant reduction in both number and percentage of Treg cell population in bone marrow accompanied with reduction of IL-10 and TGF-beta expression were observed. Furthermore, treatment with estrogen in these OVX mice was efficiently to recover Treg cells reduction in bone marrow. Considering that the development of Treg cells is not dependent on estrogen production, we supposed that Treg cells migrated towards bone marrow was depend on the estrogen expression and then investigated the chemokines expression in bone marrow of OVX mice. Following results suggested Treg cells from OVX mice expressed less CXCR4 on its surface compared with that from control group. However, the expression levels of CXCL12 in bone marrow were similar in both the groups. Since CXCR4-CXCL12 axes is the only known chemokine which regulates T cells migration towards bone marrow, we concluded that estrogen loss affects Treg cells trafficking towards bone marrow. Moreover, lack of estrogen did not result in intrinsic impairment of Treg cells because that Treg cells exhibited similar migration ability towards bone marrow when they were transferred into a estrogen rich host. A possible explanation maybe that the trafficking ability of Treg cells was shaped by the suppress mechanisms in bone marrow, which was regulated by estrogen. The observation that CXCL12 expression in bone marrow was not effected by estrogen thus provided further support to this hypothesis.

In conclusion, we reported herein estrogen deficiency affected Treg cells bone marrow trafficking and the reduction of Treg cells in bone marrow microenvironment specifically promoted pathogenic osteoclasts differentiation. Besides, we also reported the down-regulation of CXCR4 on Treg cells was recoverable, which provided now target for clinical therapies of PMO. In addition, further studies will be needed to determine the effects of estrogen or other unknown mechanisms in bone marrow on regulation of CXCR4 expression in Treg cells. And finally, our findings set the scene for the incorporation of the therapeutic manipulation of Tregs in PMO patients.

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