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Immunomodulatory effect of vitamin K2: Implications for bone health

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Abstract

Objective—In women with postmenopausal osteoporosis, Vitamin K2 appears to decrease the incidence of hip, vertebral and non-vertebral fractures. Women with post-menopausal osteoporosis have more circulating activated T cells compared to healthy post-menopausal and pre-menopausal women, but the effects of Vitamin K2 on T-cells has not been studied. In this study, we have looked at T-cell suppression by Vitamin K2.

Materials and methods—Peripheral blood mononuclear cells (PBMCs) from three healthy donors were used. The PBMCs were stimulated with the mitogens phytohemagglutinin and concanavalin A, and T-cell proliferation was analyzed using flow cytometry based on carboxyfluorescein succinimidyl ester (CSFE) dye dilution.

Results—Vitamin K2 (60 and 100 μM) inhibited T-cell proliferation. Vitamin K1 at the same concentrations did not inhibit T-cell proliferation.

Conclusion—Vitamin K2 has immunomodulatory activities.

Keywords

Vitamin K2; Immunomodulation; T-cells; Osteoporosis

Introduction

Vitamin K is a fat-soluble vitamin. Two vitamin K species are known: vitamin K1 (phylloquinone), vitamin K2 (menaquinones). Vitamin K1 activates blood clotting factors. Vitamin K2, acts on extra-hepatic tissues (bone, brain, vasculature, testis, pancreas, kidneys and lungs) to activate K2 dependent proteins such as osteocalcin and matrix gla protein. The most common forms of vitamin K2 in the human diet are MK-4 and MK-7, short- and long-chained molecules, respectively (Beulens et al., 2013; Booth, 1997, 2012; Myneni and Mezey, 2016; Shearer and Newman, 2008). The effect of vitamin K2, mainly MK-4, on the

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

VM designed the study, analyzed the data and wrote the manuscript. EM reviewed the data and edited the manuscript.

bone health of women with post-menopausal osteoporosis has been studied by a number of clinical investigators (Inoue et al., 2009; Iwamoto et al., 2003; Knapen et al., 2007; Tanaka and Oshima, 2007; Ushiroyama et al., 2002; Yonemura et al., 2004; Yonemura et al., 2000). A meta-analysis of studies in which MK-4 was used at a dose of 45 mg/day revealed that vitamin K2 reduced hip, vertebral and non-vertebral fractures (Cockayne et al., 2006). Women with post-menopausal osteoporosis have more circulating activated T cells compared to healthy post-menopausal and pre-menopausal women (Adeel et al., 2013; D'Amelio et al., 2008). T-cells are known to play a critical role in promoting bone loss in postmenopausal osteoporosis as well as bone cancers (Zhang et al., 2011), rheumatoid arthritis (Kong et al., 1999), and periodontitis (Teng et al., 2000). Given the protective effect of vitamin K2 on bone health and the role of activated T-cells in osteoporosis, we asked if vitamin K2 could suppress T-cell proliferation. This could contribute to the activities described above. We report that vitamin K2 indeed can suppress T-cell proliferation. This activity is specific to vitamin K2, and not shown by vitamin K1.

Materials and Methods

Reagents

All reagents used in this study were obtained from Sigma-Aldrich (St Louis, MO) unless mentioned. CFSE, phytohemagglutinin and Concanavalin A were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). CD3 APC antibody and 7-AAD were purchased from eBioscience.

PBMC staining and culture

Human PBMCs from healthy adult volunteers were obtained from the NIDCR core facility. Healthy volunteer blood was collected in accordance with the Declaration of Helsinki received from the NIH blood Bank. PBMCs were cultured in RPMI-1640 media with 10% Heat inactivated FBS, 2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 1× nonessential amino acids and 1% penicillin-streptomycin. PBMCs were stained with 2.5 μM CFSE for 15 min at room temperature in the dark at a cell density of 10 $\times 10^6$ PBMC/ml. The reaction was stopped by adding an equal amount of cold RPMI-1640 media with 10% Heat inactivated FBS and incubated on ice for 5 min and centrifuged at 400×g for 5 min. The cells are washed twice with cold media and resuspended at a density of 1×10^6 PBMCs/ml. The CFSE labelled PBMCs were used for the T-cell proliferation assay.

T-cell proliferation assay

The CFSE-labeled PBMCs were cultured in triplicate at a density of 5 $\times 10^4$ cells/ml in a round bottom 96-well plate. T-cell proliferation was induced with phytohemagglutinin (PHA-5 μg/ml) or concanavalin A (ConA-5 μg/ml) for 96 hr with or without vitamin K2 (MK-4) or vitamin K1 in a total volume of 200 μl. Cells were cultured in a humidified incubator at 37°C and 5% CO_2. T-cell proliferation was determined by CFSE dye dilution of CD3 positive cells using AccuriC6 flow cytometer. The gating strategy is shown in Supplemental Fig 1. The Flow cytometry data were analyzed using Flow Jo software.

Statistical analysis

All values are expressed as standard error of the mean (SEM) of three donors. Statistical significance was assessed by unpaired ANOVA followed by Tukey's post hoc testing using Prism 7. *P* values are as follows: ***p>0.001, ****p>0.0001.

Results and Discussion

In an initial series of experiments, we determined optimal concentrations of the mitogens PHA and ConA to promote T-cell activation using three donors to balance individual immune response variability (Supplemental Fig.2). A concentration of 5 μg/ml was chosen for both PHA and ConA, and was used for the rest of the study. We initially tested the effect of vitamin K2 (MK-4) on T-cell proliferation at concentrations ranging from 0 μM to 10 μM, concentrations most commonly reported in literature to affect the function of cells such as osteoblasts (Ichikawa et al., 2007), osteoclasts (Koshihara et al., 2003), hematopoietic stem cells (Miyazawa and Aizawa, 2004), and erythroid and myeloid cells (Sada et al., 2010). Vitamin K2 at these concentrations did not inhibit T-cell proliferation (Supplemental Fig.3). Vitamin K2 concentrations greater than 10 μM were shown to induce testosterone production (Ito et al., 2011). For the next set of experiments we used vitamin K2 concentrations as high as 100 μM. Vitamin K2 significantly inhibited T-cell proliferation at 60 and 100 μM in both PHA (Fig.1. a, b) and ConA (Fig.1. c, d) stimulated PBMCs. 30 μM of K2 significantly inhibited ConA-stimulated, but not PHA-stimulated PBMCs (Fig.1. b, d). We next asked whether vitamin K1 also inhibits T-cell proliferation. As shown in Fig.2. a, b, c, d, vitamin K1 did not inhibit T-cell proliferation at any of the concentrations used. These results suggest that inhibition of T-cell proliferation is specific to Vitamin K2.

It has been reported that vitamin K2, but not vitamin K1, promotes osteoblasts differentiation from mesenchymal stem cells, preventing osteoclast formation. This is similar to the effect on T-cell proliferation that we observed. Our results also suggest that immunomodulation might contribute to the overall anabolic effect of vitamin K2 on bone. In clinical trials with vitamin K2, investigators used a dose of 45 mg/day. This is the minimal effective dose to improve bone health, and there were no side effects reported when it was given continuously for three years (Inoue et al., 2009; Iwamoto, 2014). In principal, this dose of K2 taken orally could achieve a maximum blood concentration of 40 to 50 μM, but this needs to be further investigated. This would have clinical impact in that by modulating the dosage of vitamin K2 T-cell function can be modulated without affecting its functions on bone cells and other cell types. In this study, we have not looked at T-cells subsets, however. Doing this will provide a better understanding of the specific cell(s) that contribute to the function of vitamin K2 in promoting immunomodulatory activities. Further studies will be needed to determine the mechanism of immune regulatory functions of vitamin K2, and to examine the function of other vitamin K2 isoforms on immune regulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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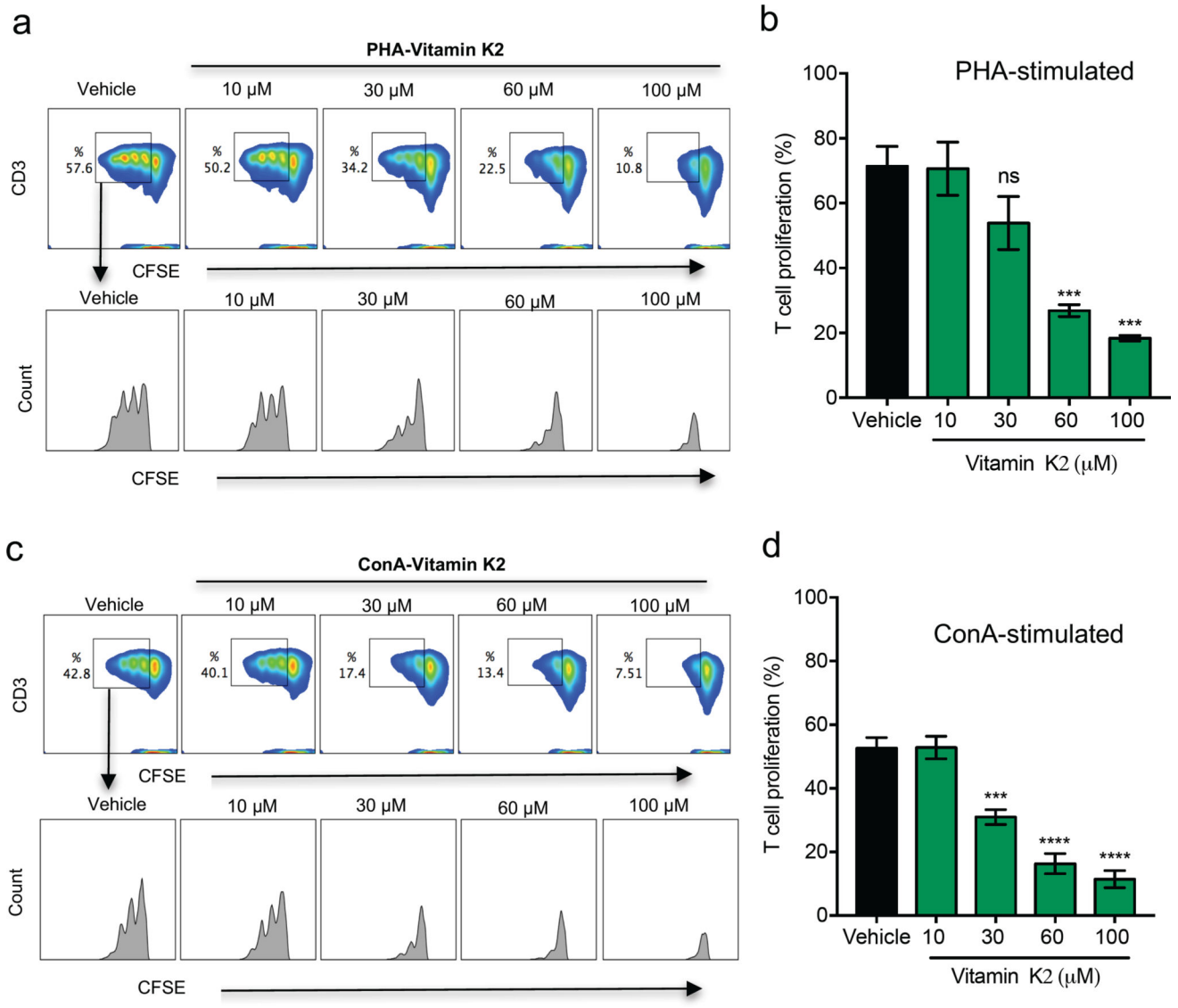

Fig. 1. Vitamin K2 inhibits T-cell proliferation

(A) Flow cytometry density plots show expression of PHA stimulated viable CSFE and CD3 positive T-cells. The gated region shows the percentage of proliferating T-cells following PHA stimulation with or without vitamin K2 for 96 hr of culture. The histogram plots of the gated regions show the suppression of vitamin K2 on PHA stimulated CD3 T-cells, each peak in the histogram represents one generation of proliferating cells. **(B)** Percentage of T-cell proliferation in presence of vitamin K2 at the indicated doses or vehicle control with PHA stimulation. **(C)** Flow cytometry density plots show expression of ConA stimulated viable CSFE and CD3 positive T-cells. The gated region shows the percentage of proliferating T-cells with ConA stimulation with or without vitamin K2 after 96 hr of culture. The histogram plots of the gated regions show the suppression of vitamin K2 on ConA stimulated CD3 T-cells. **(D)** Percentage of T-cell proliferation in presence of vitamin K2 at the indicated doses or vehicle control with ConA stimulation. n=3 donors; Error bars represent standard error of mean (SEM); ns-not significant ;***p<0.001; ****p<0.0001.

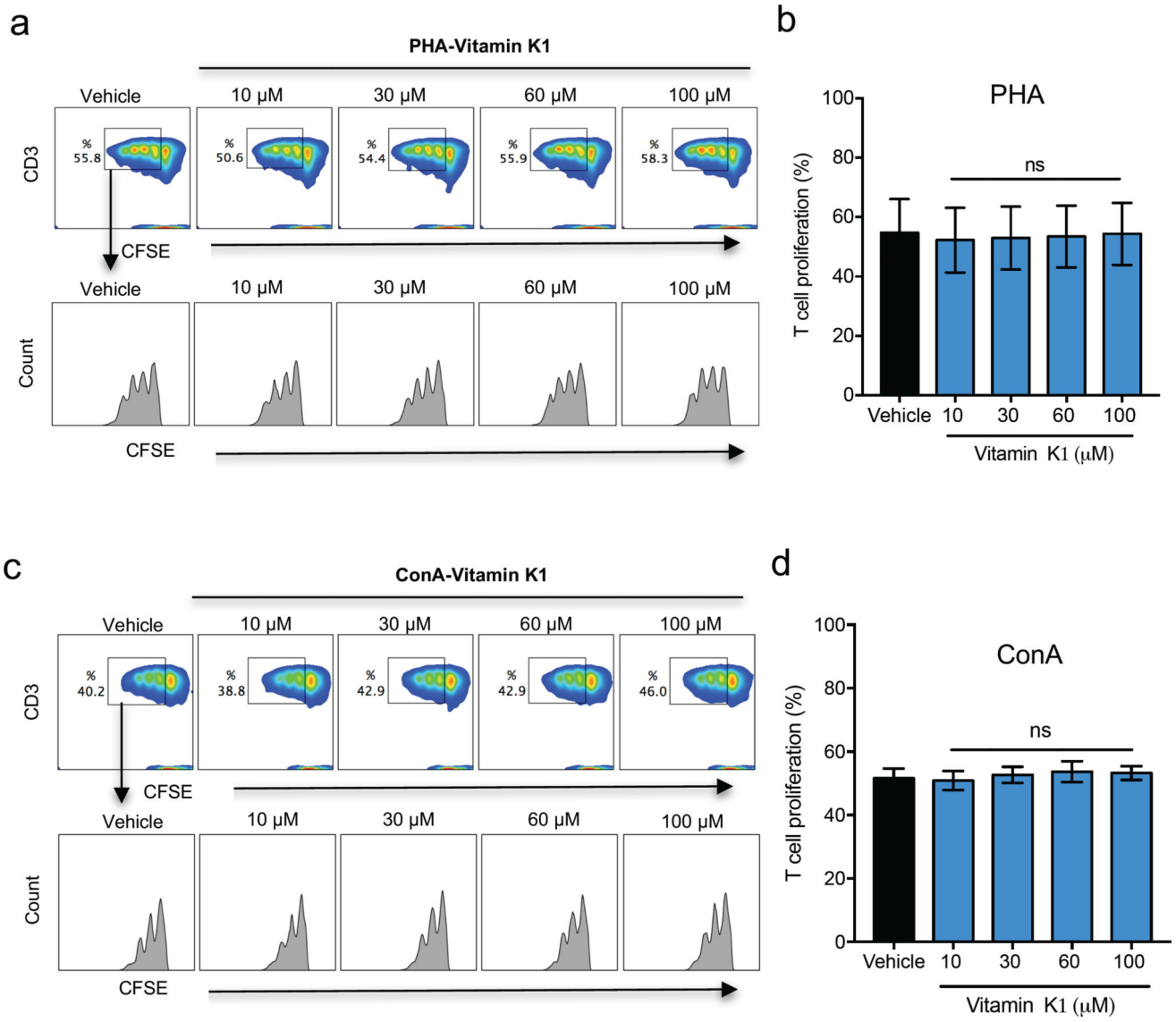

Fig. 2. Vitamin K1 has no inhibitory effect on T-cell proliferation

(A) Flow cytometry density plots show expression of PHA stimulated viable CSFE and CD3 positive T-cells. The gated region shows the percentage of proliferating T-cells following PHA stimulation with or without vitamin K1 after 96 hr of culture. The histogram plots of the gated regions show the lack of suppression by vitamin K1 on PHA stimulated CD3 T-cells, each peak in the histogram represents a generation of proliferating cells. **(B)** Percentage of T-cell proliferation in presence of vitamin K1 at the indicated doses or vehicle control following PHA stimulation. **(C)** Flow cytometry density plots show expression of ConA stimulated viable CSFE and CD3 positive T-cells. The gated region shows the percentage of proliferating T-cells with ConA stimulation with or without vitamin K1 after 96 hr of culture. The histogram plots of the gated regions show the lack of suppression by vitamin K1 on ConA stimulated CD3 T-cells. **(D)** Percentage of T-cell proliferation in

presence of vitamin K1 at the indicated doses or vehicle control with ConA stimulation. n=3 donors; Error bars represent standard error of mean (SEM); ns-not significant.