

## $\alpha$ -Tocopherol induces hematopoietic stem/progenitor cell expansion and ERK1/2-mediated differentiation

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### ABSTRACT

Tocopherols promote or inhibit growth in different cell types. In the hematopoietic system, the radioprotective property of tocopherols is thought to act through the expansion of primitive hematopoietic cells. However, the mechanisms activated by tocopherols and which HPs are affected remain poorly understood. To better address these questions, mice were treated with  $\alpha$ -tocopherol, and its effects were investigated in the BM microenvironment.  $\alpha$ -Tocopherol induced increased proliferation in HSC/HP cells, leading to BM hyperplasia. In addition, differentiation to the granulocytic/monocytic lineage was enhanced by  $\alpha$ -tocopherol treatment.  $\alpha$ -Tocopherol treatment resulted in decreased basal phosphorylation of ERK1/2, PKC, and STAT-5 in HSC/HP cells. In contrast,  $\alpha$ -tocopherol enhanced ERK1/2 activation in response to IL-3 stimulation in HSC/HP cells without altering the expression of IL-3Rs. Moreover,  $\alpha$ -tocopherol-induced differentiation and ERK1/2 activation were abolished in mice pretreated with a MEK inhibitor (PD98059); however, pretreatment with PD98059 did not reduce the  $\alpha$ -tocopherol-mediated increase in HSC/HP cells but instead, further enhanced their proliferation. Therefore,  $\alpha$ -tocopherol induces expansion of HSC/HP cells by a nonidentified intracellular pathway and granulocytic/monocytic differentiation through ERK1/2 activation. *J. Leukoc. Biol.* 90: 1111–1117; 2011.

### Introduction

$\alpha$ -Tocopherol is a member of the vitamin E family of compounds ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  tocopherols and tocotrienols), which possesses numerous biological properties [1, 2]. Studies during

the last decade have provided strong support for a specific role for  $\alpha$ -tocopherol in cell signaling and the regulation of gene expression.  $\alpha$ -Tocopherol produces significant effects on inflammation, cell proliferation, and apoptosis, which are not shared by other vitamin E isomers with similar antioxidant properties [3].

$\alpha$ -Tocopherol supplementation has been shown to have beneficial effects in numerous disorders [4]. Free tocopherol is absorbed in the intestine, together with lipids, and transported to the liver, and the products derived from tocopherol are released into the circulation for use by peripheral tissues [5].

The molecular and cellular effects of vitamin E have been explained by the compound's antioxidant functions, including the scavenging of ROS and reactive nitrogen species [6]; however, antioxidant-independent activity of  $\alpha$ -tocopherol has also been observed [7].

Initial studies of  $\alpha$ -tocopherol in the hematopoietic system have shown its effects on hemolytic and iron-deficiency anemia, during which its antioxidant action increases the resistance of erythrocytes to lysis [8]. In addition, several studies performed in irradiated mice have shown that the application of  $\alpha$ -tocopherol prior to irradiation increases the survival of these animals. This effect has been related to the increase in CFUs observed in  $\alpha$ -tocopherol-treated mice [9–11]; however, neither the type of HPs affected nor the intracellular mechanisms triggered by  $\alpha$ -tocopherol treatment have been described.

The regulation of HSC proliferation and differentiation involves complex signaling events linking the intrinsic genetic processes of blood cells and their environment. This interplay determines whether HSC/HP cells remain quiescent, proliferate, self-renew, differentiate, or undergo apoptosis [12]. The most well-known environmental regulators of hematopoiesis are the cytokines, which bind defined receptors and activate a variety of signaling pathways [13–15]. Other important envi-

Abbreviations: BM=bone marrow, FL1=fluorescence 1, Gm=geometric mean, HP=hematopoietic progenitor, HSC=hematopoietic stem cell, LSK=Lin<sup>−</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup>, p=phosphorylated, S/G2/M=synthetic phase/gap 2 phase/mitotic phase

The online version of this paper, found at [www.jleukbio.org](http://www.jleukbio.org), includes supplemental information.

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ronmental regulators include the extracellular matrix components, free radicals, purines, nutrients, and vitamin E [16–19].

In this study, the effects of  $\alpha$ -tocopherol treatment on BM progenitors and on the intracellular mechanisms that regulate these events were evaluated. We observed that  $\alpha$ -tocopherol treatment induced BM hyperplasia, which was related to an increase in the proliferation of HSC/HP cells. In addition,  $\alpha$ -tocopherol induced granulocytic/monocytic differentiation through IL-3-dependent ERK activation.

## MATERIALS AND METHODS

Detailed methodologies are fully described in the online Supplemental Material.

### Extraction of BM, $\alpha$ -tocopherol treatment, and CFU-GM assay

Three-month-old male C57BL/10 mice were treated with 40 mg/kg RRR- $\alpha$ -tocopherol (Sigma-Aldrich, St. Louis, MO, USA). Eight doses of  $\alpha$ -tocopherol were i.p.-administered every other day. Control animals received only the vehicle. The experiments were performed 24 h after the last  $\alpha$ -tocopherol dose. BM from the femurs was subsequently collected in PBS.  $\alpha$ -Tocopherol was administered i.p. to ensure the integrity of the compound by allowing direct insertion inside of the body in a region that allows a large absorption surface from which the compound can enter the circulation rapidly.

CFU-GM assays were performed by placing whole BM cells ( $2 \times 10^4$ ) in Methocult medium (Stem Cell Technologies, Beverly, MA, USA) in 35-mm dishes, according to the manufacturer's instructions. The total number of BM cells was counted in a Neubauer chamber.

### Histochemical staining

Mouse femurs were isolated, fixed, processed, and stained with H&E. Photomicrographs were obtained using a Nikon DS-Fi1 digital camera.

### BrdU assay

Control and  $\alpha$ -tocopherol-treated mice received 1 mg/mouse BrdU (Sigma-Aldrich) once/day on the last 3 days of  $\alpha$ -tocopherol treatment [20]. BM was extracted 24 h after the last injection. BrdU incorporation was evalu-

ated by immunohistochemistry and flow cytometry using Becton Dickinson's BrdU kits, according to the manufacturer's instructions.

### Flow cytometry

The following antibodies were used to identify hematopoietic BM populations: Ter-119, Mac-1, Gr-1, B220, and CD3 (Lin), c-Kit and Sca-1. Ter-119<sup>+</sup> cells were defined as erythroid cells. Mac-1<sup>+</sup>Gr-1<sup>+</sup> cells were defined as myeloid cells. B220<sup>+</sup> cells were defined as B cells. CD3<sup>+</sup> cells were defined as T cells. LSK were defined as HSC/HP cells. Lin<sup>−</sup>c-Kit<sup>+</sup>Sca-1<sup>−</sup> cells were defined as HP cells.

To measure the basal expression of the activated forms of proteins, whole BM cells were collected, fixed, permeabilized, and labeled with antibodies specific for the activated (phosphorylated) forms of AKT, PKC, STAT-5, and ERK1/2 (Cell Signaling Technology, Beverly, MA, USA). A goat anti-rabbit IgG Alexa Fluor 488-conjugated antibody (Invitrogen, Carlsbad, CA, USA) was used as a secondary antibody. To verify the activation of STAT-5 and ERK1/2, BM cells were stimulated with 10 ng/ml IL-3 (Sigma-Aldrich) at 37°C for 5 min in a physiological solution before fixation. HSC/HP cells were identified as described in the Supplemental Material.

Cell-cycle analysis was performed by flow cytometry. BM cells were fixed, permeabilized, and incubated with RNase. The cells were labeled with 1  $\mu$ M SYTO Green (Invitrogen) and with antibodies to recognize HSC/HP cells.

### Statistical analysis

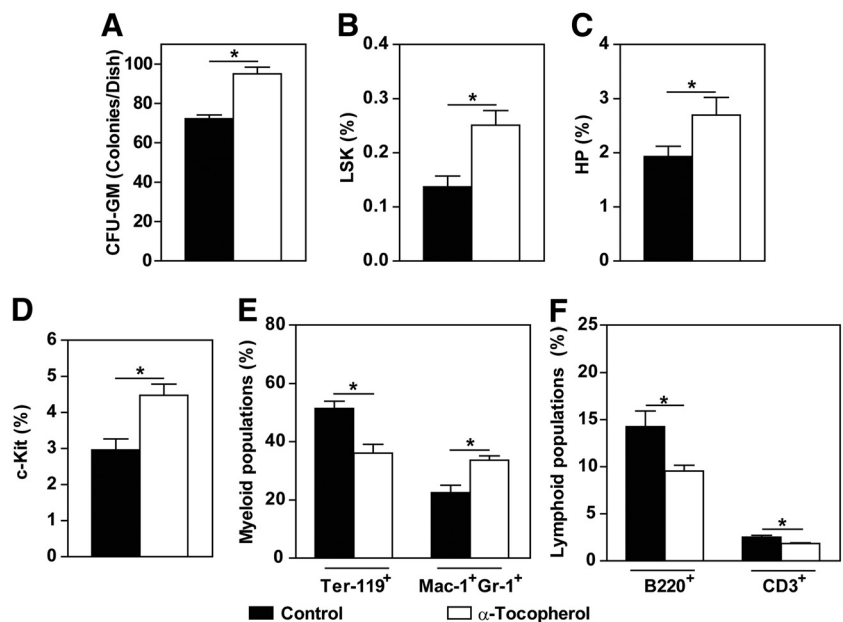
Fluorescence intensity was quantified using Gm. The results of BrdU assays are presented as the percentage of BrdU<sup>+</sup> cells. Student's *t* tests were used to compare data from two groups. To compare data from more than two groups, ANOVA was performed and followed by Bonferroni's test. Values are presented as means  $\pm$  SEM. Differences were considered significant when *P* < 0.05.

## RESULTS AND DISCUSSION

### $\alpha$ -Tocopherol increases the number of HSC/HP cells and induces granulocytic/monocytic differentiation

As a result of the antioxidant capacity of  $\alpha$ -tocopherol, previous reports have investigated the beneficial effects of treatment with a daily dose of 40 mg/kg  $\alpha$ -tocopherol for 1–4

**Figure 1.  $\alpha$ -Tocopherol increases HSC/HP cells and promotes differentiation to the granulocytic/monocytic lineage.** Mice were treated with 40 mg/kg  $\alpha$ -tocopherol i.p. every other day for 2 weeks. (A) The capacity to form primitive CFU-GM was evaluated by cultivating  $2 \times 10^4$  whole BM cells in methylcellulose for 7 days. (B–F) Whole BM cells were labeled with mAb specific for Gr-1, Mac-1, Ter-119, B220, CD3, c-Kit, and Sca-1 and analyzed by flow cytometry. The percentage of BM cells within the (B) LSK, (C) HP, (D) c-Kit<sup>+</sup>, (E) Gr-1<sup>+</sup>Mac-1<sup>+</sup> and Ter-119<sup>+</sup>, and (F) B220<sup>+</sup> and CD3<sup>+</sup> populations is shown. The data are expressed as mean  $\pm$  SEM of six independent experiments. \**P* < 0.05, Student's *t* test.

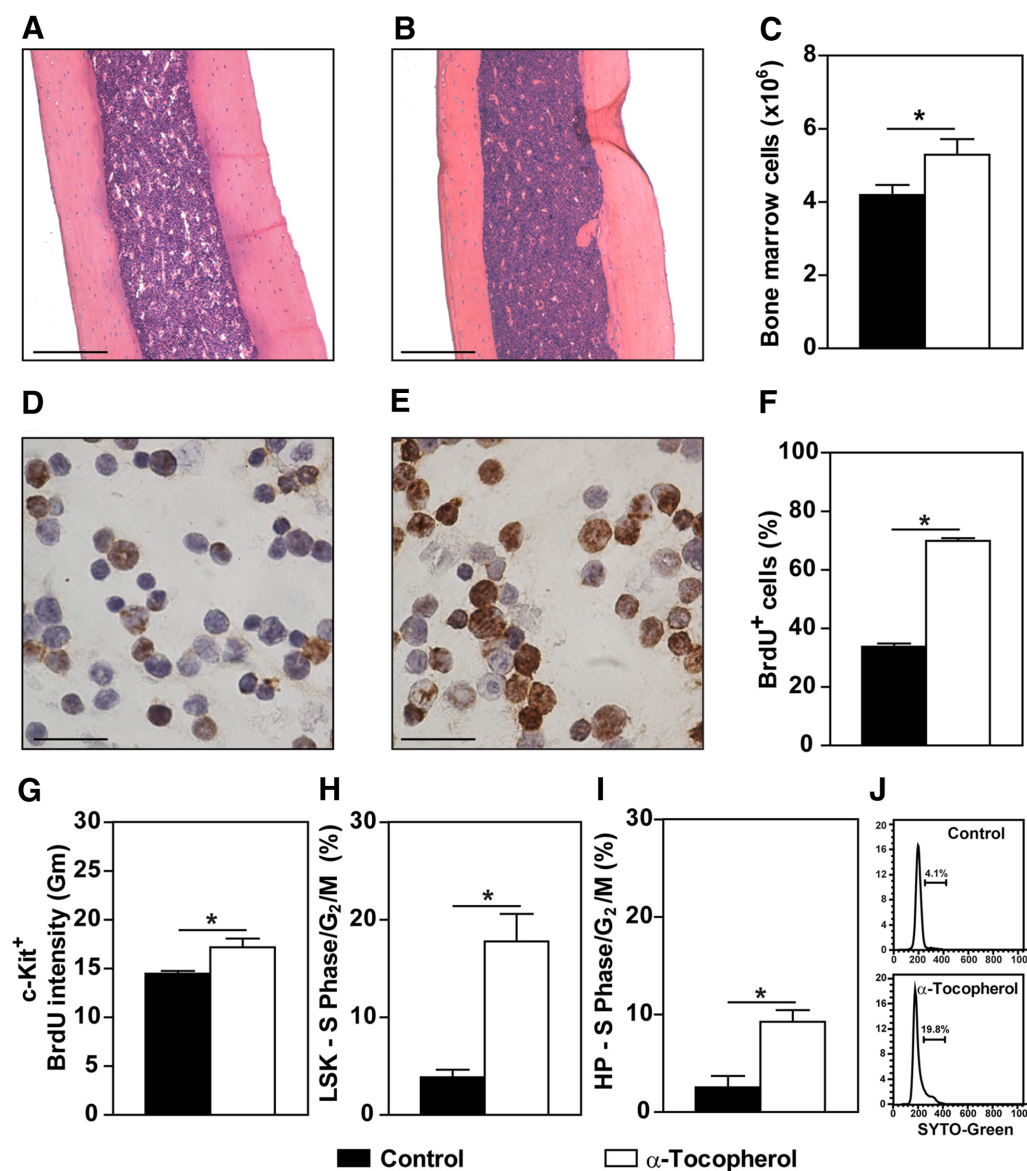


weeks [21]. In BM, the beneficial effects of  $\alpha$ -tocopherol have been evaluated after mice treatment with a single dose of 9 mg/kg  $\alpha$ -tocopherol administered immediately postirradiation or after treatment of 20 mg/kg for 5 days preirradiation, and both treatments resulted in increased numbers of HPs, as demonstrated by CFU-GM assays [9, 10, 22]. To investigate the beneficial effects of  $\alpha$ -tocopherol on HSC/HP cells, BM hematopoietic populations and intracellular hematopoietic signaling mechanisms were evaluated.

Administration of 40 mg/kg  $\alpha$ -tocopherol for 2 weeks increased the amount of  $\alpha$ -tocopherol in the plasma membrane (Supplemental Fig. 1). In all figures, the control group designation refers to the untreated group. In accordance with previous findings, this dose also increased the number of hematopoietic precursors, as determined by CFU-GM (Fig. 1A) [9–11]. In addition, we quantified the c-Kit<sup>+</sup>, LSK, and HP populations, as well as the mature hematopoietic cells (see Supplemental Fig. 2). The LSK population contains the most

primitive HPs, long-term and short-term HSCs, as well as multipotent progenitor HSCs [23]. The Lin<sup>−</sup>c-Kit<sup>+</sup>Sca-1<sup>−</sup> population contains the HP cells, common lymphoid progenitor, and common myeloid progenitor cells [20, 24, 25]. The c-Kit<sup>+</sup> population contains all primitive hematopoietic cells. In  $\alpha$ -tocopherol-treated mice, increases in the c-Kit<sup>+</sup>, LSK, and HP populations were observed (Fig. 1B–D).

Among mature hematopoietic populations, the Gr-1<sup>+</sup>Mac-1<sup>+</sup> population, which is composed of granulocytic, monocytic, and myeloblastic cells, increased upon treatment with  $\alpha$ -tocopherol (Fig. 1E). In contrast, erythroid (Ter-119<sup>+</sup>), B (B220<sup>+</sup>), and T (CD3<sup>+</sup>) cells decreased in  $\alpha$ -tocopherol-treated mice (Fig. 1E and F). The increase in the number of Gr-1<sup>+</sup>Mac-1<sup>+</sup> cells (Fig. 1E) suggests that  $\alpha$ -tocopherol treatment also induces myeloid differentiation, particularly along the granulocytic/monocytic lineage. This is in agreement with other reports that have shown that high doses of tocopherols (400 mg/kg) induce increases in the numbers of myeloid cells



**Figure 2.  $\alpha$ -Tocopherol induces medullary hyperplasia by enhancing the proliferative state of HSC/HP cells.** Mice were treated with 40 mg/kg  $\alpha$ -tocopherol i.p. every other day for 2 weeks. (A and B) Cellularity in the BM cavity was assessed by staining with H&E. The images are representative of three independent experiments. Original bars = 100  $\mu$ m. (C) Whole BM cellularity;  $n = 10$ . (D–G) BrdU assays were used to verify the enhanced, proliferative state of BM cells. Mice received 1 mg/mouse BrdU 1 h after  $\alpha$ -tocopherol (or vehicle) injection. (D and E) Immunohistochemical labeling was performed using anti-BrdU and visualized under a light microscope. Original bars = 25  $\mu$ m. The images are representative of four independent experiments. (F) The percentage of BrdU<sup>+</sup> (brown) cells in the whole BM cavity was determined by counting 300 cells/sample;  $n = 4$ . (G) The intensity of anti-BrdU-FITC staining was measured in the c-Kit<sup>+</sup> population. Data are presented as the Gm of FL1 intensity;  $n = 4$ . (H and I) DNA was stained with SYTO Green. Cell-cycle status was determined by quantifying the DNA content in the (H) LSK and (I) HP populations. (J) Representative cell-cycle histograms of control and  $\alpha$ -tocopherol-treated groups in the LSK population are shown;  $n = 6$ . The data are expressed as mean  $\pm$  SEM. \* $P < 0.05$ , Student's  $t$  test.

and HPs in peripheral blood by increasing the levels of cytokines such as IL-6 and G-CSF [1, 26]. In these previous studies, however, the BM cavity was not evaluated.

Next, histological analysis of the BM was performed after treatment with  $\alpha$ -tocopherol. Histological evaluation showed hyperplasia in the BM (Fig. 2A and B), as corroborated by the increase in total BM cellularity (Fig. 2C). Subsequently, we evaluated the proliferative state of hematopoietic cells. Immunohistochemistry and flow cytometry revealed an increase in the frequency of BrdU<sup>+</sup> cells within whole BM cells (Fig. 2D–F) and within the c-Kit<sup>+</sup> population (Fig. 2G). In addition, measurement of the DNA content of HSC/HP cells using SYTO Green demonstrated that an increased proportion of these cells was in the S/G<sub>2</sub>/M phase, confirming the proliferative state (Fig. 2H–J). Therefore, the increase of HP cells [9, 10, 22] in BM (Fig. 1) is manifested as BM hyperplasia (Fig. 2).

### Changes in intracellular signaling upon $\alpha$ -tocopherol treatment

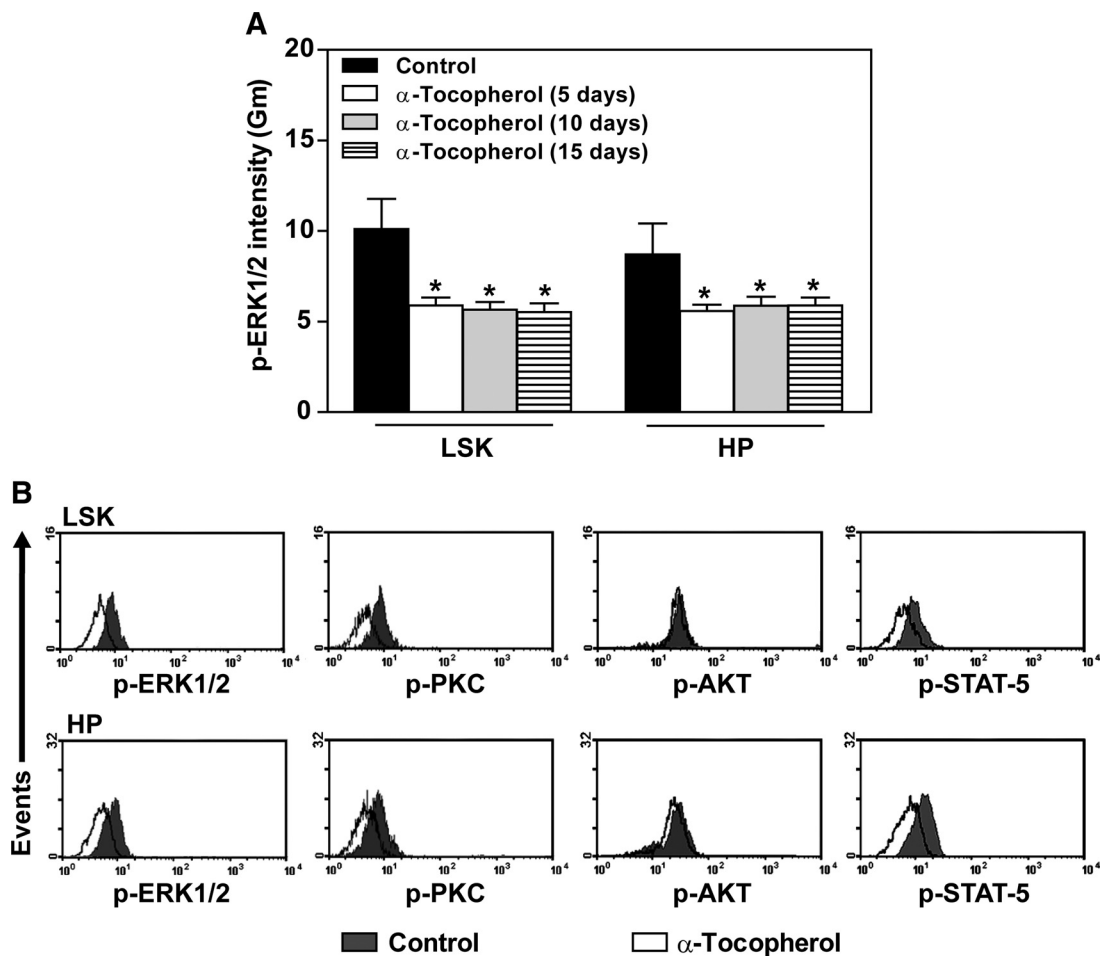
Cytokines are a group of molecules that couple with their receptors to trigger many intracellular signaling pathways that regulate hematopoiesis [27]. The binding of cytokines to their receptors induces receptor dimerization, and depending on the cytokine receptor, cytokine binding can also induce the

activation of a family of tyrosine kinases called JAKs [27]. IL-3 activates JAKs, which phosphorylate receptors on target tyrosine residues. This tyrosine phosphorylation can be recognized by other proteins containing Src homology 2 domains, such as STATs, Src kinases, PI3K, PLC $\gamma$ , and adaptor proteins, such as Shc and Grb2 [27]. Downstream signal mediators, such as MEK/ERK, PI3K/Akt, and PLC $\gamma$ /PKC, further impart signal specificity in the hematopoietic system [15, 28, 29]. Tocopherols can control the cell cycle by altering the activated forms of AKT, ERK1/2, and PKC in several cell types [7, 30–33].

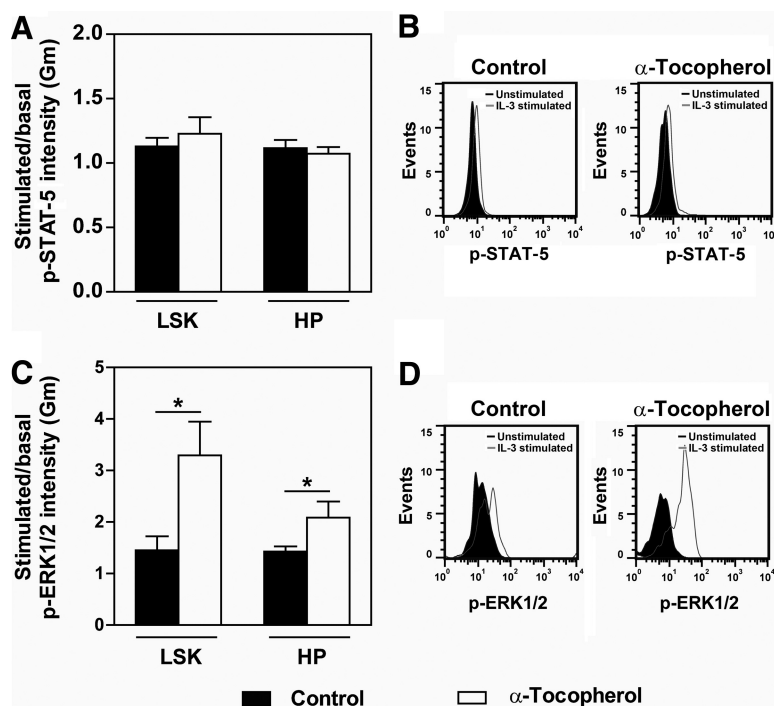
To explain the effects of  $\alpha$ -tocopherol in BM cells, we evaluated the expression of p-AKT, p-ERK1/2, p-PKC, and p-STAT-5 under basal conditions. Unexpectedly,  $\alpha$ -tocopherol treatment did not increase p-ERK1/2 during the days of treatment but instead, decreased it (Fig. 3A). Similar results were observed for p-PKC and p-STAT-5 but not for p-AKT (Fig. 3B).

Furthermore, we investigated the possibility that the activation of STAT-5 and ERK1/2 by IL-3 could have been altered in HSC/HP cells *ex vivo*. Thus, BM cells were stimulated with 10 ng/ml IL-3 for 5 min in physiological solution, and p-STAT-5 and p-ERK1/2 were quantified. The  $\alpha$ -tocopherol treatment did not induce changes in the activation of p-STAT-5 in response to IL-3 (Fig. 4A and B), whereas ERK1/2 activation in response to IL-3 was enhanced in LSK

**Figure 3.**  $\alpha$ -Tocopherol reduces the activation of proteins related to hematopoietic signaling in stem/progenitor cells. Mice were treated with 40 mg/kg  $\alpha$ -tocopherol i.p. every other day for 2 weeks. (A) The phosphorylated form of ERK1/2 was measured at different times in HP populations by flow cytometry. Treatment with  $\alpha$ -tocopherol reduced the activation of ERK1/2. Data are presented as the Gm  $\pm$  SEM of FL1 intensity values;  $n = 6$ . \* $P < 0.05$ , ANOVA. (B) In addition, other proteins related to hematopoietic proliferation, such as PKC and STAT-5, but not AKT, showed reduced activation on the 15th day of  $\alpha$ -tocopherol treatment. Filled histograms, Control; open histograms,  $\alpha$ -tocopherol-treated group. The images are representative of six independent experiments.







**Figure 4.** Treatment with  $\alpha$ -tocopherol enhances ERK1/2 activation in response to IL-3 in HSC/HP cells. Mice were treated with 40 mg/kg  $\alpha$ -tocopherol i.p. every other day for 2 weeks. After treatment, hematopoietic cells were collected, stimulated with 10 ng/ml IL-3 for 5 min, fixed, permeabilized, and labeled. The fluorescence intensity of p-STAT-5 and p-ERK1/2 was measured, and data are presented as the Gm.  $\alpha$ -Tocopherol treatment did not significantly affect the activation of (A and B) STAT-5 but enhanced the activation of (C and D) ERK1/2 in response to IL-3 in LSK and HP cells. The data were normalized to the basal phosphorylation levels of each protein. The data are expressed as mean  $\pm$  SEM. \* $P$  < 0.05, Student's  $t$  test;  $n$  = 7. (B and D) Representative histograms of p-ERK1/2 and p-STAT-5 are shown. Filled histograms, Unstimulated cells; open histograms, IL-3-stimulated cells.

and HP cells (Fig. 4C and D). In addition, the expression of IL-3Rs in HSC/HP cells was not altered upon treatment with  $\alpha$ -tocopherol (Supplemental Fig. 3), suggesting that ERK1/2 activation in response to IL-3 is enhanced by the modification of intracellular signaling pathways.

### The MEK inhibitor PD98059 abolishes $\alpha$ -tocopherol-induced differentiation but not proliferation

In the hematopoietic system, the MAPK family plays an essential role in controlling the proliferation and differentiation of hematopoietic cells and myeloid progenitors [15, 28, 29, 34, 35]. To confirm the role of ERK1/2 in  $\alpha$ -tocopherol-dependent effects on LSK and HP cells, the MEK inhibitor PD98059 was used. Mice were injected i.p. with PD98059, 1 h before each injection of  $\alpha$ -tocopherol.

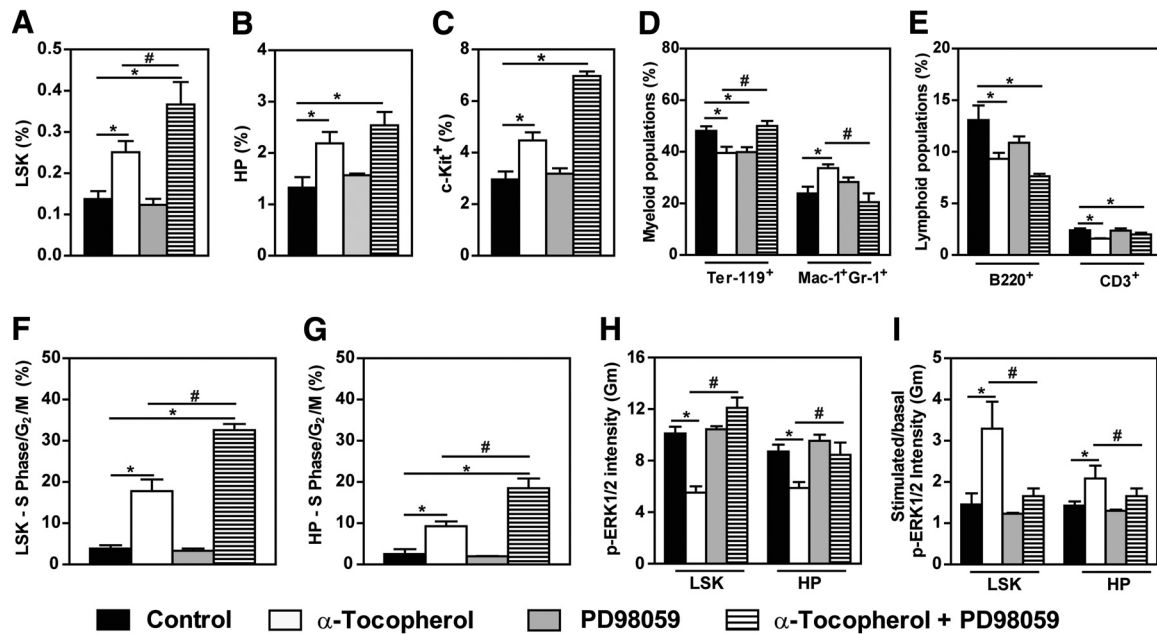
The presence of PD98059 inhibited some of the effects observed in  $\alpha$ -tocopherol-treated mice. PD98059 restored the normal proportions of Ter-119<sup>+</sup> and Gr-1<sup>+</sup>Mac-1<sup>+</sup> cells (Fig. 5D) but failed to restore the normal proportion of lymphocytes (Fig. 5E). Interestingly, PD98059 did not restore the proportion of primitive hematopoietic cells (Fig. 5A–C) or basal-proliferative LSK or HP cells (Fig. 5F and G). In contrast, the proportions of LSK and HP cell populations were increased (Fig. 5A and B), and the percentage of these populations in the S/G<sub>2</sub>/M phase of the cell cycle was also enhanced (Fig. 5F and G).

In addition, the activation of ERK1/2 by IL-3 was quantified *ex vivo* in mice treated with PD98059. The BM cells of each group were stimulated by IL-3 in a normal, physiological solution. The pretreatment of mice with the MEK inhibitor restored the normal basal phosphorylation of ERK1/2 (Fig. 5H) and its typical activation in response to IL-3 (Fig. 5I). Thus, the enhancement

of ERK1/2 activation in response to IL-3 by  $\alpha$ -tocopherol seems to be related to myeloid differentiation, as the inhibition of MEK, which is upstream of ERK1/2, restored the normal proportion of myeloid cells (Fig. 5D), the normal basal phosphorylation of ERK1/2 (Fig. 5H), and the typical activation of ERK1/2 in response to IL-3 (Fig. 5I). These findings are in agreement with those of Geest et al. [28], who described the role of MEK activation in the expansion of progenitor cells and neutrophil differentiation. Therefore, compensatory activation of ERK1/2 in response to IL-3 can explain the enhanced granulocytic/monocytic differentiation observed upon  $\alpha$ -tocopherol treatment. Similar effects on myeloid differentiation have also been observed in mice treated with  $\alpha$ -tocopherol succinate and exposed to  $\gamma$ -radiation [26, 36, 37].

The high proliferative state of HSC/HP cells in  $\alpha$ -tocopherol-treated mice did not seem to be related to the ERK1/2 pathway, as the MEK inhibitor PD98059 neither reduced the elevated numbers of HSC/HP cells nor reversed their enhanced, proliferative state; rather, PD98059 enhanced the increase in stem/progenitor cells in BM (Fig. 5F and G). The inability of this MEK inhibitor to affect proliferation could be related to the presence of other cytokines released upon  $\alpha$ -tocopherol treatment. Tocopherols induce an increase of the serum levels of cytokines, such as G-CSF, resulting in increased numbers of HSC/HP cells in the peripheral blood [1, 26]. The release of G-CSF or other cytokines is likely responsible for the expansion of HP cells independent of the ERK1/2 pathway. Thus, PD98059 did not abolish the  $\alpha$ -tocopherol-dependent expansion of hematopoietic stem/progenitor cells.

The importance of  $\alpha$ -tocopherol as a biological antioxidant is widely recognized [38]. As  $\alpha$ -tocopherol can act as an antioxidant to decrease ROS levels, oxidative stress markers and the activation



**Figure 5. The MEK inhibitor PD98059 abolishes  $\alpha$ -tocopherol-induced differentiation to the granulocytic/monocytic lineage but not proliferation.** Mice were treated with 40 mg/kg  $\alpha$ -tocopherol i.p. every other day for 2 weeks. PD98059 (10 mg/kg) was administered i.p. 1 h before each dose of  $\alpha$ -tocopherol. (A–E) Percentages of cells within different hematopoietic populations were quantified by staining with mAb. The MEK inhibitor PD98059 restored the normal percentages of (A) LSK, (B) HP, (C) c-Kit<sup>+</sup>, and (D) erythroid and granulocytic/monocytic cells but did not restore the percentage of (E) lymphoid cells. (F and G) Evaluation of the cell cycle in HSC/HP cells using SYTO Green showed increased proliferation upon treatment with  $\alpha$ -tocopherol plus PD98059. (H and I) Treatment with  $\alpha$ -tocopherol plus PD98059 restored (H) the normal basal phosphorylation of ERK1/2 and (I) ERK1/2 activation in response to IL-3 in HSC/HP cells. The data are expressed as mean  $\pm$  SEM of four to six independent experiments. \*,  $P < 0.05$ , ANOVA. \*Statistical analysis was performed against the control group; #statistical analysis was performed against the  $\alpha$ -tocopherol-treated group.

of antioxidant enzymes were quantified. Despite the previously demonstrated antioxidant activity of  $\alpha$ -tocopherol, neither alterations in ROS levels nor activation of antioxidant mechanisms were observed in the different hematopoietic populations upon  $\alpha$ -tocopherol treatment (Supplemental Figs. 4 and 5). This result suggested that the effects induced by  $\alpha$ -tocopherol in BM cells are independent of its antioxidant capacity; however, further investigation about the role of ROS in the effects of  $\alpha$ -tocopherol in the hematopoietic stem/progenitor population is necessary. It is evident that the biological role of  $\alpha$ -tocopherol needs to be re-examined, as its antioxidant and pro-oxidant properties are not sufficient to explain all of its effects. It is possible that discovering novel signaling pathways and genes influenced by  $\alpha$ -tocopherol treatment may help clarify the relationship between molecular and clinical events.

## CONCLUDING REMARKS

In conclusion, we report here that  $\alpha$ -tocopherol affects the hematopoietic compartment without altering the redox state of the BM cells.  $\alpha$ -Tocopherol induces BM hyperplasia, increasing the proportion of HSC/HP cells and inducing granulocytic/monocytic differentiation. Myeloid differentiation occurs through the increased activation of ERK1/2 in response to IL-3, whereas the proliferation of stem/progenitor cells occurs by an unidentified mechanism.

## AUTHORSHIP

A.N.P. and C.M.V.B. performed flow cytometry. A.N.P., L.L., and V.D'A. performed redox assays. A.A.F.S.M. and A.M. performed HPLC and analyzed data. H.R.C.S. performed histologic evaluation. A.N.P. and E.J.P-G. analyzed the data and wrote the paper. E.J.P-G. and A.T.F. designed and conducted the study.

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## KEY WORDS:

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