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Cryptotanshinone and tanshinone IIA enhance IL-15-induced natural killer cell differentiation

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ABSTRACT

Natural killer (NK) cells are a subset of lymphocytes crucial for innate and adaptive immune responses. Here we show a stimulatory effect of cryptotanshinone (CTS) and tanshinone IIA (TS), isolated from *Salvia miltiorrhiza Bunge*, on the differentiation of NK cells. In the presence of IL-15, tanshinones increased NK cell maturation, NK cell differentiation and the expression of several transcription factors, including Id2, GATA3, T-bet, and Ets-1. Additionally, tanshinones increased p38 MAPK phosphorylation during NK cell differentiation. Furthermore, the p38 inhibitor SB203580 blocked the developmental effects of the tanshinones and suppressed Id2, T-bet, and Ets-1 expression during NK cell differentiation. These results suggest that tanshinones significantly increased IL-15-induced NK cell differentiation via enhancing the p38 phosphorylation and the expression of transcription factors.

1. Introduction

Natural killer (NK) cells are innate lymphocytes specialized in cytokine production and cytotoxicity toward tumor and virus-infected cells. NK cells develop in the bone marrow (BM) from hematopoietic stem cells (HSCs) via lymphoid precursors [1]. Their cytotoxic function is crucial to many processes, such as tumor immunosurveillance [2] and elimination of microbial infection [3]. The BM is the main site for NK cell development in the adult mouse and is where $CD122^{+}NK1.1^{-}$ NK cell precursors (NKPs) derived from HSCs give rise to immature NK (iNK) and mature NK (mNK) cells. IL-15 is essential for the priming of NK cells and to maximize their effector functions [4,5]. However, the action of IL-15 is not confined to NK cells and the molecular basis of NK cell development is not well understood [6,7].

Several transcription factors (TFs), such as Ets-1 [8], Id2 [9], GATA-3 [10], PU.1 [11], T-bet [12], and IRF2 [13], have been reported to regulate NK cell maturation. For example, spleens of $Id2^{-/-}$ mice have only 10% of the number of mNK cells that are found in wild-type spleens, but have an equal number of NKPs or iNK cells in the BM. Additionally, the loss of GATA-3 has been shown to hinder the development of mNK cells and lead to defective IFN-γ production.

Tanshinones, such as cryptotanshinone (CTS) and tanshinone IIA (TS), are the major lipophilic constituents extracted from the rhizome of *Salvia miltiorrhiza Bunge,* which has been used in Chinese traditional medicine for multiple therapeutic remedies and has been shown to have antibacterial, antioxidant, and antineoplastic activities. Additionally, tanshinones have anti-inflammatory and anti-immunological effects. TS, CTS, and dihydrotanshinone significantly inhibit interleukin-12 (IL-12) and interferon-gamma (IFN-γ) production [14]. Furthermore, tanshinones induced differentiation of human acute myeloid leukemia HL60 cells into monocyte/macrophage cells. Recently, it has been reported that TS enhances BMP-2-stimulated commitment of C2C12 cells into osteoblasts via p38 activation [15]. This evidence indicates that the tanshinones may have wide effects on immunological system. However, there have been no studies on whether the tanshinones have effects on NK cell differentiation.

To elucidate whether tanshinones affect NK cell differentiation, we examined the effects of TS and CTS on *in vitro* NK cell differentiation. The results show that IL-15-induced NK cell maturation was significantly augmented by these tanshinones. In addition, it was observed that tanshinones increased expression of several TFs and activation of p38 MAPK during NK cell differentiation.

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2. Materials and methods

2.1. Mice

C57BL/6 mice were purchased from KoaTech (Pyeongtaek, Korea). The mice were housed under specific pathogen-free (SPF) conditions. All animal procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee, Korea Research Institute of Bioscience and Biotechnology (KRIBB-AEC-11050).

2.2. Reagents and antibodies

Tanshinones (TS and CTS) were kindly provided by Dr. Byoung-Mog Kwon (Laboratory of Chemical Genomics and Biology, Korea Research Institute of Bioscience and Biotechnology). Anti-mouse NK1.1-PE, CD3-FITC, CD122-FITC, Streptavidin-PE, NKG2A/C/E-FITC, NKG2D-PE, and DX5 (CD49b)-PE were purchased from Becton Dickinson (BD) Pharmingen (San Diego, CA). Recombinant murine IL-2, IL-12, SCF, Flt3L, IL-7, and IL-15 and human IL-2 and IL-12 were purchased from PeproTech (Rocky Hill, NJ). Antibodies against STAT3, p-STAT3^{Tyr705}, STAT5, p-STAT5^{Tyr674}, ERK, p-ERK^{Thr202,Tyr204} were purchased from Cell Signaling Technology. Antibodies against cyclin E, p38, p-p38^{Thr180,Tyr182}, and β-actin were purchased from Santa Cruz Biotechnology, Inc. Goat-anti-rabbit and anti-mouse horseradish peroxidase (HRP) conjugates were purchased from Jackson ImmunoResearch Laboratories, Inc. The p38-specific inhibitor SB203580 was purchased from Calbiochem.

2.3. In vitro differentiation of NK cells from HSCs

NK cell differentiation from HSCs was performed as described previously [16]. In brief, HSCs were isolated from total BM cells of mice. The erythrocytes in the BM cells were removed by treatment with an ACK solution. Then, c-Kit^{+}Lin^{-} (depletion of B cells [B220], T/NK cells [CD2], NK/NKT cells [NK1.1], monocytes [CD11b], granulocytes [Gr-1], and erythrocytes [TER-119]) HSCs were purified using the MACS Cell Separation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Thereafter, the depleted cells were incubated with c-Kit microbeads and sorted by MACS. The indicated antibodies and microbeads were purchased from BD Pharmingen. The purified HSCs were plated onto a 24-well plate (BD) at 1×10^6 cells/well and cultured with RPMI media containing 10% heat-inactivated fetal bovine serum (FBS), indometacin (2 μg/ml, Sigma–Aldrich, St. Louis), gentamicin (20 μg/ml, Sigma–Aldrich), 30 ng/ml SCF, 50 ng/ml Flt3L, and 0.5 ng/ml IL-7 for 7 days. To generate the mNK cells, pNK cells were harvested and re-plated at 1×10^6 cells/well. These cells were cultured in the presence of IL-15 (25 ng/ml) alone or combined with tanshinones (CTS/TS) for another 5–6 days.

2.4. Quantitative real-time PCR

Total RNA was extracted from 1 to 3×10^6 cells using TRIzol (Invitrogen, Carlsbad, CA). Reverse transcription was performed using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) with random primers (Takara Bio, Otsu, Japan). Quantitative PCR analysis was performed using a Dice TP 800 Thermal Cycler and SYBR Premix Ex Tag (Takara Bio). Values for each gene were normalized to the amount of the GAPDH transcript. The sequences of the primers used for the PCR reaction are as follows: ID2, 5′-GCAAAAGAAAGAGAAAGTAAGA-3′ and 5′-GAACACGGACATCAGCATC-3′; GATA-3, 5′-TCACACACTCCCTGC CTTCT-3′ and 5′-CACCCCATTACCACCTATCC-3′; T-bet, 5′-CCAAGAC-CACATCCACAAAC-3′ and 5′-CAACCAGCACCAGACAGAGA-3′; PU.1, 5′-GGTCATCTTCTTGCGGTTCT-3′ and 5′-CCTTCCAGTTCTCGTCCA-3′; TRAF, 5′-CAGACAGAGGAGACAGCAGGA-3′ and 5′-CACACAG AAGAGGAACTA-3′; ETS-1, 5′-CAGCCTCAAGATCATCAGCA-3′ and 5′-GTCTTCGGGTGGCAGTGAT-3′.

2.5. NK cell functional assays

Cytotoxicity was examined using a standard 4-h ^{51}Cr-release assay. ^{51}Cr-labeled target cells (1×10^4 cells/well) and serial diluted cells were used in triplicate. The radioactivity of the supernatant containing ^{51}Cr was measured using a γ-counter. The percentage of specific lysis was calculated using the formula: (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100. For evaluation of IFN-γ production, IL-15-activated cells were stimulated in triplicate for 20 h with plate-bound antibodies to IL-12. The secretion of IFN-γ (Assay Designs) into the supernatant was measured by ELISA.

3. Results

3.1. Effect of tanshinones (TS/CTS) on IL-15-mediated NK cell differentiation from HSCs

To determine the effects of tanshinones on IL-15-mediated NK cell differentiation, we treated cells with tanshinones during the pNK stage of the *in vitro* culture system. After 6 days, we analyzed the NK cell populations by flow cytometry. When pNK cells were treated with tanshinones in the presence of IL-15, the population of CD3-NK1.1 + NK cells increased approximately 1.5-fold compared with those treated with IL-15 alone (Fig. 1A). IL-15 induced NK cell differentiation in dose-dependent manner (Supplementary Fig. S1). However, at low concentrations of IL-15, NK cell viability was decreased in the presence of tanshinones (data not shown) [16]. Next, to measure the optimal concentration of CTS and TS, pNK cells were cultured with various concentrations of CTS and TS in the presence of IL-15. NK cell maturation was highest at 5 μM CTS and 10 μM TS (Fig. 1B; 2 μM CTS treated versus vehicle treated, $p < 0.05$; 5 μM CTS treated versus vehicle treated, $p < 0.05$; 10 μM CTS treated versus vehicle treated, $p < 0.05$; 10 μM TS treated versus vehicle treated, $p < 0.005$). In addition, we analyzed NK populations by other NK markers, including CD122, NKG2A/C/E, NKG2D, and DX5 (Fig. 1C). As a consequence of the increased maturation of NK cells, the expression of CD122, NKG2A/C/E, NKG2D, and DX5 were also considerably increased in CTS and TS treated differentiating NK cells. These results indicated that tanshinones enhanced differentiation of NK cells *in vitro*.

3.2. Tanshinone (TS/CTS) enhancement of NK cell maturation is dependent on IL-15

Next, to determine whether the effects of tanshinones on NK differentiation were IL-15-dependent, we evaluated tanshinone-induced NK cell differentiation in the presence or in the absence of IL-15 (Fig. 2A; IL-15 treated versus vehicle treated, $p < 0.005$; CTS treated versus vehicle treated, $p < 0.05$; IL-15/CTS treated versus vehicle treated, $p < 0.005$; IL-15/TS treated versus vehicle treated, $p < 0.005$). Compared to the tanshinone alone-treated cells, NK cell differentiation was significantly increased when IL-15 was added. We measured cell viability during NK cell differentiation. Cell viability decreased gradually during the NK cell differentiation (Fig. 2B), and compared with the IL-15-treated groups, non-IL-15-treated groups showed much less cell viability. These results confirmed that IL-15 is important for NK cell survival [17,18]. In the

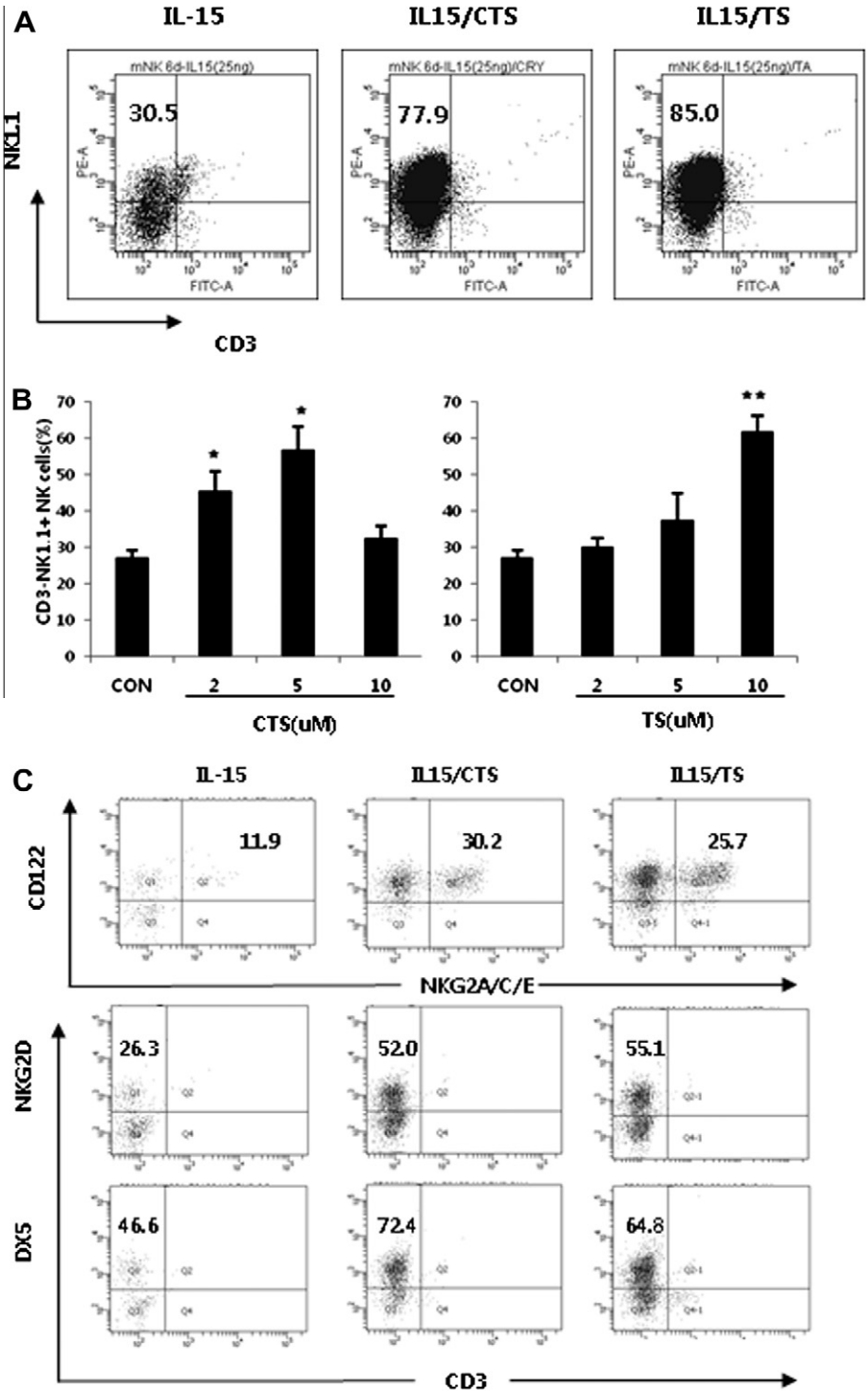

Fig. 1. Tanshinones enhance IL-15-driven NK cell differentiation. (A) Purified HSCs were cultured with SCF, Flt3L, and IL-7 for 7 days. These pNK cells were harvested and further differentiated into mNK cells in the presence of IL-15 (25 ng/ml), or tanshinones (TS, 10 μM and CTS, 5 μM). After 6 days, the cells were analyzed by FACS. (B) pNK cells were cultured at various concentrations of tanshinones in the presence of IL-15 (25 ng/ml) for 6 days. The percentage of $NK1.1^{+}CD3^{-}$ cells was assayed by FACS. $^{*}p < 0.05$, $^{**}p < 0.005$. (C) These cultured cells were stained with anti-NK1.1, CD3e, CD122, NKG2A/C/E, NKG2D, and DX5 mAbs. Then, the cells were analyzed by flow cytometry. The data are representative of three independent experiments, and the error bars represent the SD of triplicates.

absence of IL-15, there were much less cell numbers of NK cells compared those in the presence of IL-15 (Fig. 2C).

To determine whether NK cells differentiated with tanshinones have full functional activity, we investigated their function by the production of IFN-γ or their cytolytic activity. The mNK cells differentiated with IL-15 in the presence of tanshinones produced much more IFN-γ and exerted higher levels of cytolytic activity than the mNK cells differentiated with IL-15 alone (Fig. 2D; TS treated versus vehicle treated, $p < 0.05$; IL-15/CTS treated versus vehicle treated, $p < 0.005$; IL-15/TS treated versus vehicle treated, $p < 0.005$). Furthermore, we treated fully differentiated mNK cells with tanshinones or DMSO (control) for 20 h and found that tanshinones had

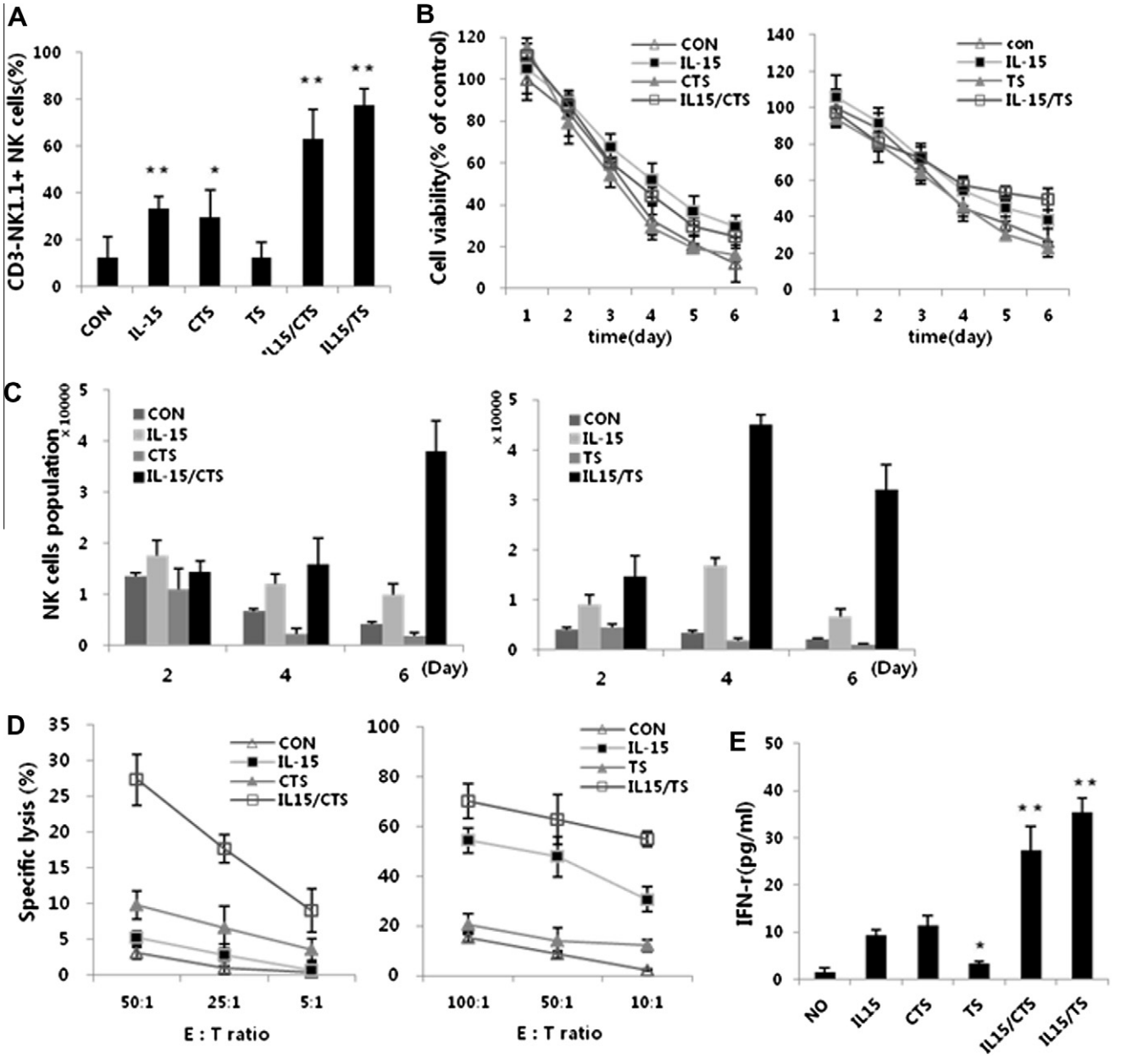

Fig. 2. Tanshinone-induced NK cell differentiation depends on IL-15. (A) pNK cells were differentiated in the absence or presence of IL-15 (25 ng/ml), and/or tanshinones (TS, 10 μM and CTS, 5 μM) for 6 days. The percentage of $NK1.1^{+}CD3^{-}$ cells was analyzed by FACS. $^{*}p < 0.05$, $^{**}p < 0.005$. (B) pNK cells (1×10^{6} cells/well) were cultured in a 24 well plate and then medium was replaced with RPMI containing 10% FBS in the absence or presence of IL-15 and/or tanshinones for 6 days. A cell viability assay was then performed during NK cell differentiation. (C) The total cell number was counted using a microscope counting chamber (hemocytometer) and $NK1.1^{+}CD3^{-}$ cell numbers were calculated depending on the percentages analyzed by FACS. (D) For IFN-γ production determination, differentiating NK cells were stimulated with IL-12 (10 ng/ml). After 20 h, IFN-γ releases into the supernatant were measured by ELISA. (E) NK cells were differentiated *in vitro* in the presence of IL-15 and/or tanshinones, and the cytolytic activity was evaluated with ^{51}Cr-release assay at the indicated ET ratio. The assay was performed in triplicate and the data represent means ± SD.

no significant effect on NK cell cytolytic activity or IFN-γ secretion (Supplementary Fig. S2). Overall, these results indicate that NK cell differentiation is induced by tanshinones in the presence of IL-15, but does not affect the functional activity of fully differentiated mNK cells.

3.3. Tanshinones (TS/CTS) treatment increased mRNA expression of TFs during NK cell differentiation

Previous studies have reported that several TFs, including Ets-1, Id2, GATA-3, T-bet, IRF2, and PU.1, are essential for the maturation of NK cells [19]. Therefore, we examined the effect of CTS/TS treatment on mRNA expression of TFs during NK cell differentiation. CTS treatment increased mRNA expression of T-bet, Id2, GATA-3, and Ets-1 in the presence of IL-15, whereas the expression of others, such as TRAF2 and PU.1, were not significantly affected (Fig. 3). Additionally, TS treatment increased mRNA expression of Id2, Ets-1, T-bet, and TNFR2 in the presence of IL-15 (Supplementary Fig. S3). Furthermore mRNA expression of Id2 was induced by CTS or TS alone. These results suggest that Id2, T-bet, GATA-3, TNFR2, and Ets-1 are involved in CTS/TS plus IL-15-induced NK cell differentiation and that the Id2 gene is a major target of CTS/TS.

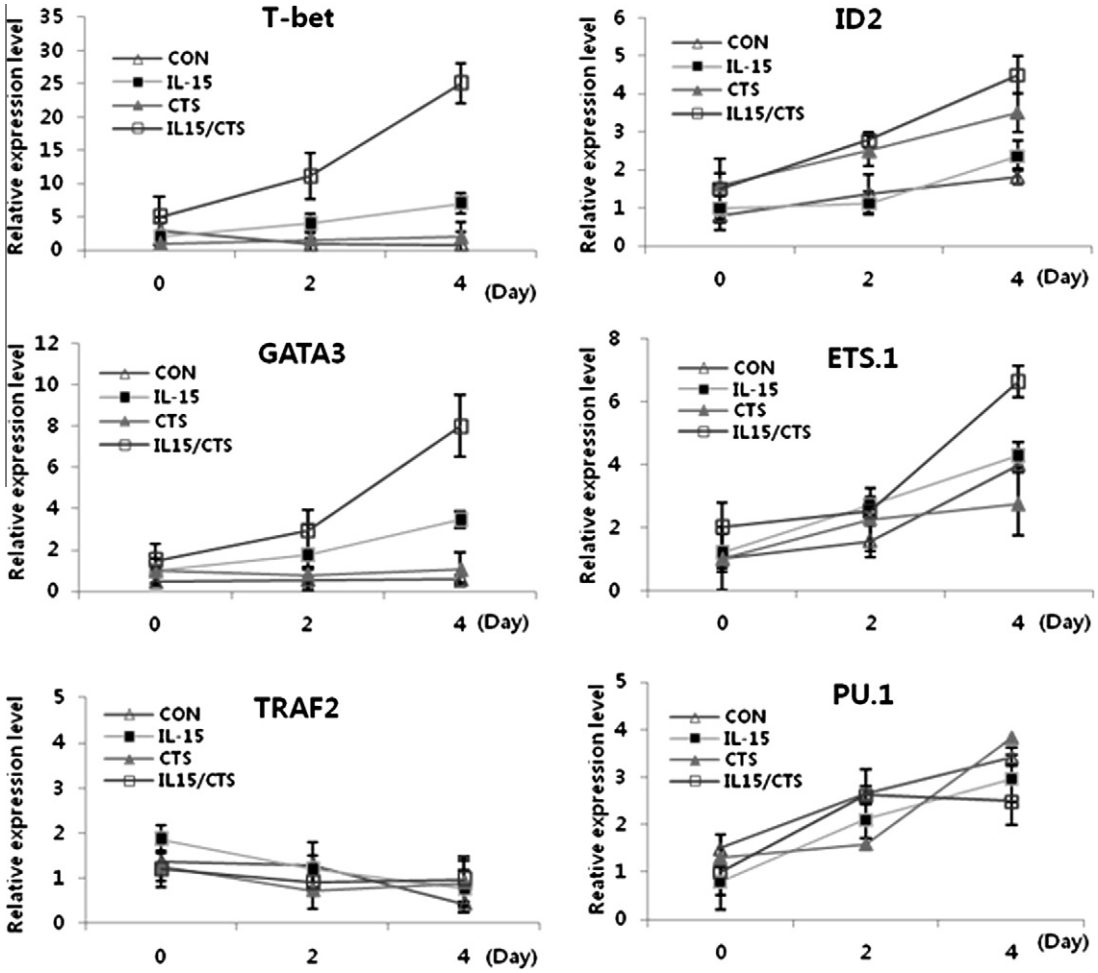

Fig. 3. Tanshinone regulates TFs related to NK cell maturation. mNK cells were differentiated *in vitro* as described. After the addition of cytokines, the cells were harvested on days 0, 2, and 4. The expression of Ets-1, TRAF-2, PU.1, Id2, T-bet, and GATA-3 was analyzed by real-time quantitative PCR. The experiment shown is representative of three independent experiments.

3.4. Tanshinones stimulate the phosphorylation of p38 MAPK during NK cell maturation

Next, to investigate the mechanisms of NK cell differentiation enhanced by tanshinones, we examined the phosphorylation of p38 MAPK in CTS- or TS-mediated NK cell maturation. It is known that TS enhances osteoblast differentiation via p38 activation [15]. Phosphorylation patterns of intracellular MAPK signaling components, including ERK, JNK, and p38, were measured by western blotting. The data showed that tanshinone treatment did not increase the total protein level or the phosphorylation of ERK, Akt and STATs (Supplementary Fig. S4), but did increase the phosphorylation of p38. Maximal activation of p38 was observed in the presence of both tanshinones and IL-15 (Fig. 4A). To determine whether enhanced p38 phosphorylation affected NK cell differentiation in tanshinones-treated cells, the cells were pretreated with p38 MAPK inhibitor SB203580 for 1 h prior to the addition of CTS or CTS plus IL-15. SB203580 significantly reduced NK cell maturation, but the MEK inhibitor U0126 had no effect on it (Fig. 4B; SB203580 treated versus vehicle treated, $p < 0.005$). Furthermore, as shown in Fig. 4C, SB203580 suppressed Id2, T-bet, and ETS-1 expression in the presence of CTS plus IL-15 and it inhibited Id2 expression in the presence of CTS alone (Fig. 4C; SB203580 treated versus vehicle treated, $p < 0.05$). Therefore, these results suggested that p38 signaling is involved in tanshinones-induced NK cell differentiation.

4. Discussion

Tanshinones regulate immune responses, such as anti-inflammatory and anti-immunological effects [14,20]. Additionally, it has been reported that tanshinones induce differentiation in human acute promyelocytic leukemia (APL) cells [21] and osteoblasts [15]. In the present study, we found that IL-15-induced NK cell differentiation was enhanced by tanshinones. Indeed, the expression of NK cell makers including NK1.1, DX5, NKG2D, and NKG2A/C/E increased in the presence of IL-15 plus tanshinones.

NK cell maturation is dependent on IL-15, which controls NK differentiation, proliferation, and survival in the periphery [6,18]. It has been reported that IL-15 and its receptor IL-15Rα are coexpressed by the same cells, which trans-present IL-15 to NK cells during development [22,23], and that IL-15$^{-/-}$ mice and IL-15 receptor α-chain (IL-15R)-deficient (Il15ra$^{-/-}$) mice [24] lack peripheral NK cells and detectable NK cell-mediated cytotoxicity. Our results show that NK cell differentiation was significantly higher when tanshinones plus IL-15 were added. In addition, tanshinones plus IL-15-driven mNK cells produced much more IFN-γ than tanshinone alone-driven mNK cells. These results indicate that IL-15 signals are augmented in the presence of tanshinones during NK-cell development.

Several transcription factors are known to control the maturation of NK cells, including GATA-3, ETS-1, PU.1, IRF2, T-bet, and

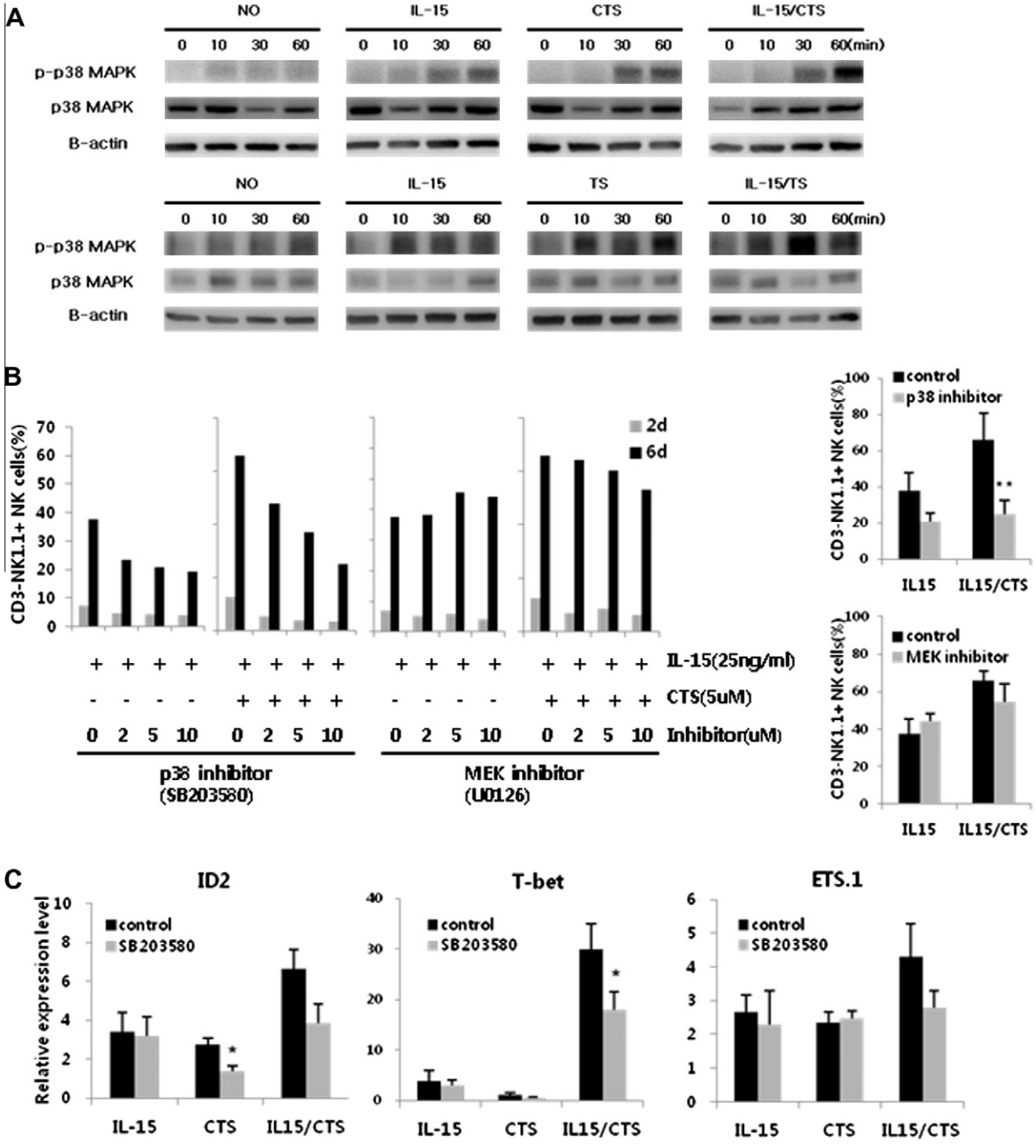

Fig. 4. Tanshinones induce phosphorylation of p38 during NK cell maturation. (A) pNK cells were treated with IL-15 and/or tanshinones and were harvested at 10, 30 and 60 min after treatment. Activation of p38 MAPK was measured by western blotting using antibodies against phospho-p38. (B) Cells were pretreated with the MEK inhibitor U0126 or p38 MAPK inhibitor SB203580 for 1 h prior to the addition of IL-15 or CTS plus IL-15, then cultured as described in the Section 2. The cells that were cultured for 6 days were stained with anti-NK1.1 and CD3 and assayed by flow cytometry. The indicated numbers show the percentages of the $NK1.1^{+}CD^{3-}$ population. $^{**}p < 0.005$. (C) Cells pretreated with the p38 MAPK inhibitor SB203580 were cultured in the presence of IL-15 and/or CTS. The cells were harvested 3 days later and the mRNA expression levels of Id2, T-bet and ETS-1 were compared using real-time quantitative PCR. The data represent means ± SD from three independent experiments. $^{*}p < 0.05$.

Id2. GATA-3 is essential for thymic NK cells [25] and modulates the function of mature NK cells. ETS-1, IRF2 [26], and T-bet [12,27] are more specifically required for the later stages of NK-cell differentiation and function. We found that the combination of tanshinones and IL-15 significantly increased T-bet, GATA-3, Id2, and ETS-1 expression. Among these factors, only Id2 expression was induced by tanshinones alone. Previous studies demonstrated that the major function of Id2 in lymphoid differentiation is to control E protein activity. During NK cell development, Id2 and Id3 may cooperatively perform this role, and Id2 becomes essential only at the mNK cell stage when Id3 mRNA is down-regulated. Thus, tanshinones may be a positive regulator for the expression of TFs during NK cell differentiation.

Next, we considered the possibility that tanshinones activate IL-15 signaling during NK cell maturation. IL-15 induces the activation of JAK kinases as well as the phosphorylation and activation of the transcription activator STAT5 in NK cells. Previous studies reported that STAT5$^{-/-}$ mice exhibited a severe loss of NK cell maturation [28]. Additionally, other studies reported that cryptotanshinone inhibits STAT3 function by blocking its dimerization in DU145 prostate cancer cells [29]. Therefore, we examined the level of STAT5 and STAT3 phosphorylation in tanshinone-treated NK cells to assess the effect of tanshinones on IL-15R signaling during NK cell development. Treatment with tanshinones did not affect the levels of phosphorylated STAT5 or inhibit STAT3 phosphorylation in differentiating NK cells (Supplementary Fig. S3). However, we found that tanshinone treatment significantly increased p38 phosphorylation during IL-15-induced NK cell maturation. p38 is a member of the mitogen-activated protein (MAP) kinase family and phosphorylation of p38 is essential for executing biological functions, including the regulation of the cell cycle, cell development, cell differentiation, tumorigenesis, apoptosis, and immune responses [30]. Like other MAP kinases, p38 kinases are primarily activated by a tri-kinase cascade. At the top of p38 signaling pathways are MAP3Ks, followed by MKKs. The MAP3Ks in this cascade include TAK1, ASK1, and MLK3 [30,31]. Previous studies reported that p38 activity is required in T cell development. The MKK6-p38 signaling pathway is critically involved in the early stages of thymocyte development [32]. Meanwhile, it is known that tanshinones enhanced neuron regeneration via upregulation of FGF2 expression. FGF2 regulates MEKK1 (MAPK kinase kinase) and MEKK1's downstream JNK, ERK, p38 for controlling uPA activity [33] . It was also proposed that tanshinone may activate p38 through some unidentified protein kinases which are sensitive to SB203580 or TAB1-associated pathways during ischemic activation of p38. In addition, tanshinone IIA enhanced BMP-2-stimulated osteoblast differentiation via p38 activation. Tanshinone IIA-induced p38 activation activates Smad-Runx2 activity for osteoblastic precursor cell differentiation via the regulation of osteogenic gene expression [15]. In this study, we found that tanshinone treatment significantly increased p38 phosphorylation during IL-15-induced NK cell maturation. SB203580 treatment inhibited tanshinone-induced NK cell differentiation and inhibited the expression of Id2, T-bet, and ETS-1. These results clearly showed that p38 is involved in the effects of tanshinones on NK cell differentiation, perhaps via MKK-p38 signaling pathway.

In conclusions, tanshinones enhanced IL-15-induced NK cell differentiation through p38 activation. These activations subsequently induced the expression of TFs, the inducers of NK cell maturation. Tanshinones, as novel modulators of NK differentiation, are potential candidates for tumor therapeutics by enhancing anti-tumor immune responses.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.093.

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