

## Thalidomide suppresses melanoma growth by activating natural killer cells in mice

AI KAWAMATA, DAISUKE ITO, TAKESHI ODANI, TOMOHIDE ISOBE,  
MASAYASU IWASE, MASASHI HATORI and MASAO NAGUMO

Department of Oral and Maxillofacial Surgery, School of Dentistry, Showa University, Tokyo, Japan

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**Abstract.** Although thalidomide (Thd) is being extensively investigated for its effects on cytokine production and T cell costimulation, it is poorly understood whether it is capable of modulating the activities of natural killer (NK) cells. In this study, Thd effects on NK cell activity were examined with a murine model of melanoma, which is mostly rejected by NK cell-dependent mechanism. Administration of Thd significantly ( $p < 0.01$  on Day 21) suppressed the growth of subcutaneous B16F1 melanoma. In Thd-treated mice, marked splenomegaly and augmented splenocyte count were observed. Additionally, the percentage of splenic NK1.1<sup>+</sup> cells was elevated to approximately 2.5-fold within 10 days after Thd treatment. The expression of interferon inducible protein (IP)-10, interferon (IFN)- $\gamma$ , interleukin (IL)-12 and IL-18 was remarkably upregulated. Production of the cytotoxic molecule perforin was also augmented. These data suggest that Thd strongly activates NK cell activity in mice, possibly resulting in enhanced tumor surveillance defense.

### Introduction

Recent reports have highlighted the resurgence of thalidomide (Thd) as a clinically effective drug (1-3) because of its anti-angiogenic (4) and anti-inflammatory (5) effects. This drug had been long withdrawn because of its strong teratogenicity (1-3), but now is the drug of choice for treating leprosy and has also been used with some success to treat rheumatoid arthritis, chronic tuberculosis, Behçet's disease, Crohn's disease (1), cutaneous lupus erythematosus (6) and various forms of malignancies, including multiple myeloma (MM) (7). Its mechanism of action has been insufficiently understood, but was initially thought to be through the suppression of

vascular endothelial growth factor, tumor necrosis factor (TNF)- $\alpha$ , and interleukin (IL)-6 (5,8,9). However, more recent studies have suggested that Thd may also act as an inducer of T cell stimulatory signals, inducing T-cell proliferation associated with interferon (IFN)- $\gamma$  and IL-2 production (10,11). Although these observations suggest that Thd may have a direct effect on T cell immune systems, the Thd effect on natural killer (NK) cell-mediated immunity is largely unknown.

The NK cell population is a major component of the innate immune surveillance mechanism and can also suppress the development of certain malignant tumors (12-15). Its development, recruitment, and cytotoxic activity are upregulated by certain immunomodulatory agents, such as poly-IC:LC, resulting in augmented tumor suppression (15,16). It was recently reported that Thd augmented the number of CD3<sup>+</sup>CD56<sup>+</sup> NK cells and *in vitro* cytotoxicity of peripheral blood mononuclear cells from patients with MM (17). However, there has been no direct evidence indicating that Thd works as an antitumor drug through augmentation of NK cell activity *in vivo*.

B16 is an aggressive and highly metastasizing melanoma cell line established from the C57BL/6 mouse (18). Its subline B16F is considered to be less immunogenic because of its lower class I MHC expression, and fails to induce adequate cytotoxic T lymphocyte activity (19,20). Therefore, the rejection of B16 melanoma has been considered to be entirely mediated by NK cell immunity. In fact, mice with impaired NK cell activity display enhanced tumor growth and metastasis of B16F10 *in vivo* (15). Thus, the B16 melanoma model is a good tool for the investigation of the Thd effect on NK cell antitumor function.

In this study, we investigated the immunomodulatory effects of Thd on NK cells and antitumor effects using the murine model of malignant melanoma.

### Materials and methods

**Animals.** C57BL/6J mice were purchased from Sankyo Laboratory Service (Tokyo, Japan). Animals were bred in specific pathogen-free conditions and used for experiments between 6- and 12-weeks of age.

**Cell line.** A B16F1 murine malignant melanoma cell line (C57BL/6) was purchased from ATCC (Manassas, VA) and maintained at 37°C in a 5% CO<sub>2</sub> incubator and grown in

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*Correspondence to:* Dr Ai Kawamata, Department of Oral and Maxillofacial Surgery, School of Dentistry, Showa University, 2-1-1 Kitasenzoku, Ohta-ku, Tokyo 145-8515, Japan  
E-mail: ai@senzoku.showa-u.ac.jp

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D-MEM (Life Technologies, Gaithersburg, MD) supplemented with heat-inactivated 10% FBS and antibiotics.

**Thd treatment of cultured cells.** B16F1 cells were seeded onto 6-well culture plates ( $2 \times 10^6$ /well) and thalidomide dissolved in dimethylsulfoxide (DMSO, Sigma, St. Louis, MO) was added to the culture (final concentration 10–50  $\mu\text{g/ml}$ ). Cells were incubated for 48 h at 37°C, collected by trypsinization, and then subjected to flow cytometry for DNA content analysis.

**DNA content analysis.** Cultured B16F1 cells were collected by trypsinization and washed twice with PBS. The cells ( $1 \times 10^6$ /test) were then pre-treated with 0.2% Triton X-100 (Sigma) in PBS containing 10 mg/ml RNase (Wako, Tokyo, Japan), and then stained with propidium iodide (Sigma). The DNA content of the cells was analyzed with a FACS-Calibur™ (Becton Dickinson, Mountain View, CA). The data were analyzed with CELL Quest™ software (Becton Dickinson).

**Antibodies (Abs).** Rat anti-mouse perforin monoclonal Ab was purchased from KYOWA Medex (Tokyo, Japan). Goat anti-mouse IL-12 polyclonal Ab was purchased from R&D Systems (Minneapolis, MN). Rat anti-mouse IL-18 monoclonal Ab was purchased from Medical and Biological Laboratories (Nagoya, Japan). Horseradish peroxidase-conjugated anti-goat and rat Ig secondary antibodies were purchased from (Amersham Biosciences, Uppsala, Sweden).

**Murine melanoma model and in vivo Thd treatment.** B16F1 cells ( $2 \times 10^5$ ) were suspended in 500  $\mu\text{l}$  of PBS and injected subcutaneously into the shaved lateral flanks of the mice. When the diameter of the tumor mass reached 5 mm, 3 mg of Thd suspended in 200  $\mu\text{l}$  of PBS was injected intraperitoneally into the tumor-bearing mice daily. The mice were euthanized on Day 21 after melanoma cell inoculation. The diameters of the resulting subcutaneous melanomas were measured with a caliper. Tumor volume ( $\text{mm}^3$ ) was calculated using the formula: volume =  $W^2 \times L/2$ , where W = short and L = long diameters (mm).

**Reverse transcription (RT)-PCR.** Total cellular RNA was extracted from the frozen samples with TRIzol® (Life Technologies) according to the manufacturer's recommendations. The RNA purity and yield were measured by the spectrophotometric absorbance at 260 and 280 nm. Total RNA (1  $\mu\text{g}$ ) was subjected to first-strand cDNA synthesis and subsequent PCR with an RNA PCR kit (AMV) ver. 2.1 using a programmed temperature control system (Astec, Fukuoka, Japan). The PCR primers used in this study were as follows: ACC ATG AAC CCA AGT GCT GCC GTC (sense) and GCT TCA CTC CAG TTA AGG AGC CCT (antisense) for interferon inducible protein (IP)-10, TGC GGC CTA GCT CTG AGA CAA TGA (sense) and TGA ATG CTT GGC GCT GGA CCT GTG (antisense) for IFN- $\gamma$ , and GGG TGG AGC CAA ACG GGT C (sense) and GGA GTT GCT GTT GAA GTC GCA (antisense) for GAPDH.

**NK cell content in the spleen.** Splenocytes were isolated by simple stomaching of the spleens, straining, and centrifugation. Erythrocytes were depleted by hypotonic shock. Freshly-

isolated splenocytes were blocked with Fc-Block (Pharmingen, San Diego, CA) and stained with FITC-conjugated anti-CD3e and PE-conjugated anti-NK1.1 antibodies (Pharmingen). Cells were then washed and subjected to flow cytometric analysis with a FACS-Calibur (Becton Dickinson). The data were analyzed with Cell Quest software (Becton Dickinson).

**Western blotting.** Snap-frozen tissue samples were homogenized and protein was solubilized in 0.25% NP-40 lysis buffer (20 mM HEPES pH 7.5, 350 mM NaCl, 25% glycerol, 0.25% NP-40, 1 mM sodium *o*-vanadate) containing Complete-Mini™ protease inhibitor (Roche Diagnostics, Mannheim, Germany). Protein was separated by SDS-PAGE and transferred to a Hybond™ PVDF membrane (Amersham Pharmacia, Uppsala, Sweden). The membrane was blocked with skim milk and reacted with primary Abs for 90 min at room temperature. The membrane was then washed and reacted with HRP-conjugated secondary antibody for 60 min at room temperature. Protein bands were visualized with an ECL™ Western blot detection system (Amersham Pharmacia).

**Immunohistochemistry.** Serial sections of 4- $\mu\text{m}$  thickness were prepared and deparaffinized with xylene and ethanol. Immunohistochemical staining was performed with a Histofine SAB-PO kit (Nichirei, Tokyo, Japan) according to the manufacturer's instructions. Briefly, sections were quenched with 0.3%  $\text{H}_2\text{O}_2$  to reduce intrinsic peroxidase activity and blocked with 10% normal rabbit serum. Sections were incubated with primary Abs for 2 h at room temperature in a humidified chamber. After incubating with biotin-conjugated secondary antibody for 30 min at room temperature, the peroxidase-conjugated streptavidin solution was applied to all the sections and incubated for 30 min. Immune complexes were detected with diaminobenzidine (DAB).

## Results

**Effect of Thd on the cell cycle and growth of B16F1.** To assess whether Thd directly affects the cell cycle and growth property of B16F1 melanoma used in this study, cells were treated *in vitro* with Thd for 24 h and DNA content analysis was carried out. As shown in Fig. 1, no notable change was induced in the cell cycle of B16F1 by Thd. On microscopic observation, an increase of dead cells in Thd culture was not found. Additionally, a cell counting assay indicated that the proliferation rate of B16F1 was not suppressed by Thd treatment (data not shown).

**Suppression of subcutaneous melanoma growth by Thd.** B16F1 cells form aggressive, highly metastatic tumors when injected subcutaneously or intravenously to the syngeneic C57BL/6 mice (15). Although Thd had no effect on the *in vitro* growth of B16F1 cells, it was expected that Thd might affect the *in vivo* development of B16F1 melanoma because of its various biological effects, such as anti-angiogenesis and immune modulation. Thus, we next investigated whether Thd suppresses the growth of melanoma.

Cells were injected to the skin of mice and Thd treatment was started when the size of visible tumor reached 5 mm in diameter. In the control (PBS-injected) mice, B16F1 formed

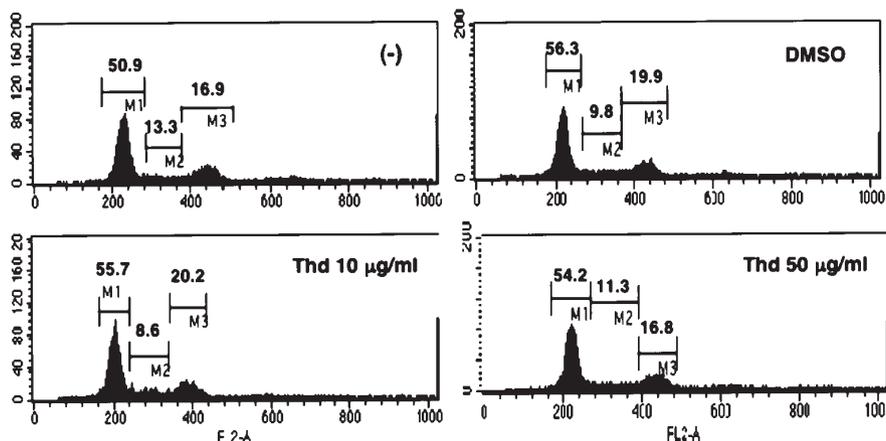


Figure 1. Effect of Thd on the cell cycle of B16F1 cells. DMSO or Thd dissolved in DMSO (final concentration 10-50 µg/ml) was added to the B16F1 culture. Cells were collected after 48 h and subjected to DNA content analysis, as described in Materials and methods. The numbers indicate the percentage of each fraction. Representative data from three individual experiments are shown.

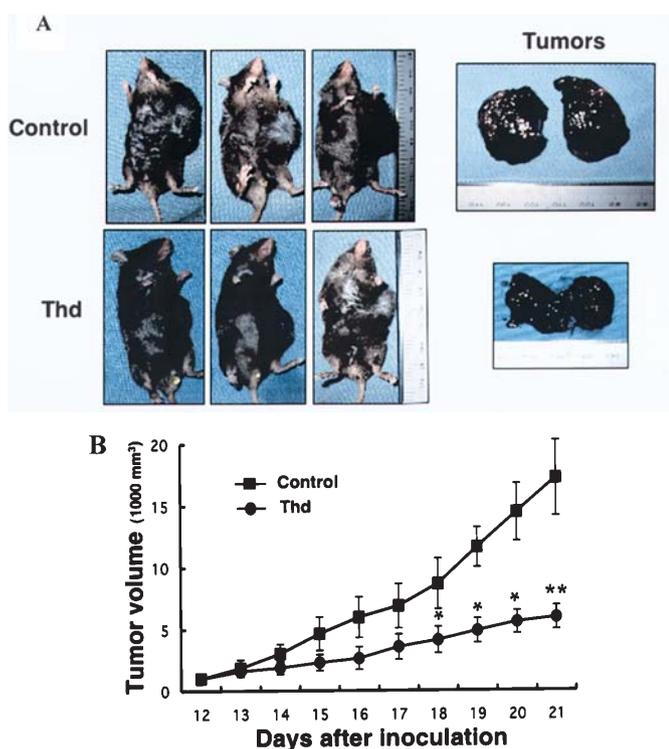


Figure 2. Suppression of *in vivo* growth of B16F1 melanoma by Thd. B16F1 cells ( $2 \times 10^5$ ) were injected into the shaved lateral flanks of C57BL6 mice (6-8 weeks of age). Intraperitoneal injection of PBS (control) or Thd was started when the tumor mass diameter reached 5 mm. (A) Melanoma-bearing mice and resected tumors. Mice were euthanized 21 days after tumor inoculation and the melanoma mass was resected. Representative mice from six mice in each group are shown. (B) Growth curve of the subcutaneous tumor. Tumor volume was calculated with the formula shown in Materials and methods. The data represent mean  $\pm$  SE of six mice in each group.

large solid masses in the lateral flank (Fig. 2A). The mice were severely sick or almost dead on Day 21 because of the progressive disease. Peritoneal invasion and/or dissemination were observed in all mice. The size of the tumors reached 40-55 mm in their longest diameter and  $17.2 \times 10^3$  mm<sup>3</sup> in their mean calculated tumor volume (Fig. 2B). Central necrosis was notable in the cross sections of all tumors. B16F1 also

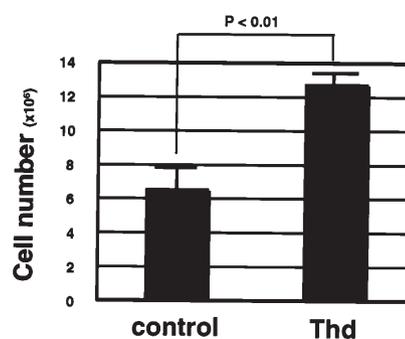


Figure 3. Number of whole splenocytes in Thd-treated mice. Spleens were harvested from mice treated or untreated with Thd for 10 days. Erythrocytes were depleted and the number of whole splenocytes was counted. The data represent the mean  $\pm$  SE of six mice in each group.

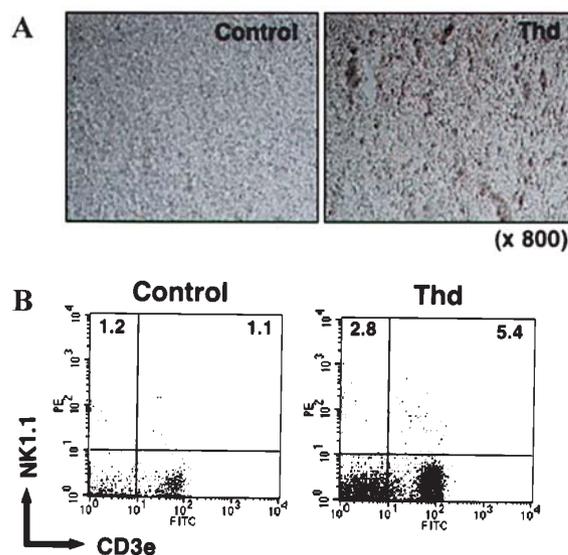


Figure 4. NK cell content in spleens from Thd-treated mice. (A) Spleens were harvested from mice treated or not treated with Thd for 10 days and subjected to immunohistochemical detection of NK1.1. (B) Splenocytes from Thd-treated and nontreated mice were double-stained with FITC-CD3e/PE-NK1.1 and analyzed with flow cytometer. The numbers indicate the percentage of each quadrant. Representative data from three individual experiments are shown.

formed tumors in Thd-treated mice; however, the tumor size was significantly smaller than the tumors in the control mice (Fig. 2). The mean tumor volume on Day 21 was  $5.95 \times 10^3$  mm<sup>3</sup>; approximately one third that of the control group. Invasion to the peritoneal cavity was found in one out of six mice examined. These findings suggested that Thd effectively suppressed the growth of subcutaneous B16F1 melanoma in C57BL6 mice.

*Augmentation of the number of whole splenocytes and NK1.1<sup>+</sup> cells by Thd.* The growth of B16 melanoma is mainly suppressed by an NK cell-dependent immune surveillance defense (15). Thus, suppressed B16F1 melanoma growth in Thd-treated mice suggested that Thd might activate the recruitment to tissues and/or the cytotoxicity of NK cells. For the next set of experiments, we examined the effect of Thd on the cellular immunity, particularly the NK cell population.

Thd (3 mg/body) was injected intraperitoneally to melanoma-free C57BL6 mice for 10 days and the whole splenocyte number was counted. In the control mice, the splenocyte count was  $6.6 \times 10^6 \pm 1.5 \times 10^6$ /spleen (Fig. 3). In the Thd-treated mice, marked splenomegaly was observed and the count was significantly ( $p < 0.01$ ) greater than that of the control;  $12.8 \times 10^6 \pm 0.8 \times 10^6$ /spleen (Fig. 3).

To determine whether the NK cell cluster was increased in spleens by Thd, immunohistochemistry and flow cytometry were carried out. As shown in Fig. 4A, spleens from the control mice contained very small number of NK1.1<sup>+</sup> cells. In contrast, the number of splenic NK1.1<sup>+</sup> cells was remarkably augmented by 10-day Thd injection (Fig. 4A). NK1.1/CD3e double staining and flow cytometric analysis of whole splenocytes indicated that the proportion of double negative and CD3e<sup>+</sup>NK1.1<sup>-</sup> cells was not changed, and that the percentage of NK1.1<sup>+</sup> cells was remarkably augmented in Thd-treated mice (Fig. 4B). Spleens from the control and Thd-treated mice contained 3.3% (1.2% of NK1.1<sup>+</sup>CD3e<sup>-</sup> and 1.1% of NK1.1<sup>+</sup>CD3e<sup>+</sup>) and 8.2% (2.8% of NK1.1<sup>+</sup>CD3e<sup>-</sup> and 5.4% of NK1.1<sup>+</sup>CD3e<sup>+</sup>) of NK1.1<sup>+</sup> cells, respectively.

It was strongly suggested from these findings that Thd enhances antitumor surveillance defense against B16F1 melanoma by augmenting the activity of NK1.1<sup>+</sup> clusters.

*Upregulated gene expression of IP-10 and IFN- $\gamma$  in spleens from Thd-treated mice.* To reveal the mechanism of NK cell activation induced by Thd, the expression of NK cell-activating cytokine and chemokine genes was analyzed by RT-PCR. Thd (3 mg/body) was injected intraperitoneally to C57BL6 mice for 10 days and the total RNA was extracted from the spleens. IP-10 is a member of the CXC chemokine subfamily and known to act as an effective chemoattractant of NK cells (21,22). Constitutive expression of the IP-10 gene was detected in spleens from normal mice, and Thd treatment drastically augmented the gene expression of this chemokine (Fig. 5). Gene expression of IFN- $\gamma$  a monocyte-derived NK cell-activating cytokine, was also upregulated by Thd (Fig. 5).

*Augmented production of IL-12, IL-18 and perforin in spleens from Thd-treated mice.* It was also speculated from these results that IFN-inducing factors other than IP-10 were

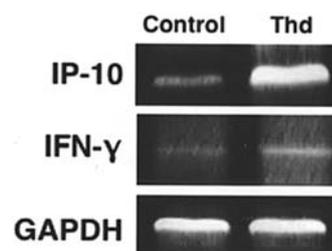


Figure 5. mRNA expression of IFN- $\gamma$  and IP-10 in the spleens of Thd-treated mice. Spleens were harvested from mice treated or not treated with Thd for 10 days. Total RNA was extracted and RT-PCR was performed as described in Materials and methods.

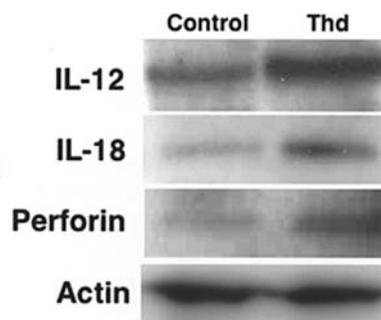


Figure 6. Protein expression of IL-12, IL-18 and perforin in the spleens of Thd-treated mice. Spleens were harvested from mice treated or not treated with Thd for 10 days. Protein was solubilized with 0.25% NP-40 lysis buffer and Western blotting was performed as described in Materials and methods.

upregulated by Thd. IL-12 is a cytokine produced by antigen presenting cells and is capable of activating IFN- $\gamma$  production (23). IL-18 also induces IFN- $\gamma$  production in cytokine-activated T cells and NK cells (24). Both of these cytokines contribute to tumor rejection by promoting the cytotoxic activity of the killer lymphocytes (23,24). Mice were similarly treated with Thd, and the production of IL-12 and 18 was examined by Western immunoblotting. As shown in Fig. 6, IL-12 was constitutively expressed and strongly increased by Thd. Similar results were obtained from IL-18 immunoblotting. IL-18 expression was almost under the detectable level in control spleens, but remarkably induced after Thd treatment (Fig. 6).

Perforin plays a key role in granule-mediated target cell lysis by cytotoxic T cells, NK cells and neutrophils (25,26). Therefore, the effect of Thd on the production of perforin was also examined. Western immunoblotting demonstrated that perforin expression was remarkably induced by Thd treatment (Fig. 6).

## Discussion

Our current results demonstrated that Thd effectively suppressed murine melanoma growth *in vivo*. Most of the mice died from large aggressive tumors soon after 21 days following subcutaneous B16F1 inoculation. In contrast, the melanoma grew significantly more slowly in mice that received Thd injection, resulting in a prolonged survival of Thd-treated tumor-bearing mice (data not shown). In the

murine melanoma model, Thd worked as an effective inhibitor of B16 melanoma growth *in vivo*. Although detailed examination of the adverse effects of Thd was not performed, notable abnormalities that might be associated with Thd injection were not observed. Thd displayed antitumor effects by interfering with angiogenesis (4) or the inhibition of tumor cell growth (27). In our experiments, B16F1 melanoma was insensitive to Thd treatment *in vitro*. Its growth and cell cycle was not affected by this agent. Thus, it was reasonable to speculate that the Thd effect on *in vivo* B16 growth was, at least partly, the result of the anti-angiogenic effect of this agent. Histological examination of peritumoral vascularization could not be carried out because of the aggressive invasion of melanoma cells into the tissue surrounding the tumors. Melanoma cells are rich in melanin pigment, which interferes with the exact evaluation of immunohistology. The expression of IP-10, which acts as an anti-angiogenic factor, as well as an NK cell chemoattractant (22), was strongly augmented by Thd. We therefore agree that an inhibited angiogenesis by Thd might have at least some contribution to the regression of B16 melanoma.

However, we have hypothesized that Thd also displayed its notable anti-B16 effect through its activation of the NK cell population because the rejection of B16F1 melanoma in syngeneic mice largely depends on NK cell tumor surveillance defense. B16 cells have lower MHC class-I expression and are not capable of inducing effective cytotoxic T lymphocytes *in vivo* (20). Additionally, B16 melanoma grew more rapidly in lymphotoxin- $\alpha$ -knockout mice with severely-disrupted NK cell activity (15). In our experiments, the splenic cell count and flow cytometric analysis revealed that the absolute number of NK1.1<sup>+</sup> cells in the spleen had elevated more than 5-fold after Thd treatment. These results were obtained from tumor-free mice, indicating that the Thd-induced NK cell increase occurs independently of tumor development and growth. The NK1.1<sup>+</sup> cell population is divided into two subpopulations by CD3e expression, NK and NK-T cells. Our results indicated that both these populations displayed 2.5-5-fold increases in their percentages. Although the differences in the contribution of NK and NK-T cells in B16 rejection has not been investigated in our study, it is strongly suggested that Thd induces enhanced NK cell recruitment and/or proliferation in the spleen.

NK cell recruitment to the various organs is effectively induced by IFN- $\gamma$  (28,29). Recent studies have demonstrated that this cytokine plays an important role in animal immunotherapy models (29,30). Results from RT-PCR indicated that IFN- $\gamma$  expression was upregulated in the spleens of Thd-treated mice. Additionally, the most potent NK cell chemoattractant, IP-10 (22) was remarkably induced by the same treatment. IP-10 production is enhanced by IFN- $\gamma$ , and it was suggested that the marked expression of IP-10 in Thd-treated mice might be due to upregulated IFN- $\gamma$  expression.

To address the mechanism of IFN- $\gamma$  upregulation and enhanced NK cell recruitment, we examined the expression of the IFN- $\gamma$ -inducing cytokines, IL-12 and IL-18. The effect of CC-4047, a Thd analog, on serum IL-12 level has been examined in a study on patients with MM (31). Our results from immunoblotting indicated that the production of these cytokines was remarkably augmented by Thd, corresponding with the previous human study. These cytokines have been the

center of attention in the field of cancer immunotherapy. The systemic administration of IL-12 resulted in the suppression of tumor growth and prolonged survival in mouse tumor models (32,33). The immunomodulatory effect of IL-12 is known to be mainly mediated by IFN- $\gamma$  upregulation by this cytokine. Watanabe *et al* (29) demonstrated that the injection of IL-12 naked DNA augmented the NK cell cytotoxic activity through an IFN- $\gamma$ -dependent mechanism. Additionally, IL-18 has been shown to activate NK cell proliferation and cytotoxicity in the spleen (34) and synergize the augmentation of IFN- $\gamma$  production by NK cells with IL-12 (35-37). Thus, it is conceivable that Thd activates NK cells through the enhancement of IL-12/18 production and subsequent upregulation of IFN- $\gamma$  and IP-10.

Perforin is produced by cytotoxic T cells (CTLs), NK cells and neutrophils, and facilitates the entry of granzymes, a group of cytolytic serin proteases, to the target cells for cell killing (25,26). Its expression is elevated in activated NK cells and CTLs, which is associated with their cytolytic activity (38,39). The data from immunoblotting showed the augmentation of perforin protein in the spleens of Thd-treated mice, suggesting that Thd enhances the NK cell cytolytic activity as well as their proliferation and/or recruitment to the organs.

The effect of Thd on NK cells has been largely unknown. Davies *et al* (17) demonstrated that therapy with Thd and its analogs increased the number of CD3-CD56<sup>+</sup> cells in peripheral blood from patients with MM. They also showed that enhanced *in vitro* cytotoxicity of peripheral blood mononuclear cells against multiple myeloma cell lines was mainly dependent on CD3-CD56<sup>+</sup> cells. In their study, the Thd effect on *in vivo* cytotoxicity was not analyzed. Using a murine experimental tumor model, in which the inoculated melanoma is mostly rejected by NK cells, we showed the effect of Thd on *in vivo* antitumor cytotoxicity. The Thd effects on NK-activating factors and cytotoxic protein in non-tumor-bearing normal animals were also demonstrated. Of course, the contribution of an anti-angiogenic effect of this agent should be carefully evaluated in our experimental model. However, our data strongly suggested that NK cell recruitment and cytotoxicity is enhanced by Thd, which might play a certain role in tumor rejection.

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