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# Antimetastatic effects of thalidomide by inducing the functional maturation of peripheral natural killer cells

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## Abstract

Thalidomide and its analogues are known as immunomodulatory drugs (IMiDs) that possess direct antimyeloma effects, in addition to other secondary effects, including antiangiogenic, antiinflammatory, and immunomodulatory effects. Although the involvement of natural killer (NK) cells in the antitumor effects of IMiDs has been reported, it is unclear whether IMiDs inhibit cancer cell metastasis by regulating the antitumor function of NK cells. In this study, we examined the protective effects of thalidomide against cancer metastasis by focusing on its immunomodulatory effects through NK cells. Using experimental lung metastasis models, we found that pharmacological effects of thalidomide on host cells, but not its direct anticancer tumor effects, are responsible for the inhibition of lung metastases. To exert the antimetastatic effects of thalidomide, both γ-interferon (IFN-γ) production and direct cytotoxicity of NK cells were essential, without notable contribution from T cells. In thalidomide-treated mice, there was a significant increase in the terminally differentiated mature $CD27^{lo}$ NK cells in the peripheral tissues and NK cells in thalidomide-treated mice showed significantly higher cytotoxicity and IFN-γ production. The NK cell expression of T-bet was upregulated by thalidomide treatment and the downregulation of glycogen synthase kinase-3β expression was observed in thalidomide-treated NK cells. Collectively, our study suggests that thalidomide induces the functional maturation of peripheral NK cells through alteration of T-bet expression to inhibit lung metastasis of cancer cells.

**KEYWORDS**

IFN-γ, immunomodulatory drug, metastasis, NK cell, T-bet

## 1 | INTRODUCTION

Natural killer (NK) cells are innate lymphocytes that play an important role in the host defense as early immune effector cells. It is well known that NK cells can eliminate nonself cells, such as virus-infected cells or transformed cancer cells, without prior stimulation.[1,2] In response to cancer cells, NK cells are activated to exert their antitumor effector function by inducing target cell death through the secretion of cytotoxic granules (perforin and granzymes), use of death ligands (Fas ligand and tumor necrosis factor [TNF]-related apoptosis-inducing ligand), or production of inflammatory cytokines (γ-interferon [IFN-γ] and TNF-α).[1,3-6] Natural killer (NK) cell-derived

cytokines also play an important role in linking innate and adaptive immunity.[1,7]

Natural killer cells can be classified into functionally distinct subsets by the expression of cell surface markers, and these NK cell subsets show distinct tissue distribution and functional diversity.[8-12] In mice, CD27 expression separates the mature $CD11b^{hi}$ NK cell population into $CD27^{hi}$ and $CD27^{lo}$ subsets, in addition to the phenotypically immature $CD11b^{lo}$ $CD27^{hi}$ NK cell subset (Mac-$1^{lo}$).[8,13,14] Although the mature $CD27^{lo}$ subset is comparatively excluded from lymphoid organs, such as bone marrow or lymph nodes, it is the dominant NK cell population in peripheral nonlymphoid tissues, including circulating blood.[8,15-17] The initial development of the NK cell lineage occurs in bone marrow from the $CD122^{+}$ early NK cell precursor lineages, although NK cells can also develop and differentiate in secondary lymphoid tissues.[18] In general, transcription factors (TFs) play important roles in the lineage specification of hematopoietic cells, thereby controlling lymphocyte development and differentiation, including NK cells.[19,20] Although the markers defining NK cell subsets differ between humans and mice, the dependence on TFs is similar in both species. The T-box TFs T-bet and Eomesodermin (Eomes) are key TFs that control NK cell differentiation and maturation.[21-23]

Metastasis is a multistep process in which cancer cells spread from the primary tumor to distant organs and establish metastatic lesions, and these processes are controlled by the tumor microenvironment, including immune responses. There are several clinical reports showing inverse correlations between the number of NK cells and the presence of metastases in different cancer types.[24-28] Consistent with these reports, the functional competence of NK cells is related to a lower risk of metastasis in multiple cancer types.[24,29-31] Furthermore, extensive preclinical studies have clarified the molecular mechanisms by which NK cells control metastasis.[32-35] Collectively, these studies suggest that NK cells are one of the major immune suppressors of cancer metastasis through their antitumor effector function.

Thalidomide and its analogues are known as immunomodulatory drugs (IMiDs) that possess direct antimyeloma effects and other secondary effects, including antiangiogenic, antiinflammatory, and immunomodulatory effects.[36,37] The mechanism of the direct antimyeloma effects of IMiDs was unclear for long time; however, there was an important finding that cereblon (CRBN) was found to be the direct target of IMiDs.[38] To exert their antimyeloma effect, IMiDs activate E3-ubiquitin ligase and induce the degradation of key TFs for myeloma survival such as IKZF1 (Ikaros) and IKZF3 (Aiolos).[39,40] Although the involvement of CRBN in the direct antimyeloma effects of IMiDs has been confirmed, the mechanisms underlying their immunomodulatory effects remain unclear. Previous studies suggested that IMiDs activate both the adaptive and innate immune cells such as T, NK, and NK T cells.[41-43] IKZF1 and IKZF3 are known to be transcriptional regulators of development of both B and T cells, whereas IKZF3 is an important TF for the terminal differentiation of B cells[44] and the maturation of NK cells.[45] Furthermore, the degradation of IKZF1 and IKZF3 through the IMiD-CRBN axis leads to changes in gene transcription, including IRF4 and increased interleukin-2 (IL-2) expression.[46] Moreover, IMiDs stimulate T cells and NK T cells to produce IL-2 and IFN-γ, which induces NK cell proliferation and cytotoxicity.[41,47,48] Although these studies strongly suggest the involvement of NK cells in the antitumor effect of IMiDs, it is unclear whether IMiDs inhibit cancer cell metastasis by regulating the antitumor function of NK cells.

In this study, we examined the antimetastatic effects of thalidomide by focusing on its immunomodulatory effects through NK cells. Our study suggests that thalidomide induces the functional maturation of peripheral NK cells likely through alteration of T-bet expression, to inhibit lung metastasis of cancer cells.

## 2 | MATERIALS AND METHODS

### 2.1 | Mice

Wild-type C57BL/6 (B6, WT) mice and BALB/c-*nu/nu* (Nude) mice were purchased from CLEA Japan. The IFN-$\gamma^{-/-}$ mice of a B6 background were kindly provided by Dr Y. Iwakura (Tokyo University of Science) and maintained at the Laboratory Animal Research Center, Institute of Medical Science, The University of Tokyo. In some experiments, groups of mice were treated with anti-Asialo-GM1 Ab (anti-asGM1, 200 μg/mouse; Wako Chemicals) on day −1 and 0. All experiments were approved and carried out according to the guidelines of the Animal Care and Use Committee of the Graduate School of Pharmaceutical Sciences of the University of Tokyo, the Care and Use of Laboratory Animals of University of Toyama, and the Animal Care and Use Committee of the Institute of Medical Science of the University of Tokyo.

### 2.2 | Cells

B16F10 melanoma cells stably expressing luciferase (B16-F10-luc-G5) were purchased from Caliper Life Sciences. The MCA205 cells and YAC-1 cells stably expressing luciferase were established as previously described.[49,50] Cells were maintained at 37°C in a 5% $CO_2$ incubator and grown in complete Eagle's minimum essential medium or RPMI-1640.

### 2.3 | Reagents

Thalidomide was purchased from Wako Chemicals, and dissolved in DMSO to create 150 mg/mL stock solutions that were maintained at 4℃. For in vivo studies, the drug was dissolved at a concentration of 15 mg/mL in 0.5% carboxymethyl cellulose before injection.

### 2.4 | Flow cytometry

Mononuclear cells (MNCs) were collected from bone marrow, peripheral blood, lungs, and spleen. To collect lung MNCs, lung

tissues were dissected, minced, and digested with 2 mg/mL collagenase (Roche Diagnostics) in serum-free RPMI-1640 for 1 hour at 37°C. Samples were further homogenized through wire mesh. For flow cytometry analysis, cells were first preincubated with anti-CD16/32 (2.4G2) mAb to avoid nonspecific binding of Abs against FcγR. The cells were then incubated with a saturating amount of fluorophore-conjugated mAb. The Foxp3 staining kit (eBioscience) was used for intracellular staining of T-bet. Antibodies against CD3ε (2C11), NK1.1 (PK136), CD11b (M1/70), CD27 (LG.3A10), and T-bet (4B10) were purchased from BioLegend, eBioscience, or Tonbo Bioscience. Flow cytometry analysis was undertaken on a FACSCanto II (BD Biosciences), and the data were analyzed using FlowJo software.

### 2.5 | Cytokine production and cytotoxicity assay

Natural killer cells were isolated from lungs using magnetic sorting (more than 80% purity, MojoSort Mouse NK cell Isolation Kit; BioLegend). To measure IFN-γ production, lung ($10^5$/well) NK cells were stimulated with PMA (50 ng/mL; Sigma) and ionomycin (500 ng/mL; Sigma) in RPMI-1640 medium. After a 24-hour incubation, the cell-free supernatants were harvested and subjected to ELISA. The amounts of IFN-γ were quantitated by specific sandwich ELISA (BioLegend). Cytotoxic activity was assessed against YAC-1 target cells using the bioluminescent imaging method previously reported with modification.[50,51] The YAC-1 target cells expressing firefly luciferase ($10^4$/well) were incubated in a total volume of 200 μL effector cells and D-luciferin (150 μg/mL; Promega) in 96-well black plates. The plates were centrifuged before incubation, and the bioluminescence after 18 hours was measured by an in vivo imaging system (IVIS Lumina II; Perkin Elmer).

### 2.6 | Western blot analysis

Cell lysates were collected in lysis buffer (25 mmol/L HEPES pH 7.7, 0.3 mol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 0.1% Triton X-100, 20 mmol/L β-glycerol phosphate, 1 mmol/L sodium orthovanadate, 1 mmol/L PMSF, 1 mmol/L DTT, 10 mg/mL aprotinin, and 10 mg/mL leupeptin). Equal amounts of protein were resolved by electrophoresis on 10% acrylamide gel and transferred to PVDF membranes. The primary Abs used were glycogen synthase kinase-3β (GSK-3β) (9315) from Cell Signaling Technology and β-actin (sc-1615) from Santa Cruz Biotechnology (Santa Cruz Biotechnology).

### 2.7 | Bioluminescence imaging of lung metastasis

B16-F10-luc-G5 cells ($5 \times 10^5$) or MCA205-luc2 cells ($5 \times 10^5$) were inoculated by i.v. injection into B6 mice from the tail vein. To obtain bioluminescence images, mice were injected with D-luciferin (150 mg/kg i.p.; Promega) and the lungs were removed to measure luminescence using an in vivo imaging system (IVIS Lumina II; Caliper Life Sciences).

### 2.8 | Statistical analysis

All data were obtained from a group of 3-6 mice and are representative of at least 2 independent experiments. Data were analyzed for significance using Student's *t* test. *P* values less than .05 were considered significant.

## 3 | RESULTS

### 3.1 | Antimetastatic effects of thalidomide through NK cells

First, we examined the antimetastatic effects of thalidomide using an experimental lung metastasis model of B16F10 melanoma cells. Mice were treated with thalidomide before or after tumor inoculation to assess the role of host response or tumor cell response, respectively, in the antimetastatic activity of thalidomide (Figure 1A). The "All" or "Pre" thalidomide treatment groups showed significant inhibition of lung metastasis of B16F10 cells, but the "Post" thalidomide treatment group did not (Figure 1). These results suggest that pharmacological effects of thalidomide on host cells, but not its direct anticancer tumor effects, are responsible for the inhibition of lung metastasis of B16F10 melanoma cells. Similar antimetastatic effects of thalidomide were also confirmed through oral administration (Figure S1A), and in a different metastasis model using the MCA205 fibrosarcoma cell line (Figure S1B). The thalidomide analogue, lenalidomide, also showed antimetastatic effects against B16F10 lung metastasis (Figure S1C).

We next examined the involvement of NK cells in the antimetastatic effects of thalidomide using NK cell-depleted mice treated with anti-asGM1 Ab. As shown in Figure 1B, the antimetastatic effects of thalidomide were lost in the NK cell-depleted mice. As NK cells exert their antimetastatic function mainly through granule-dependent cytotoxicity and/or IFN-γ production,[52] we further examined the role of IFN-γ in the antimetastatic effects of thalidomide. As thalidomide treatment resulted in comparable inhibition of B16F10 lung metastasis in athymic nude mice and immunocompetent B6 mice (Figure S1D), we concluded that T cells play no role in the antimetastatic effects of thalidomide. Although thalidomide treatment partially inhibited the lung metastasis of B16F10 cells in IFN-$\gamma^{-/-}$ mice, it was not comparable to the inhibition in WT mice (Figure 1C); therefore, both IFN-γ production and direct cytotoxicity of NK cells were important for the metastasis suppression in thalidomide-treated mice.

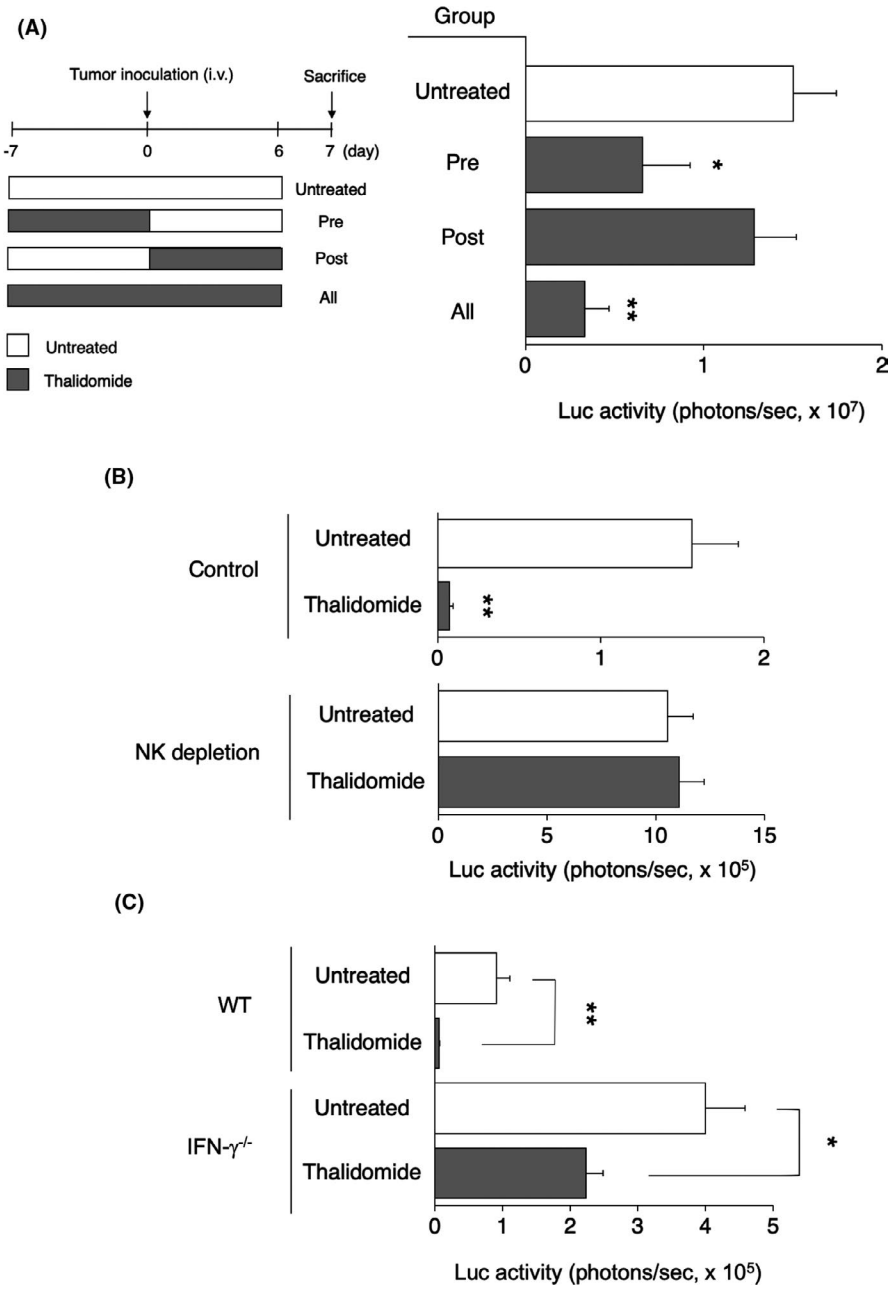


**FIGURE 1** Natural killer (NK) cell-dependent antimetastatic effects of thalidomide. A, B16-F10-luc-G5 cells ($5 \times 10^5$ cells/mouse) were inoculated i.v. into C57BL/6 (B6) mice on day 0. Mice were treated with or without (untreated) thalidomide (6 mg/mouse/d, i.p.) on days −7 to −1 (Pre), days 0 to 6 (Post), or days −7 to 6 (All). Lung metastases were quantified on day 7 by bioluminescence imaging. B, B16-F10-luc-G5 cells ($5 \times 10^5$ cells/mouse) were inoculated i.v. on day 0 into B6 mice with or without NK cell depletion using anti-Asialo GM1 Ab (200 μg/mouse, day −1, 0, i.p.). Mice were treated with or without thalidomide (6 mg/mouse/d, i.p.) on days −7 to 2. Lung metastases were quantified on day 3 by bioluminescence imaging. C, B16-F10-luc-G5 cells ($5 \times 10^5$ cells/mouse) were inoculated iv into B6 wild-type (WT) or γ-interferon-deficient B6 mice (IFN-$\gamma^{-/-}$) on day 0. Mice were treated with or without thalidomide (6 mg/mouse/d, i.p.) on days −7 to 2. Lung metastases were quantified on day 3 by bioluminescence imaging. Data are presented as mean ± SEM. *$P < .05$, **$P < .01$ compared with untreated mice. Luc, luciferase

## 3.2 | Induction of mature CD27$^{lo}$ NK cells in peripheral tissues by thalidomide treatment

To assess the pharmacological effects of thalidomide on NK cells, we analyzed NK cell populations in different organs of thalidomide-treated mice using flow cytometry. There was a significant increase in the proportion of NK cells (determined as CD3$^-$ NK1.1$^+$ lymphocytes) in the peripheral blood and a similar increase was observed in the lungs of thalidomide-treated mice, although there was no notable change in the bone marrow (Figure 2A). We further examined which subset of NK cells was responsible for the increase in NK cells in the lungs and peripheral blood of thalidomide-treated mice using NK cell subset markers CD11b and CD27. As shown in Figure 2B, there was a significant increase in the terminally differentiated mature CD27$^{lo}$ NK cells in the lungs and peripheral blood of thalidomide-treated mice; therefore, thalidomide treatment induced a mature CD27$^{lo}$ NK cell population in the peripheral tissues.

## 3.3 | Functional maturation of peripheral NK cells by thalidomide treatment

In order to clarify whether thalidomide treatment alters the antitumor effector function of NK cells in addition to inducing the mature CD27$^{lo}$ NK cell population in peripheral tissues, we examined the effects of thalidomide on NK cell cytotoxicity and IFN-γ production. Purified lung NK cells from control or thalidomide-treated mice were

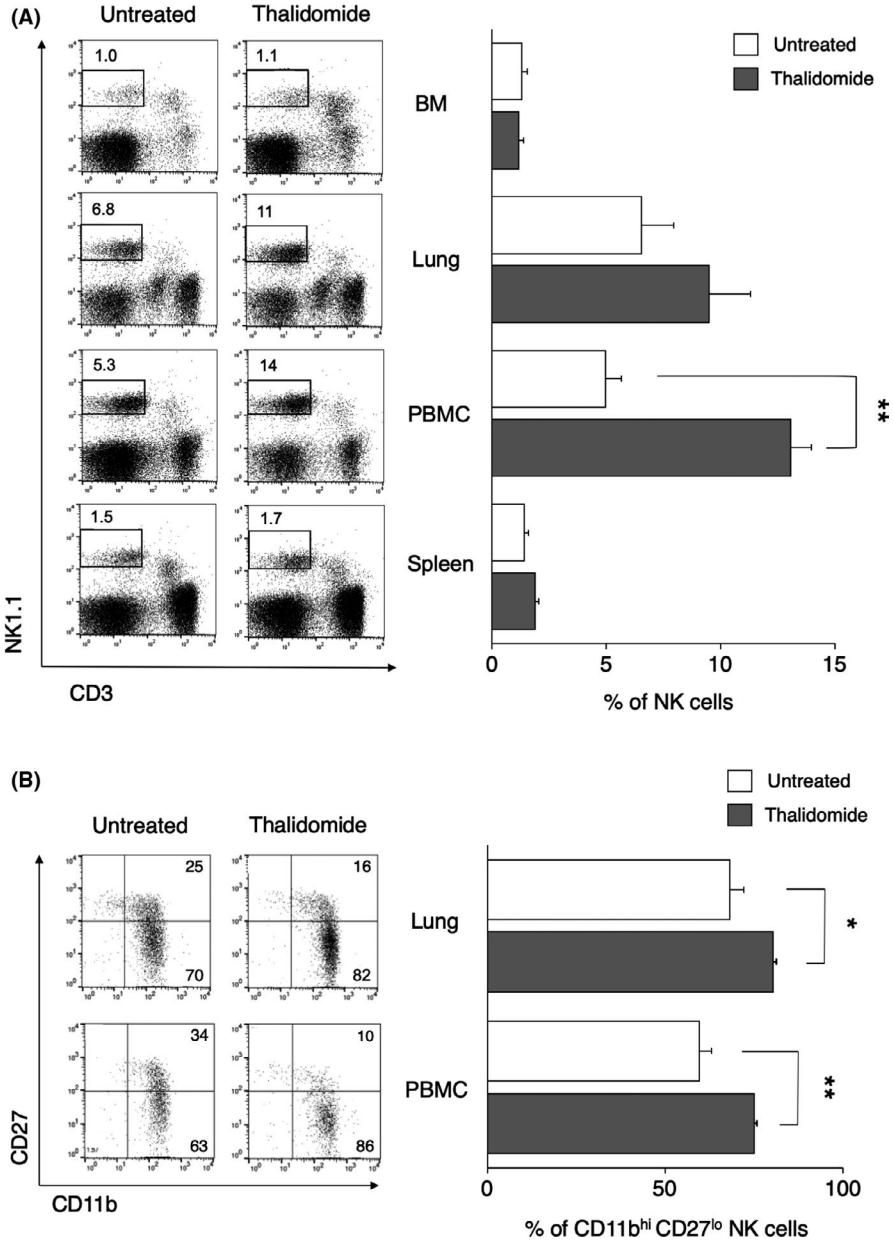


**FIGURE 2** Induction of peripheral $CD27^{lo}$ natural killer (NK) cells by thalidomide treatment. A, Representative dot plots of NK1.1 and CD3 expression lymphocytes, and summarized quantitative data of NK cells ($NK1.1^+$ $CD3^-$) in the bone marrow (BM), lung, peripheral blood (PBMC), or spleen from untreated or thalidomide-treated B6 mice (6 mg/mouse/d, i.p., 14 d) are shown. B, Representative dot plots of CD11b and CD27 expression and summarized quantitative data of NK cell subsets in lung and peripheral blood (PBMC) (electronically gated as $NK1.1^+$ $CD3^-$ cells). Data are shown as mean ± SEM. * $P < .05$, ** $P < .01$ compared with untreated mice

subjected to in vitro cytotoxicity assay against YAC-1 target cells. As shown in Figure 3A, the lung NK cells from thalidomide-treated mice had significantly higher cytotoxicity against YAC-1 cells. Moreover, the lung NK cells from thalidomide-treated mice produced more IFN-γ following in vitro stimulation with PMA and ionomycin than those from control mice (Figure 3B). This suggested that thalidomide induces not only the maturation, but also the functional competence of peripheral NK cells.

We further investigated the mechanism by which thalidomide induces the functional maturation of peripheral NK cells. As T-box transcription factors, such as T-bet and Eomes, are known to control peripheral NK cell maturation,[53] the expression of T-bet and Eomes was examined using intracellular staining. The expression of T-bet (Figure 4A), but not Eomes (data not shown), in lung NK cells was upregulated by thalidomide treatment. As we also observed the up-regulation of T-bet expression in T cells after thalidomide treatment (data not shown), such thalidomide-induced T-bet expression is not restricted to NK cells. Furthermore, in addition to T-bet induction, the down-regulation of glycogen synthase kinase 3β (GSK-3β) expression was noted in NK cells treated with thalidomide (Figure 4B). As GSK-3β is known to negatively control T-bet expression,[54] thalidomide could induce functional maturation of peripheral NK cells through the induction of T-bet expression by inactivating the GSK-3β pathway.

## 4 | DISCUSSION

Although the importance of NK cells in the pharmacological action of IMiDs has been well reported, the precise mechanism by which IMiDs exert NK cell-dependent antitumor effects is unclear. In this study, we showed that thalidomide inhibits lung metastasis of cancer

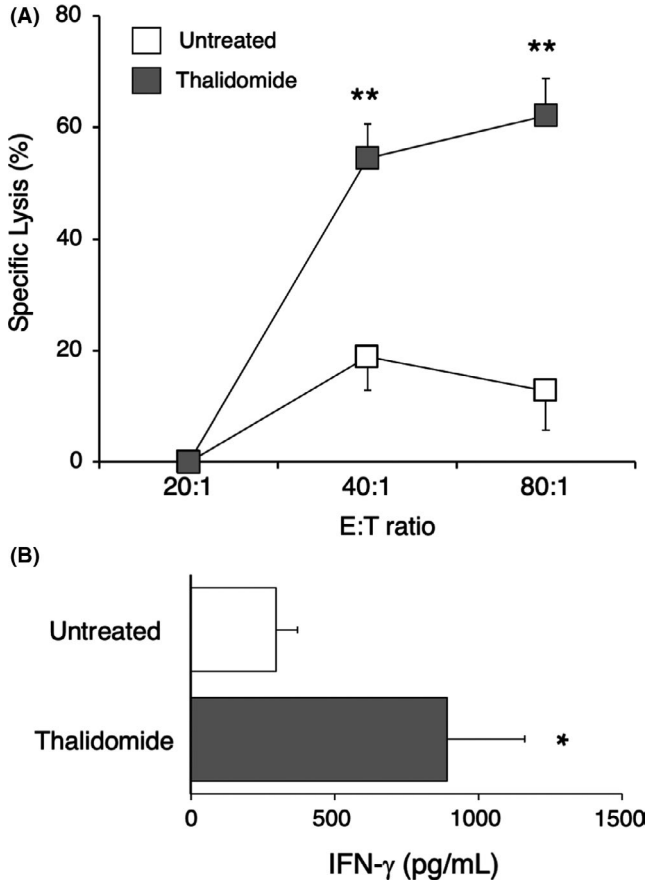


**FIGURE 3** Induction of natural killer (NK) cell function by thalidomide. Purified NK cells from lung mononuclear cells from untreated or thalidomide-treated B6 mice (6 mg/mouse/d, i.p., 7 d) were isolated. A, The cytotoxicity of NK effector (E) cells against YAC-1 target (T) cells was tested. B, NK cells were cultured with or without PMA/ionomycin for 24 h and the cell-free supernatants were harvested. γ-Interferon (IFN-γ) release was measured by ELISA. Results are shown as mean ± SEM. $^{*}P < .05$, $^{**}P < .01$ compared with untreated mice

cells in an NK cell-dependent mechanism. As thalidomide treatment induced a population of mature $CD27^{lo}$ NK cells and their functional competence in the peripheral tissues, we conclude that the antimetastatic effects of thalidomide are a result of inducing the functional maturation of peripheral NK cells.

Regarding the maintenance and control of NK cell homeostasis, the transcription factor T-bet is known to be important for their development and functional competence, and the lack of T-bet expression leads to a defect in the terminal differentiation of mouse NK cells into $CD27^{lo}$ NK cells.[22,23,55] In the absence of T-bet, the susceptibility to cancer metastasis is increased due to impaired NK cell function and survival in vivo[22]; therefore, T-bet is important for maintaining the antimetastatic activity of NK cells. Of note, GSK-3β is known as the negative regulator of T-bet[54] and the inhibition of GSK-3β was reported to improve the effector function of NK cells in acute myeloid leukemia patients.[56] In this context, thalidomide treatment significantly increased the expression of T-bet in NK cells, in addition to inducing mature $CD27^{lo}$ NK cells and their functional competence. The expression of another T-box transcription factor, Eomes, which is also known to regulate the development and terminal maturation of NK cells in combination with T-bet,[21,22] did not change in thalidomide-treated mice (data not shown); therefore, thalidomide preferentially induces T-bet expression in peripheral NK cells. T-bet is known to regulate T helper 1 (Th1) cell differentiation by directly regulating the expression of IFN-γ and other genes specific to Th1 cells.[53,57] Moreover, the acquisition of $CD8^{+}$ T cell effector functions and the development of $CD8^{+}$ T cell memory are regulated by T-bet.[58] In addition to the importance of T-bet in acquired immune cells, it also plays an important role in innate lymphoid cells (ILCs), which are a newly described type of immune cells with many functional similarities to effector T cell subsets.[59] Amongst these ILCs, NK cells and group 1 ILCs are known to express T-bet to a similar degree as type 1 T cells (Th1 or $CD8^{+}$ T cells).[59] Although the regulatory mechanism of T-bet expression in lymphocytes is not completely understood, T-bet can be induced by IL-15 to control intraepithelial lymphocyte development.[60] Furthermore, it was reported that mice deficient in T-bet are more susceptible to metastatic dissemination than WT mice and this phenotype can be largely prevented by adoptive transfer of bulk NK cells or purified $KLRG1^{+}$ $CD27^{lo}$ NK cells.[61,62] Similar protection was also conferred by treating T-bet-deficient mice with IL-15, resulting in the induction of $KLRG1^{+}$ NK cells expressing Eomes.[61] Collectively, T-bet is not only important for the development and maturation of NK cells, but also essential for the maintenance of NK cell functional competency, particularly that of the terminally differentiated peripheral $CD27^{lo}$ NK cells.

There are several studies on the immunomodulatory effects of IMiDs that suggest the induction of IL-2 and IFN-γ secretion by T cells after IMiD treatment,[42,47] and increased serum IL-2 and IFN-γ levels associated with the increased numbers of NK cells in the peripheral blood of IMiD-treated patients.[41,63] These studies suggested that IMiD treatment indirectly regulates NK cells through T cell-dependent secretion of NK cell-activating cytokines. Of note, it was previously reported that IMiDs inhibit IL-2-mediated generation of regulatory T cells (Tregs).[43] As the effector function of NK cells can be negatively regulated by Tregs, it is also possible that IMiDs alter NK cell antitumor activity indirectly through the inhibition of Tregs. Our present data indicate that T cells played no role in the antimetastatic effects of thalidomide through NK cells; therefore, they are not mediated by a T cell-dependent mechanism. Although we did not see any T cell contribution in the antimetastatic effect of thalidomide, the effect of IMiDs on T cell-dependent antitumor immunity has been reported elsewhere.[64,65] Treatment with IMiD reduces the threshold for NK cell activation through CD16 or NKG2D, possibly through actin remodeling at immune synapses.[66] It is also important to further examine the effect of IMiDs on other functions of NK cells, such as Ab-dependent cellular cytotoxicity, considering a potential combination with Ab therapies. Even though thalidomide did not exert a direct antiproliferative effect on B16 melanoma cells, thalidomide or its related compounds are known to show antiangiogenic effects.[67]

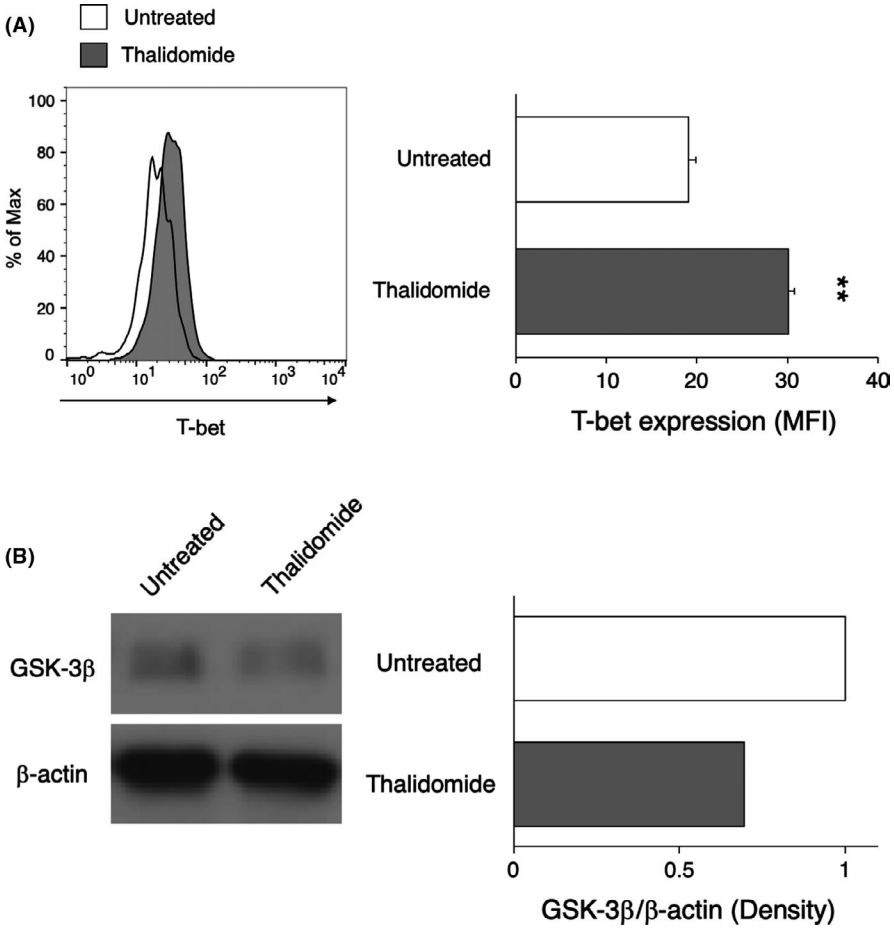


**FIGURE 4** Natural killer (NK) cell expression of T-bet and glycogen synthase kinase-3β (GSK-3β) after thalidomide treatment. A, Expression of intracellular T-bet in mature lung NK cells with or without thalidomide treatment (6 mg/mouse, i.p., 14 d) was measured (electronically gated as NK1.1$^{+}$ CD3$^{-}$ CD11b$^{hi}$ cells). Data are shown as mean ± SEM. * $P < .05$, ** $P < .01$ compared with untreated control mice. MFI, mean fluorescence intensity. B, Expression of GSK-3β in splenic NK cells was measured by western blotting. The band intensities of GSK-3β were assessed relative to β-actin using ImageJ software

Considering the immunomodulatory effect of thalidomide was somewhat preferentially seen in lung and peripheral blood NK cells (Figure 2), we presume the mature CD27$^{lo}$ NK cell subset in peripheral nonlymphoid tissue could be responsible for the antimetastatic effect of thalidomide. In this context, tissue specificity of the antimetastatic effect of thalidomide is of great interest. Further studies are required to answer the remaining questions regarding the exact mechanism explaining how IMiDs induce mature CD27$^{lo}$ NK cells in the periphery and the relevance of T-bet induction by IMiDs for NK cell-dependent antimetastatic effects. However, our study revealed that IMiDs can be a therapeutic tool for treating distant metastasis of cancer by regulating peripheral NK cell homeostasis to improve their antitumor effector function.

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## CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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