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Differential Surface Expression of CD18 and CD44 by Neutrophils in Bone Marrow and Spleen Contributed to the Neutrophilia in Thalidomide-treated Female B6C3F1 Mice[1]

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Abstract

Previously, we have reported that thalidomide (Thd) can enhance neutrophil function in female B6C3F1 mice. The present study was intended to evaluate the mechanisms underlying the enhanced neutrophil responses following Thd treatment intraperitoneally (100 mg/kg) for 14 or 28 days. Treatment with Thd increased the numbers of neutrophils in the spleen, peripheral blood, bone marrow, peritoneal cavity and lung of female B6C3F1 mice when compared to the vehicle control mice. Thd treatment for 14 days increased the percentages and the number of neutrophils in the spleen in the first eight hours (peaking at 2 h) after the last Thd treatment, and it returned to the baseline after 24 h. However, Thd treatment for 28 days increased the percentages and number of neutrophils in the spleen even at the 24-h time point after the last Thd treatment. These neutrophils were demonstrated to be functional by the myeloperoxidase activity assay. Further studies have ruled out the possibility of an increased bone marrow granulopoiesis following Thd treatment. Flow cytometric analysis of the surface expression of adhesion molecules suggested that Thd treatment for either 14 or 28 days decreased the surface expression of either CD18 or CD44 by bone marrow neutrophils. On the other hand, the surface expression of both CD18 and CD44 by splenic neutrophils was increased following Thd treatment for 28 days but not for 14 days. No effect was produced for other cell surface molecules such as CD62L and CD11a. It was possible that decreased surface expressions of CD18 and CD44 facilitated neutrophils' release from the bone marrow; increased surface expressions of CD44 and CD18 by splenic neutrophils after 28 days of Thd treatment increased their ability to remain in the periphery. Taken together, Thd treatment increased neutrophils in female B6C3F1 mice, at least partially, through differentially modulating the surface expression of CD18 and CD44 by the neutrophils in the bone marrow and spleen.

Keywords

Thalidomide; neutrophils; adhesion molecules

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INTRODUCTION

Thalidomide [N(α)-phthalimidoglutarimide; Thd] has been identified as an effective immunomodulatory drug with therapeutic potentials for a variety of diseases including multiple myeloma and myelodysplastic syndromes (MDS) despite its toxic potencies (Nogueira *et al*., 1994; Davies *et al*., 2001). Patients with MDS often develop neutropenia and are associated with a high mortality rate due to recurrent bacterial infections (Mufti *et al*., 1985; Kouides and Bennett, 1997). It is currently unknown how Thd treatment in MDS patients has improved the patient's overall health, although it was hypothesized that an increased neutrophil function may be responsible (Musto, 2004; Raza *et al*., 2004; Lubbert *et al*., 2004).

In our previous studies, we have provided evidence that Thd treatment enhanced host resistance to both primary and secondary *L. monocytogenes* infections by a neutrophil-related mechanism in female B6C3F1 mice (Guo *et al*., 2005). Neutrophil numbers in the spleen and liver were increased in Thd-treated and *L. monocytogenes*-infected mice. These observations highlighted the relevance of Thd modulation of neutrophil function in its mechanisms of action. Although it was hypothesized that an increased granulopoiesis might be involved (Invernizzi *et al*., 2005), the exact mechanism that Thd treatment increased neutrophils in these organs was not clear. There is evidence that Thd downregulates the expression of surface adhesion molecules such as L-Selectin (CD62L) by neutrophils (Nogueira *et al*., 1994; Geitz *et al.,* 1996). An increased infiltration of neutrophils in the liver after *L. monocytogenes* infection was also observed in LFA-1 (CD11a/CD18)-deficient mice (Miyamoto *et al.,* 2003). It was possible that Thd modulated the interaction between neutrophils and endothelial cells by altering the adhesion cascade and, thus, changed the distribution of neutrophils (Zwingenberger and Wnendt, 1995–96).

The present study was intended to test the hypothesis that Thd-mediated increases in neutrophil function could be related to its effects on the surface expression of adhesion molecules by neutrophils and bone marrow granulopoiesis. To this end, B6C3F1 mice were treated intraperitoneally (i.p.) with Thd for 14 or 28 days. One dose of Thd (100 mg/kg) was used in this study since our previous studies have demonstrated that Thd at 100 mg/kg was an optimal dose for modulating the immune responses and host resistances (Karrow *et al*., 2003; Guo *et al*., 2005). Additionally, this amount of Thd in mice is equivalent to a clinical human treatment dose (approximately 400 mg/day) in terms of milligram per square meter of body surface (Marriott *et al*., 1999), which usually gives more accurate interspecies extrapolation (Hodgson, 1997). The blood level of Thd is approximately 0.13 μg/ml in mice treated for a single time at 100 mg/kg for eight hours (Karrow *et al*., 2000), which is comparable to the concentration found in patients (Eriksson *et al*., 2003; Tohnya *et al*., 2004). The 14- and 28-day treatment regimens were selected for several reasons. Our experience working with animals in our immunotoxicology studies with the National Toxicology Program has suggested that a daily treatment for 14–28 days is optimal for the identification of an immunotoxic agent. In clinical studies, patients take Thd for several weeks or months. There is evidence that a prolonged treatment rather than a short trial is necessary to produce a positive response in MDS patients (Raza *et al*., 2004). Our previous reports have shown that treatment with Thd at 30–150 mg/kg (i.p.) for either 14 or 28 days could increase host resistance to *L. monocytogenes* infection by modulating neutrophil function (Karrow *et al.,* 2003; Guo *et al.,* 2005). Therefore, both the 14-day and 28-day treatment regimens were employed in this study to further determine how Thd treatment increased neutrophil responses. In our studies, mice were sacrificed at different time points after the last Thd treatment since the changes in neutrophils depend greatly on the time (Nakagawa *et al*., 1998). Mature neutrophils have the shortest life span of all leukocytes and die rapidly through apoptosis; thus, the analyses of neutrophil responses were performed within 24 hours after the last Thd treatment.

MATERIALS AND METHODS

Animals

Six-to-eight week old female B6C3F1 mice (Taconic, Germantown, NY) were quarantined for a minimum of two weeks prior to use. Mice were randomized by weight, ear-punched for identification, and transferred to plastic shoebox cages (3–4 animals per cage) for testing. The cages were bedded with hardwood sawdust and covered with a filter bonnet; mice were provided with tap water and fed certified Zeigler rodent chow *ad libitum* for the duration of the study. Room temperature was maintained at 22–24°C, and the relative humidity between 40 and 70%. The light/dark cycle was maintained on 12-h intervals. Mice were determined to be free of hepatitis and Sendai virus by serology testing. All animal procedures were conducted under an animal protocol approved by the VCU Institutional Animal Care and Use Committee (IACUC).

Treatment with Thd

Thd was provided by Celgene Corp. (Warren, NJ). Stock solutions of Thd (10 mg/ml) were prepared daily as a suspension in sterile distilled water as reported by Karrow *et al*. (2000). Female B6C3F1 mice were dosed (i.p.; 0.1 ml/10 g bw) with vehicle or 100 mg/kg of Thd for 14 or 28 days.

Cell isolation and determination of cell number

The quantification of splenocyte subsets was performed as previously described (Guo *et al*., 2002). Single cell suspensions of individual spleens were prepared by mashing the excised organs between the frosted ends of two microscope slides. After washing, cells were resuspended in RPMI complete medium and counted using a Coulter Counter ZII with the red blood cells lysed by a ZAP-O-GLOBIN II lytic reagent (Coulter Corporation, Miami, FL). Peritoneal cells were obtained by lavage using 10 ml of Hank's Balanced Salt Solution (HBSS). Bone marrow cells were obtained by flushing both femurs of mice.

Determination of neutrophils in the lung

Neutrophils were recruited to the lung using lipopolysaccharide (LPS; Becton Dickinson, San Diego, CA). Mice were anesthetized with an injection (i.p.) of a mixture of ketamine and xylazine in saline; they were then treated intranasally with LPS (0.3 mg/kg) in 50 μl of phosphate buffered saline (PBS). After 24 h, mice were sacrificed, and recruited lung neutrophils obtained by lavage using a total of 3 ml of HBSS (3 × 1 ml).

Assessment of DNA synthesis by bone marrow cells

DNA synthesis was determined by measuring the incorporation of ^{3}H-thymidine into bone marrow cells. The bone marrow cells (6×10^5 cells/well) were cultured in triplicate at 37°C, 5% CO_2 and 95% humidity in the presence of ^{3}H-thymidine (1 μCi/well) for 3 h. The incorporation of ^{3}H-thymidine into the proliferating cells was expressed as CPM/6×10^5 cells.

Assessment of colony-forming ability by bone marrow cells

The colony-forming ability of bone marrow cells was assayed according to the protocol from StemCell Technologies Inc. (Vancouver, British Columbia, Canada) with slight modification. Briefly, 1×10^5 bone marrow cells were cultured in methylcellulose medium (StemCell Technologies Inc.) at 37ºC and 5% CO_2 in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF); colonies were enumerated at the end of a 10-day incubation period. GM-CSF induces formation of all the non-lymphoid blood cells, including erythrocytes, monocytes, granulocytes (neutrophils, basophils and eosinophils) and megakaryocytes (Nelson, 1990). Colonies per femur (total activity) and per 1×10^5 bone

marrow cells (specific activity) were calculated and used as criteria for comparison among treatment groups.

The second method employed to measure the granulocyte colony-forming cells (G-CFC) was the HALO Kit Platform (HemoGenix, Inc., Colorado Springs, CO). Briefly, 2×10^4 cells of bone marrow were cultured in methylcellulose medium at 37ºC and 5% CO_2 in the presence of granulocyte colony-stimulating factor (G-CSF) for 5 days. The cells were lysed for 15 min in the incubator and the luminescence measurement performed using Trilux from Perkin Elmer (Wellesley, MA) after the addition of luciferin in the presence of the luciferase. To standardize the assay, an ATP dose-response curve was generated.

Myeloperoxidase activity

Measurement of myeloperoxidase (MPO) activity was performed as described (Auttachoat *et al*., 2004). Briefly, 5×10^6 cells were homogenized in 0.5 ml buffer containing 100 mM sodium acetate, 0.5% hexadecyltrimethylammonium bromide (HETAB; Sigma, St. Louis, MO), and 5 mM EDTA. The homogenates were sonicated and centrifuged at 13,000 × g for 2 min. The supernatants were collected and serially diluted from 1:2 to 1:2,048 with assay buffer in a 96-well plate. The final volume was 75 μl per well. Seventy-five microliters of tetramethylbenzidine substrate were added into each well. The plates were read within 2 min at 450 nm after adding 50 μl of the stop solution (2N H_2SO_4). The results were expressed as midpoint tier at the optical density (OD) of 0.5.

Hematology

On the day when the animals were sacrificed, blood was collected by retro-orbital bleed into EDTA tubes after the animals were anesthetized by CO_2 inhalation. The following hematological parameters were assessed: the number of erythrocytes and leukocytes, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). The number of reticulocytes was evaluated using the RNA stain, thiazole orange (Becton Dickinson, San Jose, CA), in conjunction with flow cytometric analysis. A blood smear was prepared at the time of blood collection and air-dried. After fixation with methanol and staining with Wright-Giemsa (Fisher Scientific, Pittsburgh, PA), the smear was used to determine leukocyte differentials in blood.

Flow cytometric analysis

Splenocytes were labeled with the appropriate monoclonal antibody (mAb), conjugated directly with a fluorescent molecule for visualization. Cells were dual stained with phycoerythrin (PE)-conjugated antibody and fluorescein isothiocyanate (FITC)-conjugated antibody or three-way stained using antibodies conjugated with FITC, PE and peridinin chlorophyll protein (PerCP). The antibodies were purchased from Becton Dickinson. The mAb used were anti-mouse Ly-6G (Gr-1) conjugated with FITC, anti-mouse IgM with FITC, anti-mouse CD8 with PE, anti-mouse CD4 with FITC or PerCP, anti-mouse Ter-119 with PE, anti-mouse CD3ε with PerCP, anti-mouse CD18 with PE, anti-mouse CD44 with PE, anti-mouse CD11a with PE, anti-mouse CD62L with PE, anti-mouse NK1.1 with PE and anti-mouse Mac-3 with PE. The antibodies were diluted (1:80) in 50% fetal bovine serum-phosphate buffered saline (FBS-PBS). Isotype-matched irrelevant antibodies were used as controls. Following the addition of the reagents, the cells were incubated at 4°C in the dark for at least 30 minutes. Thereafter, the cells were washed 2X, and enumeration performed on a Becton Dickinson FACScan Flow Cytometer in which log fluorescence intensity was read with a forward scatter threshold high enough to eliminate red blood cells. The data were analyzed using CELLQuest software. A minimum of 5,000 events was acquired. For the neutrophils in the spleen whose percentage was low, a gate was set on Gr-1^+ cells, and a minimum of 500 Gr-1^+ cells was

acquired, which was applicable to the analysis of light scattering and cell surface molecule expression.

Statistics

The data were expressed as mean ± SE. The results were tested for variance homogeneity using Bartlett's Test, and all the data reported here were homogeneous. Homogeneous data were analyzed for differences among three or more independent groups using one-way analysis of variance (Fisher's F-distribution); if there was difference we then determined the difference using the Dunnett's two-tailed t Test (Jump 5.1), which could prevent a type I error for data that were not normally distributed. Statistical significance was set at $P \leq 0.05$.

RESULTS

Terminal body weight and organ weights following Thd treatment for either 14 or 28 days

In the 14-day studies, mice were treated with vehicle or Thd (100 mg/kg) daily for 13 days. On day 14, the day of sacrifice, mice were treated again with Thd, and then they were sacrificed by CO_2 inhalation at 2, 4, 8 and 24 h after the last Thd treatment. No significant change was observed for body weight (data not shown); however, increased spleen weights (both absolute and % body) were observed at both the 2- and 4-h time points (Figure 1A and 1B). Increased liver weights were also observed at the 2-h time point (Figure 1C and 1D). In the 28-day studies, mice were treated with the vehicle or Thd daily for 27 days. On day 28, mice were treated again with Thd, and then, they were sacrificed at 3 and 24 h after the last Thd treatment. No significant changes were observed for body weight, or the weights of the liver, lung, thymus and kidneys at any time points (Table 1 and data not shown); however, significant increases in both the absolute and relative spleen weights were observed at both 3- and 24-h time points (Table 1 and data not shown).

Thd treatment for 14 or 28 days increased the numbers of neutrophils in the spleen, peripheral blood, bone marrow, peritoneal cavity and lungs

The effect of Thd on splenic white blood cell counts was examined in mice that had been treated with Thd daily for 14 or 28 days (Figure 2). Thd treatment for 14 days had no effect on the number of white blood cells (Figure 2A). Flow cytometric analysis was employed to determine the percentages of neutrophils ($Gr\text{-}1^+Mac\text{-}3^-$). Increased percentages of neutrophils were observed at the 2-, 4- and 8-h time points (Figure 2D). When the number of neutrophils in the spleen was calculated using the percentage and the total white blood cell counts, a similar pattern of increases was produced (Figure 2G). Additionally, an increase (22%) in the percentage of natural killer (NK) cells ($NK1.1^+CD3^-$) was observed at the 4-h time point, which might partially contribute to a decrease in the percentage of $CD3^+$ T cells (15%) at the 4-h time point (data not shown). Thd treatment had no effects on the percentages of $Mac\text{-}3^+$ cells, $CD4^+CD8^-$ T cells, $CD4^-CD8^+$ T cells and IgM^+ B cells (data not shown). In mice that had been treated with Thd for 28 days, the number of white blood cells in the spleen was increased at the 24-h but not at the 3-h time point (Figures 2B and 2C). The percentages and total number of neutrophils were increased at both the 3- and 24-h time points (Figures 2E, 2F, 2H and 2I). Thd treatment for 28 days had no effects on the percentages of $CD4^-CD8^+$ T cells, $CD4^+CD8^-$ T cells, NK cells and $CD3^+$ T cells at either the 3-h or the 24-h time points (data not shown). There was a slight decrease for the percentage of $Mac\text{-}3^+$ cells (from 3.36 ± 0.18% to 2.65 ± 0.27%) and that of IgM^+ B cells (from 50.81 ± 0.66% to 47.40 ± 0.65%) at the 3-h time point but not at the 24-h time point (data not shown), which might explain why there was an increase in the number of white blood cells in the spleen at 24 h but not at the 3-h time point.

To determine whether the increase of neutrophils in the spleen was due to a redistributing effect, the numbers of neutrophils in the peripheral blood, bone marrow, peritoneal cavity and

lungs were examined. Our results on the neutrophil counts in the spleen (Figure 2) suggested that the time of 3 hours after the last Thd treatment was an optimal time point to assess the redistributing effect because increases in both the percentage and number of splenic neutrophils were observed in both the 14-day and 28-day Thd-treated mice. Therefore, with a few exceptions that were clearly stated in the text, all other results reported below were performed at this time point.

Various hematological parameters in mice that had been treated with the vehicle or Thd for 28 days were examined in the peripheral blood, which included the number of erythrocytes and leukocytes, hemoglobin, hematocrit, MCV, MCH, MCHC, the percentage of reticulocytes and differential white blood cell counts. Among these parameters, the number of neutrophils was significantly increased in Thd-treated mice at both 3- and 24-h time points (Table 2 and data not shown). Additionally, an increase in the platelet number at the 24-h time point was also observed (Table 2).

Bone marrow cells were obtained by flushing both femurs of the mice that had been treated with the vehicle or Thd. In mice that had been treated with Thd for 14 days, a significant increase in the percentage of Gr-1^+ cells in the bone marrow from 31.1 ± 1.0 in vehicle mice to 44.7 ± 2.1 in Thd mice was observed at the 3-h time point after the last Thd treatment (data not shown). In mice that had been treated with Thd for 28 days, an increase in the number of nucleated bone marrow cells, but not the red blood cells, was observed (Figure 3A). Bone marrow cells could be separated into three major populations by staining with anti-Gr-1 and Ter119 (erythroid cells) antibodies. An increase in the percentage of Gr-1^+ cells, while a decrease in the percentage of Ter119^+ cells, was observed in Thd-treated mice (Figure 3C) as compared to the vehicle controls (Figure 3B).

Thd treatment for 14 days significantly increased the number of neutrophils in the peritoneal cavity when compared to the controls at the 3-h time point after the last Thd treatment (Figure 4A). We also determined the effect of Thd treatment for 14 days on LPS-induced neutrophil recruitment into the lung. As shown in Figure 4B, treatment with LPS significantly increased the neutrophil number in the lung when compared to the naïve mice, and treatment with Thd further increased LPS-recruited neutrophils in the lung when compared to the vehicle mice.

Thd treatment for 14 days increased the activity of myeloperoxidase

We further determined if Thd treatment increased the activity of MPO - an indicator of neutrophil function. As shown in Figure 5, an increase in MPO activity was observed in both the spleen (Figure 5A) and bone marrow (Figure 5B) in the 14-day Thd-treated mice.

Thd treatment for either 14 or 28 days on bone marrow proliferation and granulopoiesis

The effect of Thd treatment on granulopoiesis of bone marrow was evaluated using two different methods. The first method measured the CFU-GM, e.g., the colony-forming units in the presence of GM-CSF. The second method measured G-CFC, using the HALO Kit Platform in which the readout of luminescence based on the cellular ATP content was produced. The second method was more quantitative since both the number and size of colonies were taken into consideration. Thd treatment for 14 days did not affect the colony-forming ability of bone marrow cells in either of the assays (data not shown). Additionally, the bone marrow cell proliferation was not affected by the 14-day Thd treatment (data not shown). Thd treatment for 28 days did not affect the G-CFC of the bone marrow cells when assayed using the HALO Kit Platform; however, a slight, albeit significant, decrease in bone marrow cell proliferation was produced (52596 ± 5258 in VH group vs. 34984 ± 3448 in Thd group).

Neutrophils in the 14-day Thd-treated mice were different from those in the 28-day Thd-treated female B6C3F1 mice

Increased neutrophil forward light scatter (FSC; neutrophil size) and side light scatter (SSC; granularity) are attributable to neutrophil activation or differentiation (Leckie *et al*., 2000; Weiss and Evanson, 2003; Hock *et al*., 2003). Therefore, we compared the changes in neutrophil FSC and SSC in the 14- and 28-day Thd-treated mice. As shown in Table 3, treatment with Thd for 14 days had no significant effects on these parameters either in the bone marrow or in the spleen. However, increased SSC and FSC were observed in splenic neutrophils after 28 days of Thd treatment. A significant increase in SSC was also observed in bone marrow neutrophils after 28 days of Thd treatment (Table 3).

There is evidence that Thd modulates the surface expression of adhesion molecules by neutrophils (Nogueira *et al*., 1994). Therefore, we evaluated the effect of Thd on the surface expression of CD18, CD62L, CD11a and CD44 by neutrophils in Thd-treated mice. In the 14-day Thd-treated mice, the expression of CD18, but not CD44, by the neutrophils from bone marrow was significantly decreased (Figure 6B, Figure 7B). No significant effects were produced on the surface expression of CD18 and CD44 by splenic neutrophils (Figure 6E, Figure 7E). However, Thd treatment for 28 days decreased the surface expression of both CD44 and CD18 on bone marrow neutrophils (Figure 6C, Figure 7C). In contrast to the bone marrow data, Thd treatment for 28 days produced an increase in the surface expression of both CD18 and CD44 by the neutrophils in the spleen (Figure 6F, Figure 7F). No effect was produced for other cell surface molecules such as CD62L and CD11a in either the 14-day or the 28-day Thd treatment (Table 4).

Effect of a single Thd treatment

To determine the effect of a single Thd treatment, mice were treated with a single injection of either vehicle or Thd (100 mg/kg) and sacrificed three hours later. No effect was produced for the body weight, or the weights of the spleen and thymus. The number of splenic neutrophils and their light scattering properties were not altered by the treatment either (data not shown).

DISCUSSION

Our investigation of Thd's effect on the immune function in adult female B6C3F1 mice led to the finding that treatment with Thd for either 14 or 28 days increased the numbers of neutrophils in the spleen, peripheral blood, lung, peritoneal cavity and bone marrow. Furthermore, these neutrophils were functional as indicated by an increased MPO activity in the spleen and bone marrow following Thd treatment, as well as our previous reports that Thd-mediated enhancement in neutrophil responses was responsible for the increased host resistance against both primary and secondary *Listeria monocytogenes* infection (Karrow *et al.*, 2003; Guo *et al*., 2005). Taken together, we can conclude that Thd enhancement of neutrophil function is one of the mechanisms of action that is responsible for Thd's immunomodulatory efficacy in a variety of diseases, which may be particularly true for MDS. In MDS, the differentiation defect in the multipotent stem cell compartment not only leads to neutropenia but also results in aberrant neutrophil function. The surface expression of the CD11b/CD18 complex, which regulates neutrophil adherence, migration and diapedesis, is decreased on peripheral neutrophils from MDS patients (Mazzone *et al*., 1996); the activity of granule enzymes and expression of neutrophilic granule membrane glycoproteins are reduced; and phagocytosis of bacteria, the production of reactive oxygen species and neutrophil chemotaxis are defective (Fuhler *et al*., 2003, 2005). Further study of the effect of Thd treatment on neutrophil response and host resistance to bacterial infection in MDS mice is warranted (Lin *et al*., 2005).

Treatment with Thd daily for either 14 or 28 days significantly decreased the surface expression of CD18 by bone marrow neutrophils; the surface expression of CD44 by bone marrow neutrophils was also decreased in the 28-day Thd-treated mice. A decrease surface expression of CD18 and CD44 by neutrophils has two potential outcomes: (a) to reduce bone marrow retention (Burdon *et al*., 2005); and (b) to increase neutrophil demargination (Lyden *et al*., 1998; Sato *et al*., 1999). It was possible that Thd modulated the surface expression of CD18 and CD44, and facilitated neutrophil release from the bone marrow because there was evidence that CD18 played an important role in neutrophil retention within the bone marrow (Burdon *et al*., 2005). Decreased CD44 expression by bone marrow neutrophils would also result in similar effects as that of CD18 (Wang *et al*., 2002). Therefore, an increase in neutrophil trafficking as a result of a decreased surface expression of CD18 and CD44 by bone marrow neutrophils may be, at least partially, responsible for the neutrophilia in Thd-treated mice.

Treatment with Thd daily for 28 days significantly increased the surface expression of CD18 and CD44 by splenic neutrophils. Increased surface expressions of CD44 and CD18 by splenic neutophils would increase their ability to remain in the periphery, which might contribute to the increased numbers and percentages of neutrophils in the spleen even at the 24-h time point after the last Thd treatment in the 28-day Thd-treated mice. This finding is consistent with the report that MDS patients treated with Thd usually have a delayed response in increasing blood neutrophil counts (Raza *et al*., 2004; Invernizzi *et al*., 2005). Increased CD44 expression by peripheral neutrophils would also increase their cytotoxic function (Pericle *et al*., 1996). Unfortunately, our MPO assay could not differentiate whether the increase in MPO activity was due to an increase in the cytotoxic function or an increase in the number of neutrophils. Nonetheless, these observations are important since it has been suggested that neutrophil dysfunction and the resultant increased susceptibility to bacterial infections observed in MDS patients may be correlated with decreased expression of these surface adhesion molecules (Mazzone *et al*., 1996; Ohsaka *et al*., 1997). However, how Thd treatment causes differential expression of CD18 and CD44 by neutrophils from the bone marrow and spleen is currently unknown. It is similarly uncertain currently how long the increased surface adhesion molecule expression and increased peripheral neutrophils can last.

Although Thd treatment for either 14 days or 28 days did not change bone marrow granulopoiesis in our studies, we can't totally rule out the possibility of an increased granulopoiesis because the granulocyte colony-forming ability by the blood and spleen has not been examined (Zhang *et al*., 2005). An increase in the spleen and liver weights is indicative of increased peripheral hematopoiesis (Hejtmancik *et al.*, 2002). Interestingly, both the weights of the spleen and liver were increased in either the 14-day or the 28-day Thd-treated mice. Genetic deficiency in CD18 can also lead to increases in spleen weight and peripheral hematopoiesis (Horwitz *et al.*, 2001). Thus, a decrease in the expression of CD18 by bone marrow neutrophils after Thd treatment might play a role in increasing the spleen weight. Furthermore, our results have demonstrated that a 28-day Thd treatment increased total white blood cells in the spleen and bone marrow, and that the neutrophils in the 28-day Thd-treated mice have increases in both the FSC (neutrophil size) and SSC (neutrophil granularity). All these changes are consistent with an increased granulopoiesis. Thus, it is still possible that Thd treatment for a longer time, e.g. 28 days, may increase the granulopoiesis in organs other than bone marrow. It should be noted that bone marrow and peripheral blood neutrophils of MDS patients exhibited a significant decrease in SSC when compared to the healthy controls, and may be responsible for increased susceptibility to bacterial infection (Stetler-Stevenson *et al*., 2001; Wells *et al*., 2003; Cherian *et al*., 2005; Shikama *et al*., 2005).

Treatment with Thd daily for 14 or 28 days had no effects on the body weight (data not shown), suggesting no systemic toxicity occurred during the treatment periods. Thd treatment did not affect the surface expression of CD11a by neutrophils in our studies, which was consistent

with the *in vitro* effect of Thd (Geitz *et al*., 1996). However, the expression of CD62L by neutrophils was not affected by Thd treatment in our studies, which was in contrast to the report that Thd downregulated the surface expression of L-Selectin by neutrophils (Nogueira *et al*., 1994; Geitz *et al*., 1996). The reason for this discrepancy is not clear, but it may be related to treatment duration, species difference and neutrophil location. In the study by Nogueira *et al*. (1994), the blood neutrophils were evaluated in two 63 year-old male volunteers that had received 6 days of Thd. In the *in vitro* study by Geitz *et al*. (1996), human peripheral blood neutrophils were treated with Thd for 24 h. In our study, the weights of the spleen and liver, and the number of neutrophils were increased in the first few hours after the last Thd treatment in the 14-day Thd-treated mice. The exact mechanism for this transient effect is currently unknown, and it might be related to the half-life of Thd.

In summary, the results presented here suggest that Thd modulation of neutrophil function is one of the mechanisms of action that are responsible for Thd's immunomodulatory efficacy in a variety of diseases. By focusing on neutrophils, further study of Thd's immunomodulatory effect will help us have a better understanding of Thd's therapeutic efficacy in various diseases and make better usage of this drug for human health benefits.

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References

Auttachoat W, Chitsomboon B, Peachee VL, Guo TL, White KL Jr. Immunomodulation by Dok Din Daeng (Aeginetia indica Roxb.) extracts in female B6C3F1 mice: II. Humoral immunity, innate immunity and hematology. Int Immunopharmacol 2004;4:1381–90. [PubMed: 15313435]

Burdon PC, Martin C, Rankin SM. The CXC chemokine MIP-2 stimulates neutrophil mobilization from the rat bone marrow in a CD49d-dependent manner. Blood 2005;105:2543–2548. [PubMed: 15542579]

Cherian S, Moore J, Bantly A, Vergilio JA, Klein P, Luger S, Bagg A. Peripheral blood MDS score: a new flow cytometric tool for the diagnosis of myelodysplastic syndromes. Cytometry B Clin Cytom 2005;64:9–17. [PubMed: 15668954]

Davies FE, Raje N, Hideshima T, Lentzsch S, Young G, Tai YT, Lin B, Podar K, Gupta D, Chauhan D, Treon SP, Richardson PG, Schlossman RL, Morgan GJ, Muller GW, Stirling DI, Anderson KC. Thalidomide and immunomodulatory derivatives augment natural killer cell cytotoxicity in multiple myeloma. Blood 2001;8:210–6. [PubMed: 11418482]

Eriksson T, Hoglund P, Turesson I, Waage A, Don BR, Vu J, Scheffler M, Kaysen GA. Pharmacokinetics of thalidomide in patients with impaired renal function and while on and off dialysis. J Pharm Pharmacol 2003;55:1701–6. [PubMed: 14738599]

Fuhler GM, Drayer AL, Vellenga E. Decreased phosphorylation of protein kinase B (PKB/Akt) and extracellular signal-regulated kinase (ERK) in neutrophils from patients with myelodysplasia. Blood 2003;101:1172–1180. [PubMed: 12529294]

Fuhler GM, Knol GJ, Drayer AL, Vellenga E. Impaired interleukin-8- and GROalpha-induced phosphorylation of extracellular signal-regulated kinase result in decreased migration of neutrophils from patients with myelodysplasia. J Leukoc Biol 2005;77:257–66. [PubMed: 15561756]

Geitz H, Handt S, Zwingenberger K. Thalidomide selectively modulates the density of cell surface molecules involved in the adhesion cascade. Immunopharmacology 1996;31:213. [PubMed: 8861747]

Guo TL, Zhang LX, Chen JP, Nguyen VA, White KL Jr, Gao B. Differential STAT5 activation and phenotypic marker expression by immune cells following low levels of ethanol consumption in mice. Immunopharmacol Immunotoxicol 2002;24:121. [PubMed: 12022440]

Guo TL, Chi RP, Karrow NA, Zhang LX, Pruett SB, Germolec DR, White KL Jr. Thalidomide enhances both primary and secondary host resistances to Listeria monocytogenes infection by a neutrophil-

related mechanism in female B6C3F1 mice. Toxicol Appl Pharmacol 2005;209:244–54. [PubMed: 15921716]

Hejtmancik MR, Ryan MJ, Toft JD, Persing RL, Kurtz PJ, Chhabra RS. Hematological effects in F344 rats and B6C3F1 mice during the 13-week gavage toxicity study of methylene blue trihydrate. Toxicol Sci 2002;65:126–34. [PubMed: 11752692]

Hock H, Hamblen MJ, Rooke HM, Traver D, Bronson RT, Cameron S, Orkin SH. Intrinsic requirement for zinc finger transcription factor Gfi-1 in neutrophil differentiation. Immunity 2003;18:109–20. [PubMed: 12530980]

Hodgson. Toxicology Testing and Risk Assessment. In: Hodgson, E.; Levi, PE., editors. A Textbook of Modern Toxicology. 2. Appleton & Lange; Stamford, Connecticut: 1997. p. 336

Horwitz BH, Mizgerd JP, Scott ML, Doerschuk CM. Mechanisms of granulocytosis in the absence of CD18. Blood 2001;97:1578–83. [PubMed: 11238094]

Invernizzi R, Travaglino E, De Amici M, Brugnatelli S, Ramajoli I, Rovati B, Benatti C, Ascari E. Thalidomide treatment reduces apoptosis levels in bone marrow cells from patients with myelodysplastic syndromes. Leuk Res 2005;29:641–7. [PubMed: 15863203]

Karrow NA, Guo TL, Zhang LX, McCay JA, Musgrove DL, Peachee VL, Germolec DR, White KL Jr. Thalidomide modulation of the immune response in female B6C3F1 mice: a host resistance study. Int Immunopharmacol 2003;3:1447. [PubMed: 12946441]

Karrow NA, McCay JA, Brown RD, Musgrove DL, Pettit DA, Munson AE, Germolec DR, White KL Jr. Thalidomide stimulates splenic antibody response and cytotoxic T lymphocyte activity and alters leukocyte subpopulation numbers in female B6C3F1 mice. Toxicol Appl Pharmacol 2000;165:237. [PubMed: 10873712]

Kouides PA, Bennett JM. Understanding the Myelodysplastic Syndromes. Oncologist 1997;2:389–401. [PubMed: 10388074]

Leckie MJ, Bryan SA, Khan J, Dewar A, Aikman SL, McGrath J, Okrongly DA, Burman JF, Barnes PJ, Hansel TT. Automated quantitation of circulating neutrophil and eosinophil activation in asthmatic patients. Thorax 2000;55:471–7. [PubMed: 10817795]

Lin YW, Slape C, Zhang Z, Aplan PD. NUP98-HOXD13 transgenic mice develop a highly penetrant, severe myelodysplastic syndrome that progresses to acute leukemia. Blood 2005;106:287–95. [PubMed: 15755899]

Lubbert M, Daskalakis M, Kunzmann R, Engelhardt M, Guo Y, Wijermans P. Nonclonal neutrophil responses after successful treatment of myelodysplasia with low-dose 5-aza-2'-deoxycytidine (decitabine). Leuk Res 2004;28:1267–71. [PubMed: 15475067]

Lyden SP, Patton JH Jr, Ragsdale DN, Croce MA, Fabian TC, Proctor KG. Transient inhibition of CD18-dependent leukocyte functions after hemorrhage and polymicrobial sepsis. Surgery 1998;123:679–91. [PubMed: 9626319]

Marriott JB, Muller G, Dalgleish AG. Thalidomide as an emerging immuno-therapeutic agent. Immunol Today 1999;20:538. [PubMed: 10562702]

Mazzone A, Porta C, Fossati G, Gritti D, Mazzucchelli I, Ricevuti G. Granulocyte dysplasia and dysfunction, and CD11/CD18 defects in myelodysplastic syndromes Leuk. Lymphoma 1996;23:267–275.

Miyamoto M, Emoto M, Emoto Y, Brinkmann V, Yoshizawa I, Seiler P, Aichele P, Kita E, Kaufmann SH. Neutrophilia in LFA-1-deficient mice confers resistance to listeriosis: possible contribution of granulocyte-colony-stimulating factor and IL-17. J Immunol 2003;170:5228. [PubMed: 12734371]

Mufti GJ, Stevens JR, Oscier DG, Hamblin TJ, Machin D. Myelodysplastic syndromes: a scoring system with prognostic significance. Br J Haematol 1985;59:425–33. [PubMed: 3970861]

Musto P. Thalidomide therapy for myelodysplastic syndromes: current status and future perspectives. Leuk Res 2004;28:325–32. [PubMed: 15109529]

Nakagawa M, Terashima T, D'yachkova Y, Bondy GP, Hogg JC, van Eeden SF. Glucocorticoid-induced granulocytosis: contribution of marrow release and demargination of intravascular granulocytes. Circulation 1998;98:2307–13. [PubMed: 9826319]

Nelson DA. The biology of myelopoiesis. Clin Lab Med 1990;10:649–659. [PubMed: 2272167]

Nogueira AC, Neubert R, Helge H, Neubert D. Thalidomide and the immune system. 3 Simultaneous up- and down-regulation of different integrin receptors on human white blood cells. Life Sci 1994;55:77. [PubMed: 7516993]

Ohsaka A, Saionji K, Igari J, Watanabe N, Iwabuchi K, Nagaoka I. Altered surface expression of effector cell molecules on neutrophils in myelodysplastic syndromes. British Journal of Haematology 1997;98:108–113. [PubMed: 9233572]

Pericle F, Sconocchia G, Titus JA, Segal DM. CD44 is a cytotoxic triggering molecule on human polymorphonuclear cells. J Immunol 1996;157:4657–63. [PubMed: 8906846]

Raza A, Buonamici S, Lisak L, Tahir S, Li D, Imran M, Chaudary NI, Pervaiz H, Gallegos JA, Alvi MI, Mumtaz M, Gezer S, Venugopal P, Reddy P, Galili N, Candoni A, Singer J, Nucifora G. Arsenic trioxide and thalidomide combination produces multi-lineage hematological responses in myelodysplastic syndromes patients, particularly in those with high pre-therapy EVI1 expression. Leuk Res 2004;28:791–803. [PubMed: 15203277]

Sato Y, Walley KR, Klut ME, English D, D'yachkova Y, Hogg JC, van Eeden SF. Nitric oxide reduces the sequestration of polymorphonuclear leukocytes in lung by changing deformability and CD18 expression. Am J Respir Crit Care Med 1999;159:1469–76. [PubMed: 10228113]

Shikama Y, Shichishima T, Matsuoka I, Jubinsky PT, Sieff CA, Maruyama Y. Accumulation of an intron-retained mRNA for granulocyte macrophage-colony stimulating factor receptor common beta chain in neutrophils of myelodysplastic syndromes. J Leukoc Biol 2005;77:811–9. [PubMed: 15728248]

Stetler-Stevenson M, Arthur DC, Jabbour N, Xie XY, Molldrem J, Barrett AJ, Venzon D, Rick ME. Diagnostic utility of flow cytometric immunophenotyping in myelodysplastic syndrome. Blood 2001;98:979–87. [PubMed: 11493442]

Tohnya TM, Hwang K, Lepper ER, Fine HA, Dahut WL, Venitz J, Sparreboom A, Figg WD. Determination of CC-5013, an analogue of thalidomide, in human plasma by liquid chromatography-mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2004;811:135–41.

Wang Q, Teder P, Judd NP, Noble PW, Doerschuk CM. CD44 deficiency leads to enhanced neutrophil migration and lung injury in Escherichia coli pneumonia in mice. Am J Pathol 2002;161:2219–28. [PubMed: 12466136]

Weiss DJ, Evanson OA. Evaluation of activated neutrophils in the blood of horses with colic. Am J Vet Res 2003;64:1364–8. [PubMed: 14620771]

Wells DA, Benesch M, Loken MR, Vallejo C, Myerson D, Leisenring WM, Deeg HJ. Myeloid and monocytic dyspoiesis as determined by flow cytometric scoring in myelodysplastic syndrome correlates with the IPSS and with outcome after hematopoietic stem cell transplantation. Blood 2003;102:394–403. [PubMed: 12649150]

Zhang P, Quinton LJ, Gamble L, Bagby GJ, Summer WR, Nelson S. The granulopoietic cytokine response and enhancement of granulopoiesis in mice during endotoxemia. Shock 2005;23:344–52. [PubMed: 15803058]

Zwingenberger K, Wnendt S. Immunomodulation by thalidomide: systematic review of the literature and of unpublished observations. J Inflamm 1995–96;46:177. [PubMed: 8878794]

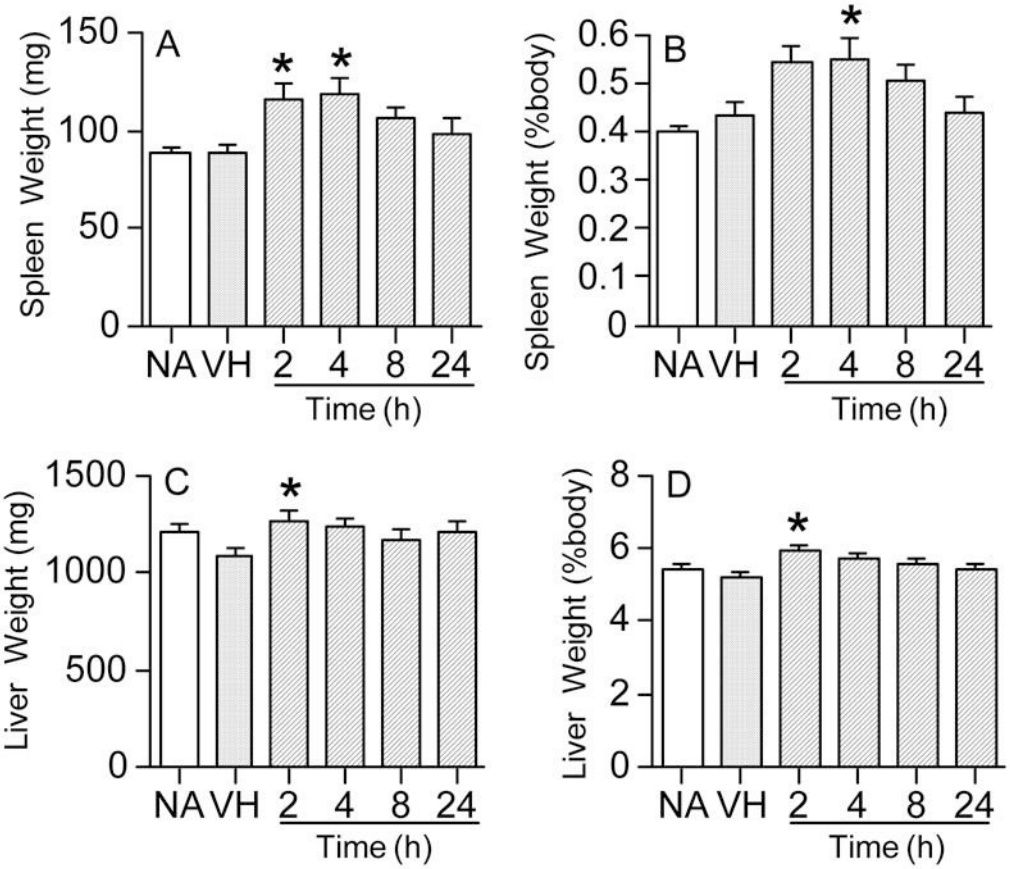

Figure 1.

Thd treatment for 14 days transiently increased the weights of the spleen and liver in B6C3F1 mice. Mice were treated with vehicle or Thd (100 mg/kg; i.p.) daily for 13 days. On day 14, the day of sacrifice, mice were treated again with Thd, and then they were sacrificed by CO_2 inhalation at the time points of 2, 4, 8 and 24 h after the last Thd treatment. Mice were weighed, and the spleen (A, B) and liver (C, D) removed and weighed. *, $p \leq 0.05$ as compared to vehicle controls. N = 8.

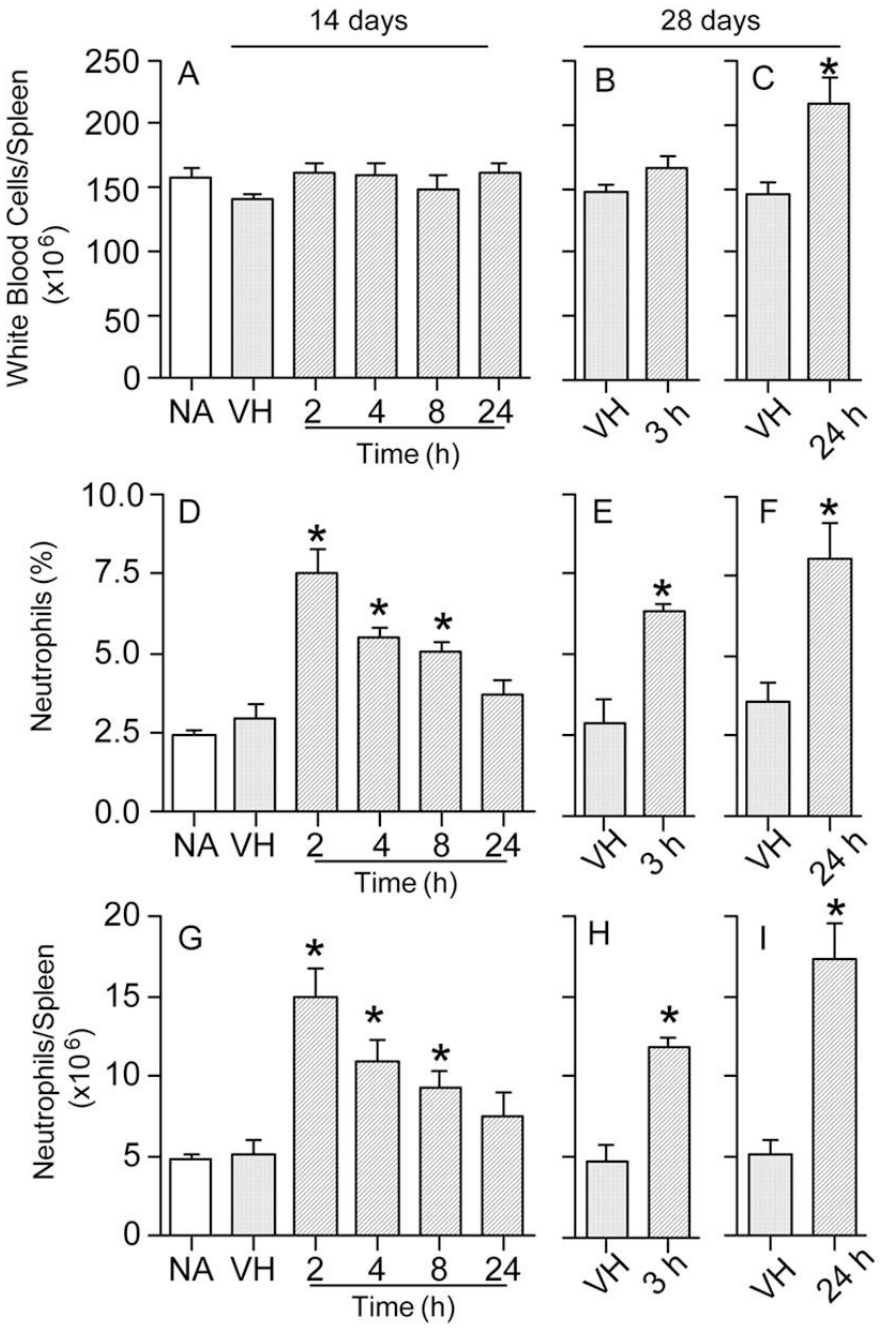

Figure 2.

Thd treatment on splenocyte counts. Female B6C3F1 mice were treated with vehicle or Thd (100 mg/kg; i.p.) for 14 (A, D and G) or 28 days (B, C, E, F, H and I). Cell counts for total white blood cells (A, B and C), and flow cytometric analysis for the percentage of neutrophils (D, E and F) were performed. The number of splenic neutrophils (G, H and I) was determined as described. Mice were sacrificed at different time points after the last Thd treatment. The spleens were removed and mashed. After washing, splenocytes were resuspended in RPMI complete medium and counted using a Coulter Counter ZII with the red blood cells lysed using a ZAP-O-GLOBIN II lytic reagent. *, $p \leq 0.05$ as compared to vehicle controls. N = 8. NA = naïve.

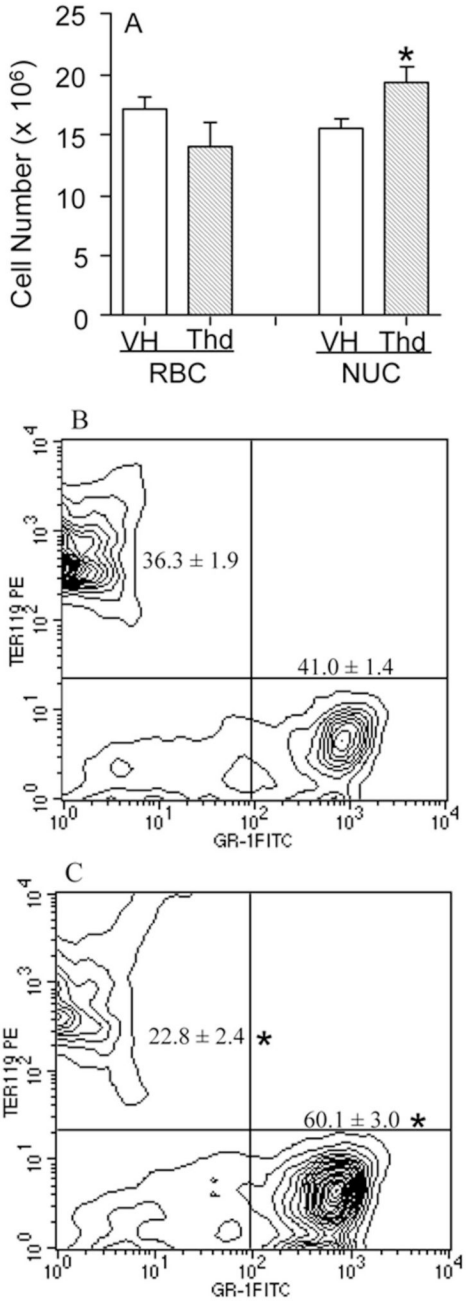

Figure 3.

Thd treatment on differential bone marrow cells. Female B6C3F1 mice were treated with VH or Thd (100 mg/kg; i.p.) for 28 days. Three hours after the last Thd treatment, bone marrow cells were obtained as described. The counts for total bone marrow cells and nucleated bone marrow cells (NUC) were obtained. The cell count in the absence of ZAP-O-GLOBIN II lytic reagent was the total cell number (both red blood cells + nucleated cells). The count in the presence of ZAP-O-GLOBIN II lytic reagent was the nucleated bone marrow cells. The number of red blood cells (RBC) was obtained by subtracting the number of NUC from the number of total bone marrow cells. Flow cytometric analysis was performed as described. A, the number of RBCs and NUCs; B, a representative contour plot (Ter119 vs. Gr-1) of flow cytometric

analysis for vehicle mice; and C, a representative contour plot (Ter119 vs. Gr-1) of flow cytometric analysis for Thd-treated mice. Also shown in panels B and C are the mean ± SE of the percent values for the upper left and lower right quadrants. *, $p \leq 0.05$ as compared to vehicle controls. N = 8.

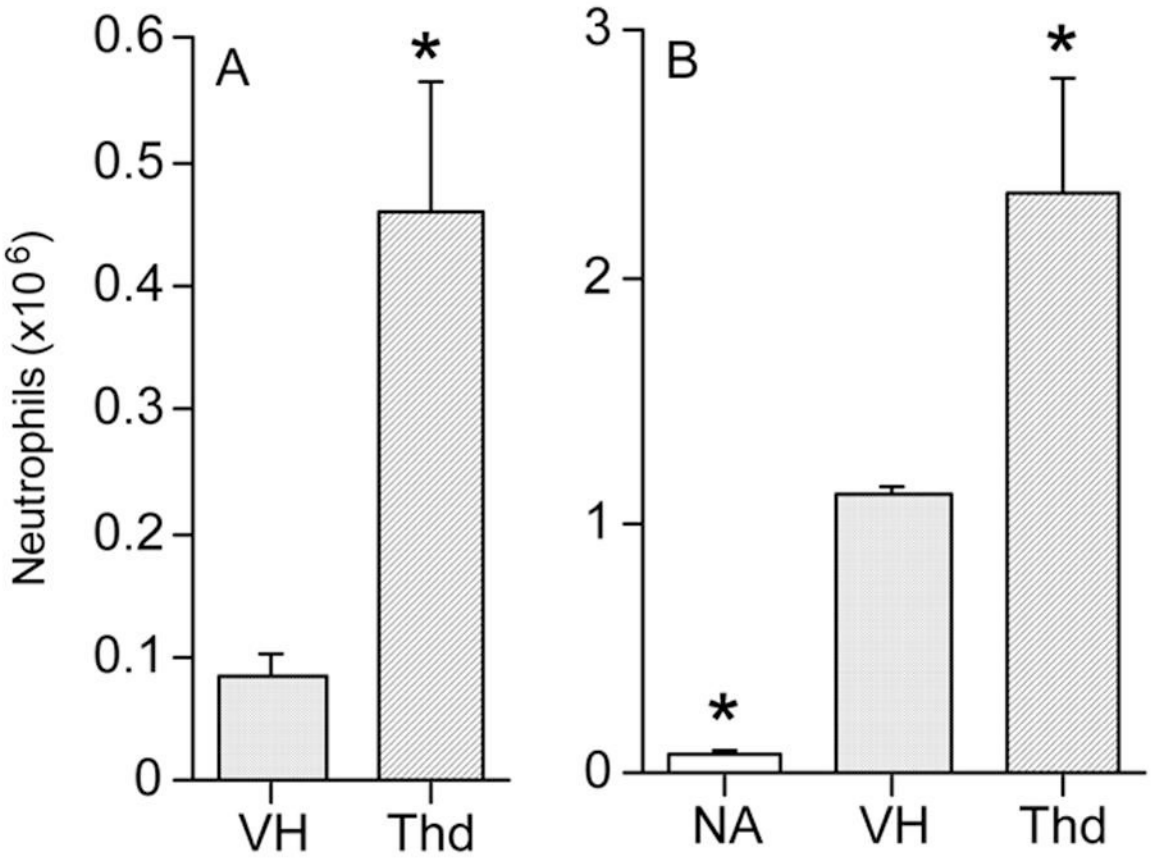

Figure 4.

Thd treatment increased the number of neutrophils in the peritoneal cavity (A) and lung (B). Female B6C3F1 mice were treated with VH or Thd (100 mg/kg; i.p.) for 14 days. Three hours after the last Thd treatment, mice were sacrificed, and the peritoneal cavity lavaged. To determine the effect of Thd treatment on neutrophil recruitment into the lung, mice (lightly anesthetized) were treated with LPS (0.3 mg/kg) intranasally three hours after the last Thd treatment. Twenty-four hours after LPS treatment, mice were sacrificed and the lung lavaged. The cell counts were obtained for the lavaged cells, and flow cytometric analysis of $Gr1^{+}Mac\text{-}3^{-}$ cells performed. *, $p \leq 0.05$ as compared to vehicle controls. N = 8. NA = naïve.

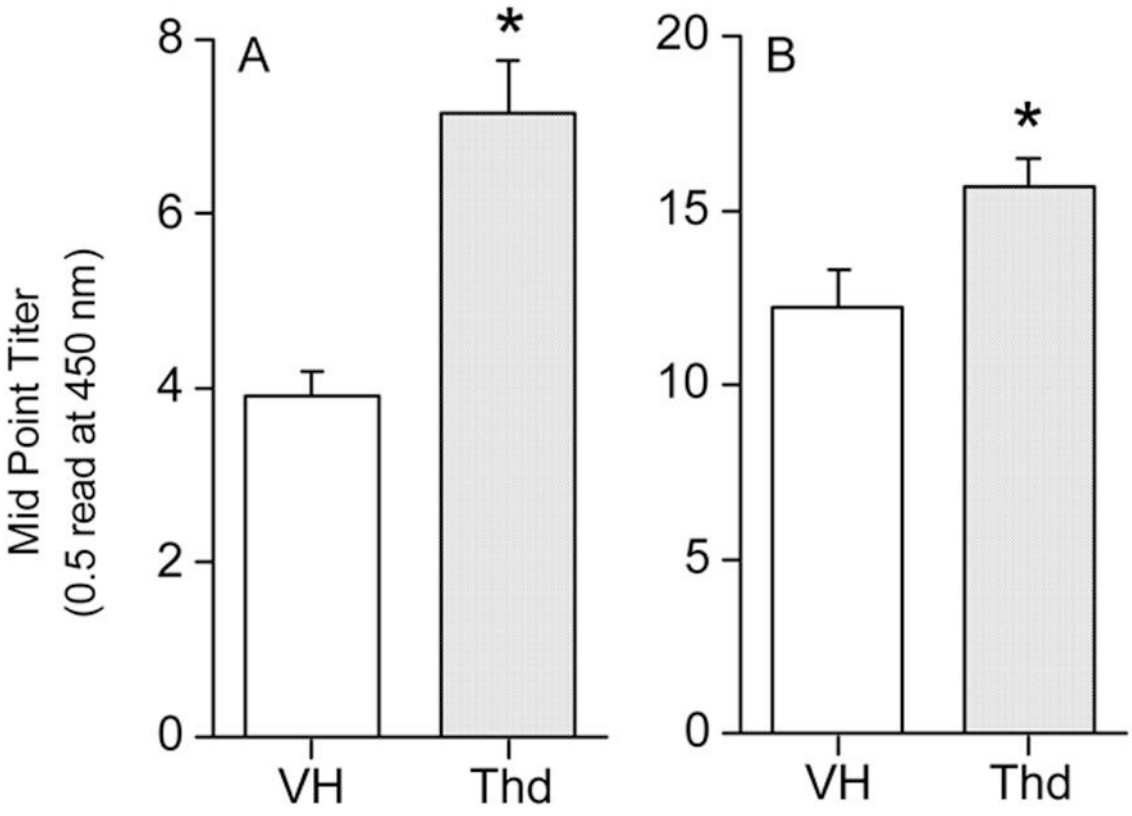

Figure 5.
Thd treatment increased the myeloperoxidase (MPO) activity in both the spleen (A) and bone marrow (B). Female B6C3F1 mice were treated with VH or Thd (100 mg/kg; i.p.) for 14 days. Three hours after the last Thd treatment, mice were sacrificed. Splenocytes and bone marrow cells were obtained. MPO activity was measured as described. *, $p \leq 0.05$ as compared to vehicle controls. N = 8.

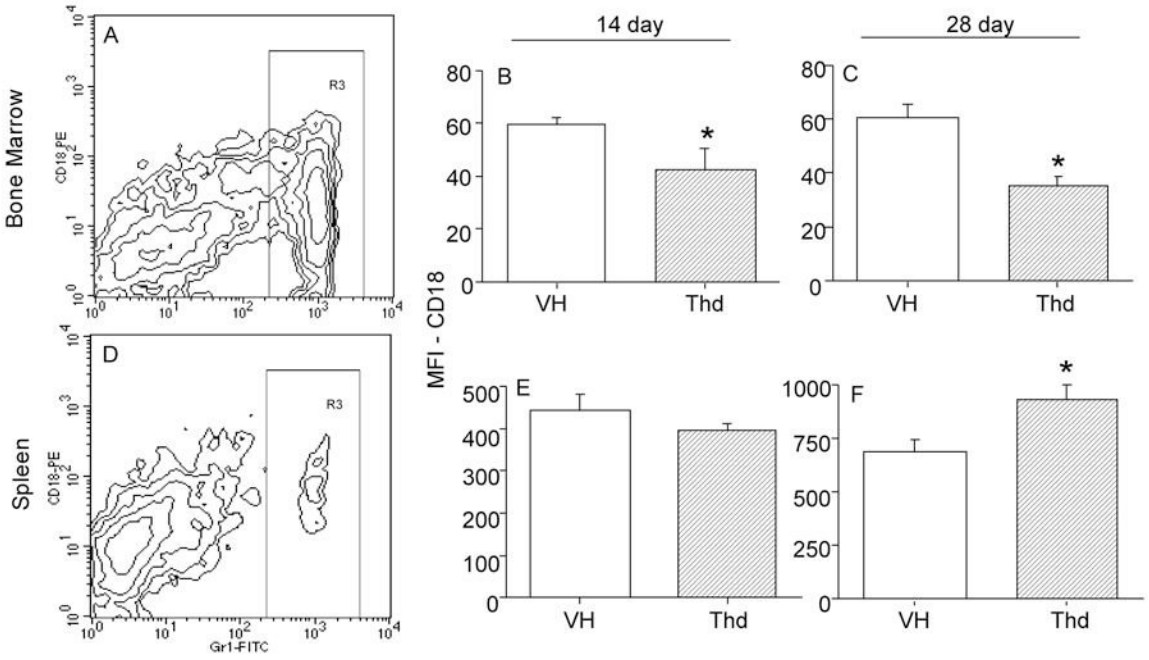

Figure 6.

Thd treatment on the surface expression of CD18 by neutrophils. Female B6C3F1 mice were treated with VH or Thd (100 mg/kg; i.p.) for 14 (B and E) or 28 days (C and F). Three hours after the last Thd treatment, mice were sacrificed, bone marrow cells and splenocytes obtained, and flow cytometric analysis performed as described. (A) A representative contour plot of bone marrow cells (CD18 vs. Gr-1) showing the gate of neutrophils (R3); (B) CD18 mean fluorescence intensity (MFI) by bone marrow neutrophils (R3) in the 14-day Thd-treated mice; (C) CD18 MFI by bone marrow neutrophils (R3) in the 28-day Thd-treated mice; (D) A representative contour plot of splenocytes (CD18 vs. Gr-1) showing the gate of neutrophils (R3); (E) CD18 MFI by splenic neutrophils (R3) in the 14-day Thd-treated mice; and (F) CD18 MFI by splenic neutrophils (R3) in the 28-day Thd-treated mice. *, $p \leq 0.05$ as compared to vehicle controls. N = 8.

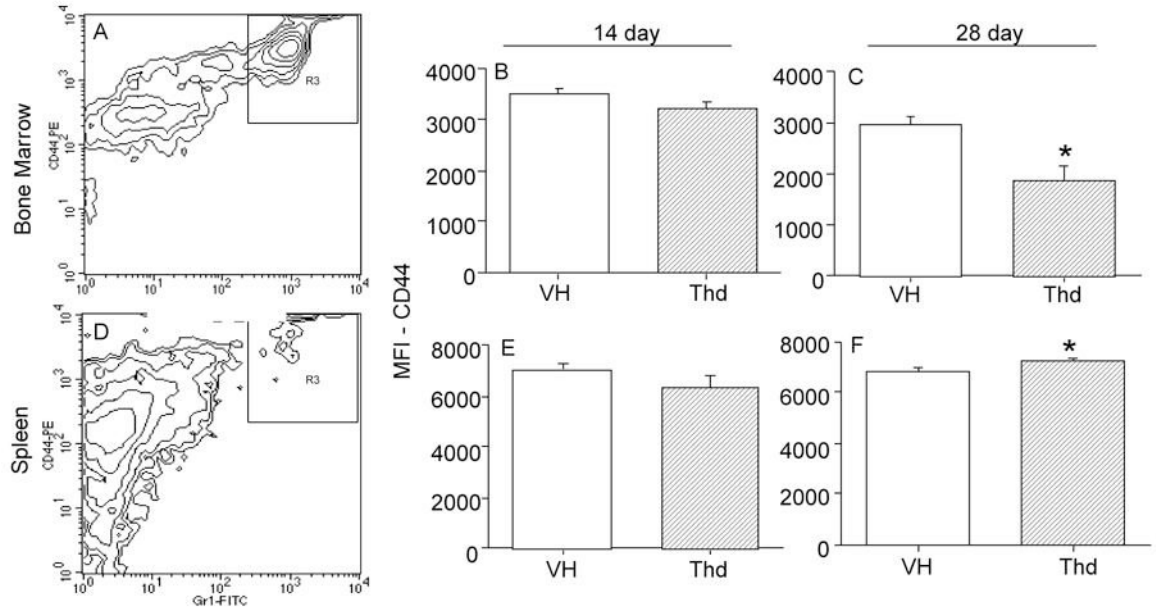

Figure 7.

Thd treatment on the surface expression of CD44 by neutrophils. Female B6C3F1 mice were treated with VH or Thd (100 mg/kg; i.p.) for 14 (B and E) or 28 days (C and F). Three hours after the last Thd treatment, mice were sacrificed, bone marrow cells and splenocytes obtained, and flow cytometric analysis performed as described. (A) A representative contour plot of bone marrow cells (CD44 vs. Gr-1) showing the gate of neutrophils (R3); (B) CD44 MFI by bone marrow neutrophils (R3) in the 14-day Thd-treated mice; (C) CD44 MFI by bone marrow neutrophils (R3) in the 28-day Thd-treated mice; (D) A representative contour plot of splenocytes (CD44 vs. Gr-1) showing the gate of neutrophils (R3); (E) CD44 MFI by splenic neutrophils (R3) in the 14-day Thd-treated mice; and (F) CD44 MFI by splenic neutrophils (R3) in the 28-day Thd-treated mice. *, $p \leq 0.05$ as compared to vehicle controls. N = 8.

TABLE 1

Thd Treatment for 28 days on the Body Weight and Organ Weights (Absolute and %) in Female B6C3F1 Mice

Parameters		Treatment	
		Vehicle	Thalidomide
Body Weight (g)		28.4 ± 0.6	28.7 ± 0.6
Liver Weight (mg)		1450.7 ± 51.4	1544.3 ± 50.7
	%	5.09 ± 0.10	5.39 ± 0.20
Spleen Weight (mg)		96.7 ± 5.1	145.7 ± 12.1*
	%	0.34 ± 0.01	0.51 ± 0.05*
Lung Weight (mg)		210.7 ± 8.7	210.0 ± 12.9
	%	0.74 ± 0.03	0.74 ± 0.05
Thymus Weight (mg)		101.9 ± 6.5	85.4 ± 6.0
	%	0.36 ± 0.02	0.30 ± 0.02
Kidney Weight (mg)		404.6 ± 9.7	410.6 ± 11.6
	%	1.42 ± 0.02	1.43 ± 0.05

Note. Mice were treated with vehicle or Thd (i.p.) for 28 days. Twenty-four hours after the last Thd treatment, mice were sacrificed, and organs removed and weighed. Values represent the mean ± SE derived from eight animals.

*, $p \leq 0.05$ as compared to vehicle controls.

TABLE 2

Thd Treatment for 28 days on the Hematological Parameters in Female B6C3F1 Mice

Parameters	Treatment	
	Vehicle	Thalidomide
Erythrocytes(10^6/mm^3)	9.13 ± 0.15	8.98 ± 0.17
Hemoglobin (g/dl)	15.47 ± 0.14	15.11 ± 0.24
Hematocrit (%)	46.30 ± 0.77	45.50 ± 0.85
MCV (fl)	50.73 ± 0.21	50.69 ± 0.22
MCH (pg)	16.96 ± 0.16	16.84 ± 0.11
MCHC (g/dl)	33.44 ± 0.30	33.20 ± 0.16
Platelet (10^3/μl)	659.86 ± 126.51	1106.14 ± 72.26*
Reticulocytes (%)	4.33 ± 0.30	4.55 ± 0.22
WBC (10^3/mm^3)	7.63 ± 0.58	7.47 ± 0.89
Lymphocytes (10^3/mm^3)	6.07 ± 0.49	4.84 ± 0.58
Neutrophils (10^3/mm^3)	1.56 ± 0.22	2.63 ± 0.39*

Note. Mice were treated with vehicle or Thd (i.p.) for 28 days. Twenty-four hours after the last Thd treatment, blood was collected by retro-orbital bleed into EDTA tubes after mice were anesthetized by CO_2 inhalation. The hematological parameters were assessed as described. Values represent the mean ± SE derived from eight animals. WBC = white blood cells.

*, $p \leq 0.05$ as compared to vehicle controls.

TABLE 3

Thd Treatment on the SSC and FSC of Neutrophils in Female B6C3F1 Mice

Parameters		14-day Thd Treatment		28-day Thd Treatment	
		Spleen	Bone Marrow	Spleen	Bone Marrow
FSC	Vehicle	605.33 ± 11.28	503.45 ± 2.23	674.28 ± 6.78	685.46 ± 2.22
	Thd	612.57 ± 7.22	504.68 ± 6.66	709.85 ± 6.80*	691.57 ± 5.40
SSC	Vehicle	240.11 ± 14.91	212.61 ± 2.23	229.05 ± 4.84	248.95 ± 1.52
	Thd	239.48 ± 7.19	211.02 ± 2.97	258.18 ± 3.82*	255.67 ± 2.49*

Note. Female B6C3F1 mice were treated with vehicle or Thd (100 mg/kg, i.p.) for 14 or 28 days. Mice were sacrificed at the 3-h time point after the last Thd treatment. Flow cytometric analysis of both FSC and SSC for neutrophils in the bone marrow and spleen was performed.

*, $p \leq 0.05$ as compared to vehicle controls. N = 8.

TABLE 4

Thd Treatment on the Surface Expression of CD62L and CD11A by Neutrophils in Female B6C3F1 Mice

Parameters		14-day Thd Treatment		28-day Thd Treatment	
		Spleen	Bone Marrow	Spleen	Bone Marrow
CD62L	Vehicle	455.55 ± 129.77	143.91 ± 15.57	302.78 ± 45.75	140.90 ± 21.19
	Thd	194.51 ± 39.67	129.89 ± 1.64	345.02 ± 78.05	129.22 ± 18.84
CD11A	Vehicle	1274.70 ± 25.06	503.54 ± 15.82	1370.15 ± 75.56	569.16 ± 13.57
	Thd	1243.70 ± 30.31	461.38 ± 14.59	1342.63 ± 75.56	531.10 ± 19.33

Note. Female B6C3F1 mice were treated with vehicle or Thd (100 mg/kg, i.p.) for 14 or 28 days. Mice were sacrificed at the 3-h time point after the last Thd treatment. Flow cytometric analysis of CD62L and CD11A expression by neutrophils in the bone marrow and spleen was performed. Statistical analysis was performed as described. N = 8.