RESEARCH ARTICLE

Thalidomide Inhibits Alternative Activation of Macrophages In Vivo and In Vitro: A Potential Mechanism of Anti-Asthmatic Effect of Thalidomide

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Abstract

Background
Thalidomide is known to have anti-inflammatory and immunomodulatory actions. However, the effect and the anti-asthmatic mechanism of thalidomide in the pathogenesis of asthmatic airways are not fully understood.

Objective
This study is designed to determine the effect and the potential mechanism of thalidomide in the pathogenesis of asthmatic airways using animal model of allergic asthma.

Methods
Six-week-old female BALB/C mice were sensitized with alum plus ovalbumin (OVA) and were exposed to OVA via intranasal route for 3 days for challenge. Thalidomide 200 mg/kg was given via gavage twice a day from a day before the challenge and airway hyperresponsiveness (AHR), airway inflammatory cells, and cytokines in bronchoalveolar lavage fluids (BALF) were evaluated. The expression levels of pro-inflammatory cytokines and other mediators were evaluated using ELISA, real time (RT)-qPCR, and flow cytometry. CRL-2456, alveolar macrophage cell line, was used to test the direct effect of thalidomide on the activation of macrophages in vitro.
Results
The mice with thalidomide treatment showed significantly reduced levels of allergen-induced BALF and lung inflammation, AHR, and the expression of a number of pro-inflammatory cytokines and mediators including Th2 related, IL-17 cytokines, and altered levels of allergen-specific IgG1/IgG2a. Of interesting note, thalidomide treatment significantly reduced expression levels of allergen- or Th2 cytokine-stimulated alternative activation of macrophages in vivo and in vitro.

Conclusion
These studies highlight a potential use of thalidomide in the treatment of allergic diseases including asthma. This study further identified a novel inhibitory effect of thalidomide on alternative activation of macrophages as a potential mechanism of anti-asthmatic effect of thalidomide.

Introduction
Allergic asthma is a chronic inflammatory disease of the airways. It is characterized by pulmonary eosinophilia, mucus hypersecretion, an increase in serum levels of allergen-specific IgE, and airway hyper-responsiveness (AHR) and Th2 cytokines, such as interleukin (IL)-4, IL-5 and IL-13 [1–5].

In asthma pathophysiology, the Th2 cytokine profile in asthmatic lung contributes to the appearance of alternatively activated or M2 macrophages. These macrophages also generate several proallergic factors, such as chemokines, chitinase-like molecules [6] and ‘found in inflammatory zone 1’ (FIZZ1, also known as Relm-α) [7,8], which all contribute to the airway inflammatory and remodeling responses during asthma [9,10]. It has also been shown that asthmatics have higher percentages of macrophages expressing mannose receptor in bronchial biopsies than in healthy subjects [11]. In addition, severe asthmatics had higher numbers of IL-13-positive M2 macrophages in bronchoalveolar lavage fluid (BALF) as compared to healthy controls [12]. Markers of M2 macrophages are correlated with severity of allergic airway disease in humans and mice, suggesting that M2 macrophages contribute to the disease. Elimination of M2 macrophages by pharmacological interventions remarkably decreased the degree of airway inflammation [13].

Recently, it has been reported that thalidomide suppressed airway inflammation and airway hyperresponsiveness in a murine model of allergic asthma by us and others [14,15]. However, little is known about the effects of thalidomide on Th2 cytokines and the mechanism of thalidomide on the airway hyperresponsiveness and eosinophilic inflammation in allergic asthma. In this study, we demonstrated that thalidomide treatment before and after each challenge with ovalbumin (OVA) almost completely suppressed allergen-induced eosinophil-dominant airway inflammation, AHR, and Th2-derived humoral responses in OVA-sensitized mice. It also suppressed the expression of a number of pro-inflammatory and Th2 cytokines including tumor necrosis factor (TNF)-α, IL-4, IL-5, IL-13, and IL-17. These results strongly suggest a potential use of thalidomide as a therapeutic agent for the treatment of asthma and other allergic diseases. In addition, we showed thalidomide had a potent suppressive effect on alternative activation of macrophages in vivo and in vitro, which revealed, for the first time, a novel regulatory role of thalidomide on alternative activation of macrophages as a potential underlying mechanism of anti-asthmatic effect of thalidomide.
Materials and Methods

Animals

Female BALB/C mice (18–20 g) were purchased from SLC Inc. (Hamamatsu, Kotoh-cho, Japan) and were maintained in specific pathogen-free conditions. Six weeks old female mice were used in all experiments. All experiments were performed with the approval of the Institutional Animal Care and Use Committee of the Institute (IACUC) of Laboratory Animal Resources at Seoul National University (SNU-09-1111-4).

Sensitization and airway challenge

BALB/C mice were sensitized by intraperitoneal injection with 75 μg of OVA (Grade V, Sigma Aldrich, St. Louis, MO, USA) and 2 mg alum (Pierce, Rockford, IL, USA) in 200 μL of phosphate-buffered saline (PBS) on day 0 and 7. Intranasal injection with 50 μg OVA in 20 μL of PBS was followed on day 21, 22, and 23 (Fig 1A).

Thalidomide treatment

Thalidomide (Sigma Aldrich, St. Louis, MO, USA) was dissolved in 0.2 mL of 0.5% carboxymethylcellulose (CMC; Sigma Aldrich) and 0.25% Tween 80 (Sigma Aldrich). Thalidomide vortexed immediately before its use was administered orally by using a stainless needle 1 h before and 6 h after each of the OVA challenges in amount of 200 mg/kg (Fig 1A). The effective dose and frequency were selected based on the pharmacokinetic of thalidomide in mice [16]. The administration was repeated six times during challenge period. Control mice were orally administered vehicle.

Measurement of methacholine hyper-responsiveness

One day after the last challenge with OVA, airway hyperresponsiveness was measured with a barometric plethysmographic chamber (OCP 3000, Allmedicus, Anyang, Korea) and the Penh (enhanced pause) value for 3 min was monitored.

Analysis of bronchoalveolar lavage and serum

Twenty-four hours after the assessment of airway hyperresponsiveness, mice were sacrificed and bronchoalveolar lavage fluid and lung tissue were obtained. BAL fluid inflammatory cells were obtained as previously described [10]. At least 300 cells per slide were evaluated to obtain a differential leukocyte count. The supernatants were decanted and immediately frozen at -80°C. BAL cell slides were stained with Diff-Quik (Sysmex Co., Kobe, Japan). The level of IL-17 in BAL fluid was measured using commercially available ELISA Kit (Quantikine, R&D Systems Inc., Minneapolis, MN, USA) as manufacturer’s guideline. IL-4 and IL-5 in BAL fluid were measured using commercially available ELISA Kit (BioSource International, Camarillo, CA, USA). Serum OVA-specific IgG1, and IgG2a were measured by using ELISA.

Histopathology

To evaluate and compare the severity and character of pathological changes in lung parenchyma, left lungs of mice were fixed in 10% neutral buffered formalin and embedded in paraffin, and 3-mm sections were stained with hematoxylin & eosin (H & E staining) and Periodic acid-Schiff (PAS) stain.
Quantitative real-time RT-PCR

Total RNA was extracted from the whole lung. Total RNA (2 μg) from each sample was reverse transcribed into cDNA with a single-strand cDNA synthesis kit (Promega, Madison, WA, USA). Quantitative real-time PCR was performed on a 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The mRNA expression of cytokines was determined by real-time PCR using SYBR Green Master Mix (Applied Biosystems). The expression of each gene within each sample was normalized against β-actin and expressed relative to the control sample using the formula 2-(ΔΔCt), in which ΔΔCt = (Ct mRNA—Ct β-actin). All used primer sequences were validated by Primer bank (Harvard, USA).

Culture of cells

CRL-2456, alveolar macrophage cell line was purchased from the American Type Culture Collection (ATCC). Cells were grown in RPMI medium with 10% FBS and antibiotics (complete culture medium) in a humidified atmosphere at 37°C with 5% CO₂, with refreshment of
medium every 2–3 day. CRL-2456 were plated in 24-well plate (5 X 10^5 per well), one day prior to exposure and then stimulated by LPS (1 mg/mL), recombinant IL-4 (20 ng/mL), recombinant IL-13 (20 ng/mL) and cultured for 18h in 24-well plate with or without the presence of thalidomide (100 μg/mL). Cells were analyzed using flow cytometry analysis. The mRNA expression was determined by Real-time RT-qPCR.

**Preparation of lung tissue, draining lymph nodes of lung, and spleen**

Lung tissues from mice were minced and digested with 1.6 mg ml^−1 of collagenase type 4 (Worthington, Lakewood, NJ) and 0.1% of DNase I (fraction IX; Sigma Aldrich) at 37°C for 1 h. Total cells were lysed for RBCs and stained for FACS analysis. Single cell suspensions of draining lymph nodes and spleen from mice were plated at a concentration of 1×10^6 cells/mL onto a 96-well plate and re-stimulated with OVA protein (100 μg/mL) for 72 hours for flow cytometry analysis.

**Flow cytometry**

Single-cell suspensions were preincubated with FcγR-specific blocking mAb (2.4G2) and washed before staining. Cells were stained with the following antibodies: PerCP-cy5-conjugated anti-CD45 (eBioscience), APC-conjugated anti-F4/80 (eBioscience), PE-cy7-conjugated anti-CD11c (eBioscience), FITC–conjugated anti-CD206 (BioLegend), APC-conjugated anti-CD3 (eBioscience), FITC–conjugated anti-CD4 (eBioscience); For intracellular staining, cells were permeabilized (Cytofix/Cytoperm kit; BD Biosciences) and incubated with PE–conjugated anti-IL-4, PE–conjugated anti-IL-5, PE–conjugated anti-IFN-γ (eBioscience), rabbit anti-Ym-1 (Stem Cell Technologies) and PE–conjugated anti-rabbit IgG (eBioscience). Cells were analyzed on a LSRII (BD Biosciences) with FlowJo ver.10 software (TreeStar).

**Statistics**

All data are expressed as mean ± standard error of the mean (SEM). Differences between groups were calculated for statistical significance using the unpaired Student’s t-test. P < 0.05 was considered as significant.

**Results**

**Thalidomide inhibits eosinophilic inflammation, mucus secretion and airway hyperresponsiveness**

WT mice were challenged with OVA intranasally and then sacrificed 48 h after the last challenge. While the number of total cells, macrophages, eosinophils, and lymphocytes in BALF was increased in OVA challenged mice, it was almost completely decreased by 200 mg/kg thalidomide treatment (p < 0.05) (Fig 1B). The recruitment of inflammatory cells into the lungs of mice was also investigated by histopathological studies. Control mice had no inflammatory cells in the lung (Fig 1C-a and b). OVA challenged mice showed infiltration of inflammatory cells around peribronchial and perivascular spaces (Fig 1C-c). The majority of the infiltrated inflammatory cells were macrophages and eosinophils. However, the infiltration of macrophages and eosinophils was significantly reduced in thalidomide-treated mice compared to OVA challenged mice (Fig 1C-d). In addition to inflammation, mucus secretion was reduced in thalidomide-treated mice compared to OVA challenged mice (Fig 1C-g and h).

We also examined the role of thalidomide in the development of AHR. AHR to methacholine was significantly increased in OVA challenged mice compared to control mice (p < 0.01).
However, AHR was significantly reduced in thalidomide treated mice compared to OVA challenged mice (p < 0.05) (Fig 1D).

**Thalidomide alters the humoral immune response**

We examined the effect of thalidomide on OVA specific IgG1 and IgG2a antibody levels. The level of OVA specific IgG1 antibody was significantly increased in OVA challenged mice compared to control mice (p < 0.05). In contrast, the level of OVA specific IgG1 antibody was significantly reduced in thalidomide treated mice compared to OVA challenged mice (p < 0.05) (Fig 2A). Opposite effects were showed on OVA specific IgG2a antibody levels (p < 0.05) (Fig 2B). The ratio of OVA specific IgG1/IgG2a antibody was also significantly reduced in thalidomide treated mice compared to OVA challenged mice (p < 0.05) (Fig 2C).

![Image of IgG1 and IgG2a levels](image-url)
Thalidomide decreases IL-4 and IL-5 levels in BALF

While the levels of IL-4, IL-5, and IL-17 from BALF were significantly increased in OVA challenged mice compared to control mice (p < 0.05), these levels were significantly reduced in thalidomide treated mice compared to OVA challenged mice (p < 0.05) (Fig 3A–3C). These data demonstrated that thalidomide inhibits the Th17 cytokine as well as the Th2 cytokines.

Thalidomide decreases IL-4 and IL-5-producing CD4+ T cells in lymph nodes and spleen

The frequencies of IL-4 and IL-5-producing CD4+ T cells in draining lymph nodes were significantly decreased in thalidomide treated mice compared to OVA challenged mice (p < 0.05) (Fig 3D and 3E). The frequency of IL-4-producing CD4+ T cells in spleen was also significantly decreased in thalidomide treated mice. On the contrary, the frequency of IFN-γ-producing CD4+ T cells in spleen was significantly increased in thalidomide treated mice compared to OVA challenged mice (p < 0.05) (Fig 3F and 3G).

**Fig 3. Effects of thalidomide on cytokine protein levels in BALF, Lung draining lymph nodes and spleen.** A-C, The upper low shows the levels of IL-4 (A), IL-5(B) and IL-17(C) 48h after the last challenge. The levels were detected with enzyme-linked immunosorbent assay. D-G, The lower low shows the frequencies of IL-4(D) and IL-5(E)-producing CD4+ T cells 72h after re-stimulated in vitro with OVA protein in draining lymph node cells. The frequencies of IL-4(F) and IFN-γ(G)-producing CD4+ T cells 72h after re-stimulated in vitro with OVA protein in spleen cells. The expressions were analyzed using Flow cytometry. n = 5–8 for each group, * indicates p < 0.05.

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Thalidomide decreases TNF-α, IL-13, Eotaxin-1, and MUC-5AC mRNA expressions in Lung

Messenger RNA expressions were examined in lung homogenates. While analysis of mRNA expressions in OVA challenged mice revealed increased levels of TNF-α, IL-13, eotaxin-1, and MUC-5AC compared to control mice (p < 0.05), these mRNA expression levels in lungs were significantly decreased by thalidomide treatment in OVA challenged mice (p < 0.05) (Fig 4A–4D).

Thalidomide inhibits in vivo and in vitro expressions of M2 macrophage

We explored the effects of thalidomide on change of M1 and M2 macrophage markers in lung homogenates. The level of Relm-α, Arg-1, CD206, and Ym-1 mRNA expressions from lung was significantly reduced when treated with thalidomide in OVA challenged mice (p < 0.05) (Fig 5A–5D). Flow cytometry performed on the lung homogenates revealed the following results.
The frequency of F4/80 expressions in CD45+ cells from lung increased in OVA challenged mice but those populations were significantly reduced with thalidomide treatment ($p < 0.05$) (Fig 6A and 6D). Similarly, the frequency of CD206 and Ym-1 expressions in F4/80+ macrophage cells increased in OVA challenged mice but showed significant decrease with thalidomide treatment ($p < 0.05$) (Fig 6B–6C and 6E–6F). On the contrary, M1 macrophages by gating on F4/80+CD11c+CD206- cell [17,18] were reduced in OVA challenged mice compared control mice ($p < 0.05$) (Fig 6G). With thalidomide treatment, this population showed a recovering tendency but the difference was not significant compared to thalidomide untreated OVA challenged mice. As a result, the high M2/M1 ratio in OVA challenged mice significantly decreased with thalidomide treatment (Fig 6H).

We also examined the in vitro effects of thalidomide on Th2 cytokines-skewed M2 macrophage markers using CRL-2456 macrophages. The mRNA expressions Relm-$\alpha$, Arg-1, and CD206 showed significant decrease in IL-4 or IL-13 stimulated CRL-2456 macrophages with treatment of thalidomide ($p < 0.05$, $p < 0.01$) (Fig 7A–7C). Thalidomide treatment also

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**Fig 5. Effects of thalidomide on the mRNA expressions of M2 macrophage markers in mouse lung tissues.** A-D, Real-time RT-PCR was performed to determine the changes in mRNA levels of Relm-$\alpha$ (A), Arg-1 (B), CD206 (C) and Ym-1 (D) from lung tissues. The levels of mRNA are represented as the ratio to $\beta$-actin. $n = 5$–8 for each group. * indicates $p < 0.05$, ** indicates $p < 0.01$.

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resulted in a significant reduction in the frequencies of CD206 or Ym-1 expressing CRL-2456 macrophages induced by stimulation of IL-4 or IL-13 (Fig 7D and 7E).

Discussion

Thalidomide was synthesized in 1954 by CIBA Pharmaceutical Company and widely prescribed as an antiemetic for morning sickness. However, it was withdrawn from most countries because of its high risk for teratogenicity. Recently, clinical investigations of thalidomide have been conducted in patients with diverse diseases, including multiple myeloma, rheumatoid arthritis, and human immunodeficiency virus infection [19,20] as the beneficial effects of thalidomide including anti-angiogenic, anti-viral, anti-inflammatory, and immunomodulatory activities were revealed [21]. Thalidomide shows anti-inflammatory activities, such as blocking...
TNF-α production by stimulated human peripheral blood mononuclear cells (PBMCs) [22] and by alveolar macrophages [23,24] and inhibiting IL-12 release [25].

Pharmacokinetic studies in healthy human male volunteers suggest a one-compartment, first order absorption and elimination model, with peak plasma levels reached within 4–5 hr and a half-life of 8.7 ± 4.1 hr [26]. In mice, peak plasma concentration of thalidomide after oral administration was attained after 1.0 hr when administered at 20 mg/kg and elimination half-life was 1.2±0.05 hr. In this study, thalidomide by oral administration (200 mg/kg) twice a day during challenge period could suppress eosinophil dominant airway inflammation in OVA-challenged mice.

Th2 cytokines such as IL-4, IL-5 and IL-13 are key mediators for the development of allergic airway inflammation and AHR. Recently, it has been shown that Th17 cells significantly enhance not only Th2-cell–mediated eosinophilic airway inflammation in airways but also Th2 cytokines–induced AHR [27]. TNF-α, which is also produced by Th17 cells [28], has been
shown to induce eotaxin production from epithelial cells [29]. The present study demonstrates the attenuation of the number of inflammatory cells, mucin secretion, and cytokine levels of IL-4, IL-5 and IL-17 in BALF, the frequency of IL-4 and IL-5 producing CD4+ T cells in lung draining lymph nodes and spleen, and AHR to methacholine in OVA-challenged mice by thalidomide. Whereas all these Th2 changes were reduced with thalidomide treatment, serum IgG2 level and the frequency of IFN-γ from spleen cells increased in thalidomide treated mice compared to OVA group mice. Considering these findings, thalidomide seems to play a role in Th1/Th2 polarization in OVA asthma model. In addition, thalidomide also suppressed the expressions of TNF-α, IL-13, Eotaxin-1, and MUC-5AC in lung. Previous study showed that thalidomide had a limited suppressive effect on the expression of IL-5 and TNF-α and AHR, but not on IL-4 and IL-13 in OVA challenged mice [15]. Considering the relatively short half-life serum levels of the thalidomide in mice (≈1.2 hr in 20 mg/ kg dose) [16], the dose and frequency of thalidomide administration could significantly affect the effectiveness of the thalidomide in in vivo setting. Previously we and Asano, et al. administrated thalidomide once daily during each OVA challenge [14,15]. Since thalidomide was administered twice daily (before and after allergen challenge) in this study, we could have more profound and consistent in vivo effect of thalidomide.

The role of macrophages in the pathogenesis of asthma and allergic inflammation is still unclear. Recently, it is known that the Th2 cytokine profile in asthmatic lung contributes to the appearance of alternatively activated macrophages which were recruited into the lung following OVA sensitization and challenge [30]. These M2 subsets transferred to naive mice increase AHR, eosinophilic inflammation, and Th-2 cytokine secretion. Recently, it was reported that several mediators from M2 macrophages could contribute to promoting Th2 responses and suppressing Th1-driven inflammatory pathology [31,32]. A human study also demonstrated that M2 macrophages from human asthmatic patients directly contribute to the proliferation of CD4+ lymphocytes [33]. Significant up-regulation of M2 markers was reported in OVA-sensitized and-challenged mice, including Arg-1, MGL-2, Ym-1, Relm-α, and IL-10 [34]. Up-regulation of Arg-1 may be of particular physiologic importance. In patients with asthma, Arg-1 mRNA expression is increased in sub-mucosal inflammatory cells [35]. Arginase expression is increased in the lungs of allergen-sensitized and-challenged mice, and inhibition of its expression attenuates airway responsiveness to methacholine in OVA-sensitized and-challenged mice [36]. The role of Relm-α in Th2-driven pulmonary disease is known to contribute to the pathology of allergic airway disease, including airway remodeling and angiogenic responses [37,38]. Additionally, Ym-1 has been implicated in experimental and clinical asthma [39]. CD206, macrophage mannose receptor 1, is the first known marker of alternative macrophage activation, is related with fibrogenic condition and found to be up-regulated at the surface of alveolar macrophages from patients with idiopathic pulmonary fibrosis [40]. CD206 in human circulating fibrocytes was reported to have a role in regulation of allergen induced allergic response in asthma [41] but its role macrophage is not clearly investigated in asthma yet.

The differentiation of M2 macrophages is mediated by IL-4—and IL-13–dependent activation of STAT6, which leads to the expression of M2 markers such as Relm-α, and arginase in macrophages in vitro [42]. In this study, the administration of thalidomide to OVA-challenged mice attenuated the expression of M2 marker, such as Relm-α, Ym-1 and CD206, which are the major products of alternatively activated macrophages. Flow cytometry analysis for the evaluation of lung macrophage subsets reconfirmed reduction of M2 macrophage population but not M1 macrophage population by thalidomide. M1 macrophage population by gating on CD11c+CD206- cells and CD11c+Ym-1- cells showed rather a tendency to increase than to decrease. Based on these findings, thalidomide seems to inhibit polarization of M2 macrophages activated by Th2 stimulation but further study is needed to verify steps which thalidomide had
an effect on. In support of in vivo finding, our in vitro studies also showed suppression of IL-4 or IL-13 induced mRNA expression of Relm-α, arginase-1, and CD206 on lung and CD206 or Ym-1 expressing macrophage population by thalidomide. These findings suggest the direct inhibitory effect of thalidomide on macrophage activation as well as Th2 responses could be a potential mechanism underlying anti-asthmatic effect of thalidomide shown in this study.

Last but not the least, the safety issue of thalidomide should be considered before application to human asthmatics. Thalidomide has had serious adverse effects when administered during pregnancy so its use cannot be justified for patients with asthma in general. However, it can be a possible candidate drug for some asthmatic patients who are refractory to traditional anti-asthmatic medication.

In conclusion, this study demonstrated that thalidomide was clearly effective in suppressing all features of allergic airway disease shown in OVA-challenged mice, including airway resistance to methacholine, inflammation, altered levels of allergen-specific IgG1/IgG2a, and the increased expression of proinflammatory cytokines and mediators associated with allergic Th2 inflammation. Thus, these studies highlight a potential use of thalidomide in the treatment of allergic diseases including asthma. This study further identified a novel inhibitory function of thalidomide on allergen- or Th2-cytokine stimulated alternative activation of macrophages as a potential mechanism of anti-asthmatic effect of thalidomide.

**Author Contributions**

Conceived and designed the experiments: HSL HSK H-R Kang YSC. Performed the experiments: HSL HSK DEP YDW. Analyzed the data: HSL H-R. Kang H-R. Kim YDW HYK. Contributed reagents/materials/analysis tools: SHC KUM YSC. Wrote the paper: HSL HSK H-R. Kang YSC.

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