



## DATURA METEL LINN AMELIORATES ASTHMA SYMPTOMS IN BALB/c MICE

Muhaimin Rifa'i <sup>\*1</sup>, Dewi Satwika <sup>1</sup>, Aulanni'A M <sup>2</sup>

<sup>1</sup> Biology Department, Faculty of Sciences, Brawijaya University, Malang 65145, Indonesia

<sup>2</sup> Chemistry Department, Faculty of Sciences, Brawijaya University, Malang 65145, Indonesia

### Abstract

**Context:** *Datura* has been recognized as viable alternate options to ameliorate asthma symptoms. Atropine contained in *Datura* is believed to be anti-asthmatic agent with its capacity to eliminate the spasms that cause asthma attacks. In Indonesian traditional medicine, the leaves of *Datura* are generally smoked either in cigarette or a pipe, and most asthma patients get benefit to relieve this disease.

**Objective:** The potentiality of *Datura metel* was tested to control immune response and ameliorate the disease in mouse models of asthma.

**Materials and Methods:** Mice were sensitized by twice intraperitoneal (i.p) injection with 10 µg ovalbumin (OVA), then exposed to 1% aerosolized OVA with nebulizer system. Mice then were orally treated by aqueous extract of *D. metel* L for 5 days with a dose of 0.56 mg/kg body weight (BW) or 1.12 mg/kg BW.

**Results:** The number of naïve CD4<sup>+</sup> T cells (CD4<sup>+</sup>CD62L<sup>+</sup>) increased in mouse model of asthma treated with *D. Metel* L. In contrast, the number of CD4<sup>+</sup>CD25<sup>+</sup> T cells decreased in mouse model of asthma after treatment. Dose variations in this experiment had no significant effect ( $p < 0.05$ ), and the optimum dose to reduce bronchioles thickness was 0.56 mg/kg BW.

**Conclusion:** *D. metel* L. has a potential to ameliorate asthma symptom by promoting naïve T cell development and reducing activated T cells.

**Key words:** BALB/c mice, Asthma, *Datura metel* Linn, Regulatory T cells.

### Introduction

Asthma is characterized by clinical symptoms of wheezing, chest tightness, dyspnea, cough, and by the presence of reversible narrowing airway and/or airway hyper responsiveness (AHR) to a variety of inhaled bronchoconstriction stimuli. People with asthma will increase in levels of T-helper-2 (Th-2) cytokines and undergo chronic airway inflammation and remodeling (Matt *et al.* 2009, Silva *et al.* 2010, Zhang *et al.* 2011). In that condition mast cells develop to an effector phase and produce Th2 cytokines, like IL-4, IL-5, and IL-9. Cell activation in turn contributes to the accumulation of pro-inflammatory cytokines. Activated T cells will lose the expression of cell surface molecule and lead to the expression of other surface molecules, such as CD69<sup>+</sup>, CD25<sup>+</sup> and CD44<sup>+</sup>. Some previous studies show that activated memory CD4<sup>+</sup> T cells has important role to produce Th2 cytokines in asthma. There is evidence that CD8<sup>+</sup> T cells are able to promote Th2 cytokines secretion and contribute to allergic inflammation and airway sensitivity. Airway inflammation is also characterized by infiltration of lymphocytes and eosinophils in both bronchial epithelium and lamina propria (Seneviratneet *et al.* 2002, Rensen *et al.* 2005, Vignola *et al.* 2000, Knight *et al.* 2007, Machura *et al.* 2010). Mononuclear cell infiltration induces structural changes in the bronchial wall, often referred to "tissue remodeling". The features of airway remodeling in asthma include thickening airway smooth muscle layer. Herbs have been chosen because they are more secure and have less negative effect compared to modern drug. Some herbs have been identified to have a benefit to cure asthma. *Datura stramonias* principle mechanism of action when the smoke is inhaled is anti-cholinergic bronchodilatation, with efficacy similar to

\* Corresponding author E-mail: rifa123@ub.ac.id

salbutamol via metered dose inhaler, although the duration of action is shorter. *Lobelia inflata* was introduced as a medication in 1813 and is a plant alkaloid with mixed antagonism to nicotinic acetyl-choline receptors. Apart from its principle mechanism of bronchial vasodilatation, it has been used as a nicotine substitute, as it acts on central dopaminergic systems. For these reasons it is often included in asthma cigarettes. Allergic inflammation in a mouse model of asthma can be ameliorated by blockage of growth factor production (Simon *et al.* 2010, Yan *et al.* 2012).

*D. metel*, commonly known as "Devil's Trumpet" or in Indonesia known as "Kecubung" is an herb able to cure various diseases and it is also recognized to have analgesic properties. It belongs to the family of *Solanaceae* widely used in phytomedicine to cure diseases such as asthma, cough, convulsion, and insanity (Wannanget *et al.* 2009, Kiruthika and Sornaraj 2011). According to Alexander *et al.* 2008 most *Datura* sp. contains high concentration of alkaloids. Anticholinergic activity of *D. metel* flower and seed is believed to relax airway smooth muscle, so that the responsiveness of airway decreases. However, *D. metel* also causes poisoning such as delirium, hallucination, dilated pupils, dry mouth, dry skin, hyperthermia, cardiac conduction abnormalities, and urinary retention (Murch *et al.* 2009). Indonesian people usually process herbs as "*jamu*"—a traditional medicine made by mixing some kinds of herbs, boiling or brewing them to get crude extract. In this study we observed the activity of T cell lymphocyte in mouse models of asthma and also examined the bronchial architecture after oral treatment with aqueous extract of *D. metel*.

## Materials and Methods

### Experimental animal

BALB/c mice (7 week-old male) were obtained from the Animal Center of Brawijaya University and maintained in our pathogen-free laboratory facility. The study complied with the recommendations of the Animal Care and Ethics Committee at Brawijaya University.

### Collection and identification of plant material

*D. metel* flower were obtained from Sukun sub district, Malang City, Indonesia. The authentication of this plant is done by Plant Taxonomy Laboratory, Faculty of Sciences, Brawijaya University, Malang, Indonesia.

### Sensitization and inhaled exposure with OVA

Mice were classified into asthma and non-asthma groups. Mouse models of asthma were obtained by twice intraperitoneal injections of 10 µg ovalbumin (OVA, Sigma-Aldrich) with alum adjuvant on day 0 and 7 of the experiment. On the day 18, 21, and 24, mice were housed in chambers connected with *Omron CompAir Compressor Nebulizer*, and exposed to 1% aerosolized OVA for 20 minutes per day. Non-asthma group only received physiological saline solution.

### Preparation of aqueous extract

Dried flowers were cut down into tiny pieces by mortar and pestle. The weighed aliquot, about 0.28 mg, was put into 500 ml sterile distilled water at 55°C for 24 hours by shaking to ensure homogeneity. The simplicia was then filtered separately using Whatman no.1 filter paper. The selected doses were 0.56 mg/kg BW and 1.12 mg/kg BW. Control groups received distilled water instead of *D. metel* extract as placebo. *D. metel* aqueous extract was given to the mice for 5 days.

### Flow cytometry analysis

Mice were challenged with OVA on day 18, 21, and 24 executed 1 day after the last challenge. Spleen cells were stained by FITC (fluorescent iso-thiocyanate) -conjugated anti-mouse CD4, PE(phycoerythrin) -conjugated anti-mouse CD25, PE-conjugated anti-mouse CD8, and PE-conjugated anti-mouse CD62L in ice box (4°C) for 30 minutes. Flow cytometry analysis was done by Cell Quest Pro software.

### Preparation of histological specimens

Lung and liver tissues were fixed by 4% PFA, and processed for H-E staining. Analysis was done by a microscope digital imaging (Microscope Olympus BX51 with Camera digital DP20).

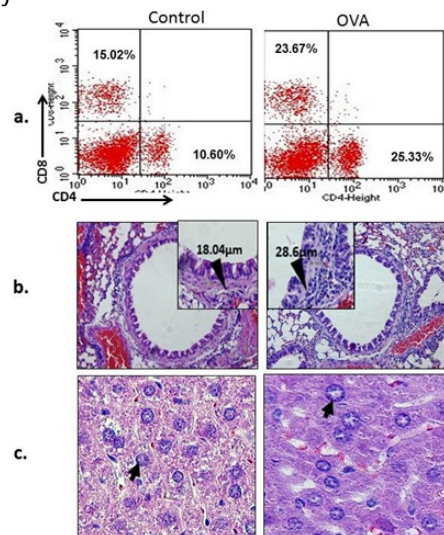
### Statistical analysis

Data were statistically analyzed by ONE-way ANOVA at  $p < 0.05$  and Tukey HSD test using SPSS 16.0 for Windows.

### Results

#### T cells and Histological Architecture in Mice Model of Asthma

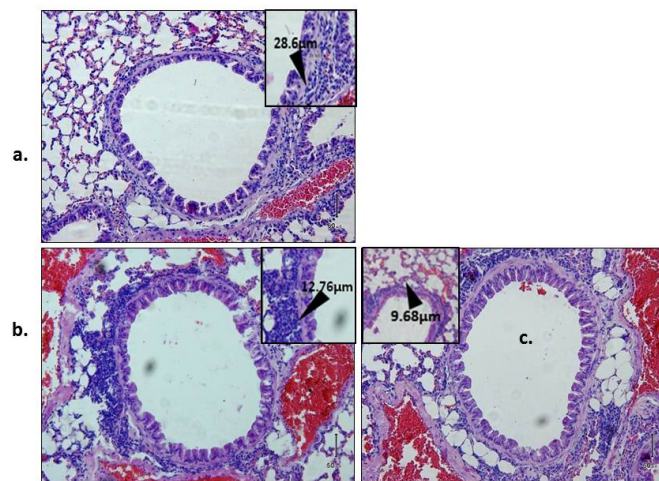
OVA challenges induced allergic airway signed by an increase in the numbers of CD4<sup>+</sup>T cells (Fig.1). Relative numbers of CD4<sup>+</sup> T cells in control group and treated mouse group were 10.60% and 25.33% respectively and absolute numbers were  $12.09 \times 10^6$  and  $20.18 \times 10^6$  respectively. OVA exposure induces proliferation and changes the status of naive CD4<sup>+</sup> T cells to an effector phase (Akbari *et al.* 2006). OVA-challenged mice developing to asthma will be safer from airway inflammation, eosinophil domination; and CD4<sup>+</sup> T cells are markedly increased (Pimentel *et al.* 2010). In this experiment we found that the percentage of CD8<sup>+</sup> also increased significantly after OVA challenge. Relative numbers of CD8<sup>+</sup> T cells in control group and treated mice were 15.02% and 23.67% respectively and absolute numbers of CD8<sup>+</sup> T cells were  $14.60 \times 10^6$  and  $43.03 \times 10^6$  respectively.



**Fig.1.** Ova challenge increased the number of T cells and the thickness of smooth muscle of bronchioles but it did not change the property of hepatocyte cells. (a) Mice were sensitized twice (i.p) on day 0 and 7 with ovalbumin (OVA). Intranasal challenge was administered on day 18, 21, and 24. Spleen cells were obtained from control group and treated group. Spleen cells were stained with FITC-conjugated anti-mouse CD4, PE-conjugated anti-mouse CD8 antibodies and analyzed by flow cytometry. Percentages of CD4<sup>+</sup> or CD8<sup>+</sup> T cells were shown in each panel. (b) Histological architecture of bronchioles and diameter of smooth muscle layer (insert) after H-E staining. (c) Histological architecture of liver stained by H-E and hepatocytes were shown by an arrow.

#### ***D. metel* ameliorates architecture of bronchioles in mice model of asthma**

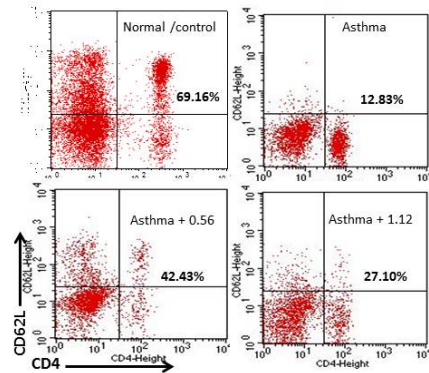
In this study, we found that aqueous extract of *D. metel* could reduce the thickness of smooth muscle significantly. The thickness of smooth muscle in mice model of asthma reached 28.60  $\mu\text{m}$  before treatment. After administering aqueous extract of *D. metel* with doses of 0.56 mg/kg BW and 1.12 mg/kg BW, the thickness of smooth muscle in mice model of asthma decreased to 12.76  $\mu\text{m}$  and 9.68  $\mu\text{m}$ , respectively. This study confirmed the importance of *D. metel* in airway remodeling and extended results obtained from BABL/c mice to make mouse models of asthma. In addition, we demonstrated that CD62L molecule underwent down regulation in mice exposure to OVA.



**Fig. 2.** Aqueous extract of *D. metel* L ameliorated bronchial architecture by normalizing the thickness of smooth muscle. Mouse models of asthma were treated by *D. Metel* L with the dose of 0.56 mg/kg BW or 1.12 mg/kg BW for each mouse. Histological analysis of bronchioles was done by H-E staining. (a) Mice model of asthma without treatment showed a thick smooth muscle (28.6  $\mu\text{m}$ ). (b). After treated with *D. metel* L at the dose of 0.56 mg/kg BW the thickness of smooth muscle was 12.76  $\mu\text{m}$ . (c) After treated with *D. metel* L at the dose 1.12 mg/kg BW the thickness of smooth muscle was 9.68  $\mu\text{m}$ . Results were the representative of two independent experiments with three mice per group.

#### ***D. metel* induced the development of naïve CD4 T cells (CD4<sup>+</sup>CD62L<sup>+</sup>)**

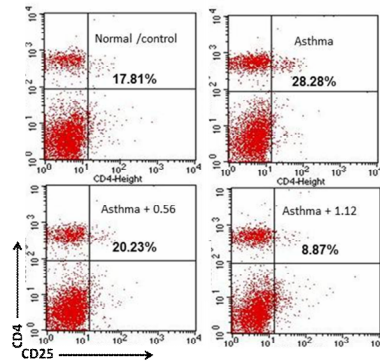
In this study we clearly showed that mice model of asthma were known to lose their CD62L molecule expression in CD4<sup>+</sup> T cells (Fig. 3). Flow cytometry analysis revealed that relative number of CD4<sup>+</sup>CD62L<sup>+</sup> T cells decreased significantly from 69.16% to 12.83% when asthma occurred. The absolute number of cell decreased from 19.25  $\times 10^6$  cells (control group) to 8.35  $\times 10^6$  cells (asthma group). The expression of L-selectin or CD62L significantly decreased in animal with asthma. In any conditions, mice treated by aqueous extract of *D. metel* at both doses of 0.56 mg/kg BW and 1.12 mg/kg BW showed significant increase in number of CD4<sup>+</sup>CD62L<sup>+</sup> T cells compared to untreated mouse models of asthma (Fig. 3).



**Fig. 3.** Aqueous extract of *D. metel* L changed the number of naive CD4<sup>+</sup> T cells (CD4<sup>+</sup>CD62L<sup>+</sup>) in mouse models of asthma. Spleen was removed from normal mice (control), mouse models of asthma, and mouse models of asthma treated by *D. metel* L. Spleen cells were then isolated and stained by FITC-conjugated anti-mouse CD4 and PE-conjugated anti-mouse CD62L and analyzed by flow cytometry. The percentage of CD62L<sup>+</sup> cells (naive T cells) was shown in each panel. Upper left panel was obtained from normal mice. Upper right panel was obtained from mouse models of asthma without any treatments. Lower left panel was obtained from mouse models of asthma treated by 0.56 mg/kg BW of *D. metel* L. Lower right panel was obtained from mouse models of asthma treated by 1.12 mg/kg BW of *D. metel* L. Data were mean ± SD values of three mice in each group.

***D. metel* decreased the development of CD4<sup>+</sup>CD25<sup>+</sup> T cells**

Mouse models of asthma had CD4<sup>+</sup>CD25<sup>+</sup> T cells higher than normal ones (Fig. 4). In mouse models of asthma treated by *D. metel* at dose of 0.56 mg and 1.12 mg/kg BW showed the decrease of CD4<sup>+</sup>CD25<sup>+</sup> T cells. The administration of *D. metel* aqueous extract reduced CD4<sup>+</sup>CD25<sup>+</sup> T cells significantly in mouse models of asthma. Relative numbers of CD4<sup>+</sup>CD25<sup>+</sup> T cells after administration of *D. metel* at dose of 0.56 and 1.12 mg/kg BW decreased from 28.28% to 20.23% and 8.87% respectively.



**Fig. 4.** Aqueous extract of *D. metel* L changed the expression of CD25<sup>+</sup> molecules in CD4 T cell in mouse models of asthma. Spleen was obtained from normal mice (control), mouse models of asthma, and mouse models of asthma treated by *D. metel* L. Spleen cells were then isolated and stained by FITC-conjugated anti-mouse CD4 and PE-conjugated anti-mouse CD25 and analyzed by flow cytometry. Percentage of CD25<sup>+</sup> cells (regulatory or memory T cells) was shown in each panel. Upper left panel was obtained from normal mice. Upper right panel was obtained from mouse models of asthma without any treatments. Lower left panel was obtained from mouse models of asthma treated by 0.56 mg/kg BW mg of *D. metel* L. Lower right panel was obtained from mouse models of asthma treated by 1.12 mg/kg BW of *D. metel* L. Data were mean ± SD values of three mice in each group.

The absolute number of CD4<sup>+</sup>CD25<sup>+</sup> T cells in the same condition was  $2.77 \times 10^6$ . After the administration with doses of 0.56 and 1.12 mg/kg BW, absolute number of CD4<sup>+</sup>CD25<sup>+</sup> T cells became  $2.81 \times 10^6$  and  $1.34 \times 10^6$  cells respectively. Here we suppose that CD4<sup>+</sup>CD25<sup>+</sup> T cells in mouse model of asthma are not regulatory T cell but rather activated memory T cell type. The decrease of CD4<sup>+</sup>CD25<sup>+</sup> T cells consistent with the result that naive type of CD4<sup>+</sup>CD62L<sup>+</sup> T cells increase after *D. metel* administration.

## Discussion

### *T cells and Histological Architecture in Mice Model of Asthma*

The increasing number of both CD4 and CD8 T cells after OVA challenge was consistent with the report stating that increasing number of CD8<sup>+</sup>-exacerbated mouse models of asthma. CD8<sup>+</sup> T cells regulated the secretion of proinflammatory cytokine such as IL-4, IL-5, and IL-9. Miyahara *et al.* (2004) states that the level of IL-13 and eosinophilic inflammation are lower in CD8-deficient mice compared to wild-type mice when observed in bronchoalveolar lavage fluid. These responses were restored by an adoptive transfer of antigen-primed CD8<sup>+</sup>. Besides increasing the level of CD4<sup>+</sup> and CD8<sup>+</sup> cells, we found that OVA challenge could change thickness of smooth muscle layer, from 18.04  $\mu\text{m}$  to 28.6  $\mu\text{m}$ . The airway remodeling in mouse models of asthma is characterized by structural changes, including mucus metaplasia, sub epithelial fibrosis, and smooth muscle hypertrophy / hyperplasia. In this experiment, however, structural changes were not found in liver tissue (Fig. 1c). H-E stained liver tissue showed a normal architecture of hepatocyte. OVA challenge via airway did not influence other tissues, except bronchioles tissue. According to Rodolfo *et al.* 2007, OVA-sensitized mice can increase hyper responsiveness, eosinophilia inflammation, and smooth muscle thickness when supplemented by creatin. Besides that, mice challenged with OVA can up the regulated CD69 expression and secrete the cytokines such as IFN- $\gamma$  and TNF- $\alpha$  (Enomoto *et al.* 2012).

This result indicated that T cell tended to be activated after OVA challenge. Based to our finding, we suggest that active compound dissolved in *D. metel* aqueous extract has a beneficial effect to relieve the alveolar inflammation by normalizing bronchial architecture and regulating effector function on T cells. Additionally, we suggest that aqueous extract of *D. metel*, at both doses of 0.56/kg BW and 1.12 mg/kg BW are able to cure mouse models of asthma by reducing smooth muscle thickness. This can be observed by looking the physical condition of mice after treatment. Treatment with doses of 0.56 and 1.12 mg/kg BW could ameliorate asthma symptoms. Histopathology examination of hepar showed the sign of necrotic cells at the dose of 1.12 mg/kg BW but not at that of 0.56 mg/kg BW (data not shown). Necrotic cells were characterized by condensed chromatin, shrinkage nuclei, pyknotic nuclei, and membrane integrity destruction. This result indicates that *D. metel* have a toxic effect in high dose and one should be careful to apply this herb for treatment in human. Although this herb has toxic effect, it is still interesting to be explored because the proper dosage is very beneficial as shown in mouse models of asthma.

In this experiment we showed that most CD4<sup>+</sup> T cells in mouse models of asthma changed their status into the memory type and developed to the effector phase (Fig. 3). According to Rifa'i *et al.* (2004, 2013) activated CD4<sup>+</sup> T cells in some circumstance also express other cell surface molecules such as CD69<sup>+</sup>, CD25<sup>+</sup>, and CD44<sup>+</sup>. In this experiment we showed that the dose of 0.56 mg/kg BW was able to increase CD4<sup>+</sup>CD62L<sup>+</sup> cell numbers up to  $35.53 \times 10^6$  compared to those asthma groups, but there were no statistically differences between doses of 0.56 and 1.12 mg/kg BW.

CD4<sup>+</sup>CD25<sup>+</sup> T cells usually known as regulatory T cells are very important to maintain the homeostasis by suppressing activated cells. In this experiment we proposed that cells are activated memory rather than regulatory T cells. The decrease of CD4<sup>+</sup>CD25<sup>+</sup> T cells in mouse models of asthma after administering *D. metel* indicated that cells were not required to suppress activated cells. Most T cell in mouse models of asthma changed the status from activated to naive type after the treatment. The decrease of CD4<sup>+</sup>CD25<sup>+</sup> T cells after treatment with *D. metel* indicated that CD25 in CD4<sup>+</sup> T cells functioned as effector molecules rather than regulatory ones. That was why after most T cells were dominated by naive type, the number of CD4<sup>+</sup>CD25<sup>+</sup> T cells decreased significantly.

## Conclusion

*D. metel* had pharmacological activities signed by its ability to ameliorate architecture of bronchioles in mice model of asthma. This herbal medicine may serve as an immunomodulator agent by reducing the number of activated T cells and maintaining T cells in naïve type status. CD4<sup>+</sup>CD25<sup>+</sup> T cells in mice model of asthma reflexed as activated T cells and the number decreased after *D. metel* administration. *D. metel* ameliorate mice model of asthma by improving bronchial architecture and normalizing the thickness of smooth muscle.

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