Inflammatory cytokines and immunoglobulin expression following dust mite allergen exposure in pregnant mice

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be hypersensitive to the same allergens due to genetic factors, while others could tolerate the allergens.\textsuperscript{5} Other authors extrapolated that such outcomes were dependent on the type of allergen, in addition to either the rate or duration of exposure.\textsuperscript{6,7}

Cytokines are required to help regulate placental implantation, but their production can change during pregnancy due to allergies or infections.\textsuperscript{8} Cytokines produced by macrophages include pro-inflammatory cytokines such as interleukin (IL)-1\textbeta, IL-6, IL-12, IL-18, and tumor necrosis factor-alpha (TNF-\textalpha).\textsuperscript{9} Neonates born to parents with a history of allergies will have an increased risk of experiencing allergies due to a lower mononuclear cell cytokine response to the causative pathogen.\textsuperscript{8} In addition, when an allergy occurs, immunoglobulin will be induced by the body, especially immunoglobulin G (IgG) as an antibody. IgG is related to the level of allergy and the response of Th2 cytokines to allergens. Early allergic symptoms may stem from the pathogenesis of IgG producing B cell clones of the allergen. The frequency of B cells in allergic children switches production from IgG to IgE.\textsuperscript{10}

The fetus may respond differently following allergen exposure.\textsuperscript{11} It is axiomatic that fetal dendritic cells are more responsive to T-helper 1 (Th1), rather than T-helper 2 (Th2) cells.\textsuperscript{1} Allergic immune reactions are thought to be transferred from the maternal to fetal circulation via the placental barrier, that is IgG and antibody. Maternal IgG is transferred to the fetus via the syncytiotrophoblast and cytotrophoblast cell barriers, which then cross the villous stroma to reach the lumen of the fetal endothelial vessels. While maternal antibody is transferred to the fetus in the womb through the placenta and through breastfeeding after the baby is born, which plays a role in protecting the baby from infection during the first months of life.\textsuperscript{12} Circulating allergens are caught by dendritic cells in placental tissues and thereafter brought to T-helper 0 (Th0) cells that react by differentiating into Th1, Th2, and Treg cells. Th0 cells predominantly become Th1 cells, which produce inflammatory cytokines namely interleukin (IL)-2 and IFN-\textgamma.\textsuperscript{13} Meanwhile, Th0 cells that differentiate into Th2 cells mainly produce IL-4 and IL-5, while Treg cells developed from Th0 produce IL-10 and TGF-\textbeta1, which could affect immunoglobulin class-switching recombination in B cells.\textsuperscript{14} The objective of this study was to evaluate various doses of mite allergens and cytokines associated with Th1, Th2, Treg, IgG, IgM, IgA, and IgE cells with regards to possible allergic tolerance in neonatal mice. We hope that the information gained from this study can be used as a reference to prevent certain allergic influences during pregnancy.

**Methods**

This study was conducted at the Laboratory of Virology & Immunology, Department of Microbiology, Faculty of Veterinary Medicine, Airlangga University from April to December 2014. The case group was the group that received high and low doses of exposure, while the control group did not get exposure. A true experimental design with a post-test only control group model was used to study 60 atopic mice (pregnant BALB/C). This study used a post-test only control group model design because there were two study groups, namely the experimental group and the control group. A total of 15 pregnant mice were sampled, 5 high-dose groups, 5 low-dose groups, and 5 control groups. While the sample of mice neonates was 45, namely 15 high-dose groups, 15 low-dose groups, and 15 control groups. We chose these mice for our study because their immunological characteristics are similar to those of humans with atopy.\textsuperscript{15} We observed the BALB/C mice (homogenous in gender, age, and body weight) for 8 months. The study time started from the preparation stage, the breeding stage of Balb/c mice, the rearing stage of the mice (neonatal period), and the laboratory testing stage. The breeding stage of mice starts from mating the parent mice aged 10 weeks by using a ratio of 1 male and 5 female. The stage of rearing mice began when the mother gave birth until the mice reach the age of 2 weeks (neonatal period), while the laboratory examination stage was in the form of examination of the serum of the mother and neonate mice.

Consumable materials used in this study included mite allergen kits, adjuvant Al(OH)\textsubscript{3}, and Freund’s incomplete adjuvant. In addition, sandwich-ELISA method kits were used to estimate the levels of IL-2, IFN-\textgamma from Th1 cells, IL-4 and IL-5 from Th2 cells, IL-10 and TGF-\textbeta from Treg cells; other ELISA kits were used to measure IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA according to optical density (OD), and to measure IgE levels. Laboratory instruments and equipment utilized were Berker glasses, Petri dishes, multichannel
pipettes and micropipettes of various sizes, pipette pumps, measuring cups, ELISA reader by Multi Science, yellow tip, blue tip, microwell plate, and incubators. The mite allergen used was *D. pteronyssinus* antigen (LTN-DPE-4) culture medium natural 1 mL. Allergen exposure is given with a low dose of 63 AU/unit and a high dose of 630 AU/unit. A low dose was defined as 63 AU/ unit in aluminum hydroxide intraperitoneally for 4 weeks. A high dose was equal to a ten-fold increase in low-exposure allergens.

Female BALB/C mice aged 10 weeks were bred with male mice. Pregnant BALB/C mice were exposed to allergens for 4 weeks’ gestation. BALB/C mice with congenital anomalies and witnessed or marked clinical conditions such as decreased body weight, abnormal breathing pattern, and diarrhea, or even death during the observation in the breeding stage were excluded. The minimum required sample size was calculated with a formula of $(t_1-1) (t_2-1) (r-1) \geq 20$. $T_1$ was total first exposure given to study subjects that is 6, $t_2$ was second exposures given to study subjects that was 2, and $r$ was repeated. So, the minimum sample size in this study was 5 for pregnant mice and 5 for neonates. Maternal serum specimens were taken during the pregnancy period after 4 weeks of treatment. After birth, neonatal blood specimens were taken at 2 weeks postnatally.

ANOVA test was used to assess homogeneity; Kolmogorov-Smirnov was used to evaluate the data distribution and normal probability plot was used to determine differences between variables in groups and between groups. ANOVA was used for normally distributed data, while Kruskal Wallis A, Brown-Forsythe, and Mann-Whitney statistical tests were used for non-normally distributed data.

### Results

Serum from maternal mice exposed to low-dose mite allergens had significantly higher expression of IL-2 and IFN-γ than control mice serum. Neonates from low-dose mothers also had significantly higher serum IL-2 and IFN-γ levels than control neonates. However, in high-dose maternal and neonatal mice, IL-2 and IFN-γ were not significantly increased (Table 1).

The IL-4 and IL-5 levels in low dose mothers were significantly higher than the control group. Neonates from low dose mothers also had significantly higher levels of IL-4 and IL-5. However, the high dose allergen groups had significantly elevated IL-5 in mothers and neonates; IL-4 was not significantly different from controls in high dose mothers and neonates (Table 1).

Regarding cytokines derived from Treg cells, IL-10 levels were not significantly different between the study and control groups for low and high dose mothers and neonates. However, TGF-β1 levels were significantly higher than controls in low and high dose mothers and neonates.

As shown in Table 2, maternal mice who received low dose of allergens had significantly higher levels of IgG-1, IgG-2a, IgG-2b, and IgG-3 than in the control group. Similarly, IgA, IgM, and IgE levels were significantly higher in the low-dose maternal group than in the control group. The high-dose maternal mice had significantly increased IgG-1, IgG-2a, IgA, IgM, and IgE compared to controls. Meanwhile, IgG3 for the high-dose maternal mice had significantly lower compared to control group. In neonates from mothers who received low allergen doses, IgG-1, IgG-2b, IgG-2a, and IgG-3 were significantly higher than in controls. In neonates from mothers who received high allergen doses, IgG-1 and IgG-3 were significantly increased compared to controls, though both measurements were lower than that of low dose neonates. In contrast, the levels of IgG-2a and IgA were significantly decreased in the high-dose neonates compared to controls. Comparing the low and high dose maternal groups, IgG-1, IgG-2a, IgG-2b, and IgG-3 were significantly higher in the low dose groups, while IgE was significantly lower in the low dose group, compared to the high dose group. In neonates, there were also significantly higher levels of IgA, as well as the IgG subclasses for the low dose group compared to high dose group.

### Discussion

We found that exposing pregnant mice, to mite allergens in low doses provides an inflammatory response in mothers and neonates. The inflammatory effect on the Th1 cytokine profile in maternal mice and neonates was also shown in previous studies. The significant increases in IL-2 and IFN-γ expression in maternal mice after low dose mite allergen exposure were in agreement with a study using lymphocyte BALB/C cultures. The implications of the results of
## Table 1. Cytokine levels after allergen exposure

| Variables | IL-2 Mean (SD), μL | IFN-γ Mean (SD), μL | IL-4 Mean (SD), μL | IL-5 Mean (SD), μL | IL-10 Mean (SD), μL | TGF-β Mean (SD), μL |
|-----------|-------------------|-------------------|------------------|------------------|------------------|-------------------|
| **Maternal mice** | | | | | | |
| High dose | 1621.82 (2020.23) | 338.69 (137.75) | 55.79 (7.80) | 165.84 (71.87) | 267.95 (12.72) | 1093.45 (468.63) |
| Low dose  | 1237.86 (62.54)  | 309.04 (32.48)  | 75.67 (3.63)  | 308.02 (46.37) | 266.55 (11.56) | 922.95 (392.73)  |
| Control   | 695.91 (304.26)  | 270.65 (17.63)  | 50.87 (3.16)  | 147.01 (15.52) | 272.23 (11.47) | 174.26 (84.74)   |

| **Neonates** | | | | | | |
| High dose    | 802.37 (448.37) | 284.43 (15.53) | 51.49 (2.78) | 473.91 (414.09) | 269.79 (9.50) | 490.11 (385.66) |
| Low dose     | 1065.68 (240.54) | 294.19 (16.54) | 70.98 (2.01) | 381.74 (104.12) | 259.80 (10.31) | 936.40 (390.34) |
| Control      | 479.74 (201.55) | 270.65 (10.09) | 50.46 (5.28) | 140.54 (7.96)  | 267.95 (8.69) | 197.26 (87.41)  |

Description P (r-t): P (low dose-high dose); * P value < 0.05 was statistically significant

## Table 2. Levels of immunoglobulins expressed after exposure to mite allergens

| Immunoglobulins | Maternal mice | Neonates |
|-----------------|---------------|----------|
|                 | High dose     | Low dose | Control | High dose | Low dose | Control |
| **IgA**         | 0.34 (0.11)   | 0.35 (0.19) | 0.15 (0.09) | 0.18 (0.10) | 0.114 | 0.10 (0.04) | 0.22* |
| **IgM**         | 1.35 (0.14)   | 1.46 (0.45) | 1.16 (0.22) | 2.21 (0.16) | 0.116 | 0.24 (0.08) | 0.32 |
| **IgE**         | 123.87 (19.05) | 112.89 (19.91) | 21.89 (0.84) | 30.26 (12.81) | 0.196 | 27.56 (13.70) | 0.403 |
| **IgG-1**       | 1.48 (0.05)   | 1.92 (0.02) | 1.26 (0.07) | 1.17 (0.10) | 1.85 (0.02) | 1.36 (0.03) | 0.004* |
| **IgG-2a**      | 1.55 (0.08)   | 2.99 (0.06) | 1.41 (0.05) | 0.72 (0.07) | 2.52 (0.30) | 1.48 (0.18) | 0.000* |
| **IgG-2b**      | 2.59 (0.14)   | 3.47 (0.03) | 2.58 (0.06) | 2.13 (0.11) | 3.06 (0.07) | 1.95 (0.18) | 0.004* |
| **IgG-3**       | 0.21 (0.05)   | 1.59 (0.64) | 0.36 (0.04) | 0.15 (0.05) | 0.011* | 1.00 (0.02) | 0.000* |

*significantly higher compared to control group; *significantly lower compared to control group; *P value < 0.05 was statistically significant
our study are different from a study conducted during the newborn period, in which Th1-derived cytokines such as IFN-γ was decreased. Decreased production of Th1 cytokines is due to attenuated macrophage responses associated with weakened innate immunity in neonates. These reports suggest that exposure to low doses of allergen mite during pregnancy can aggravate the expression of Th1 cytokines in mothers and neonates, but the number of mite allergens does not directly effect an increase in IL-2 and IFN-γ levels. Low dose mite allergens were associated with increased Th2 cytokines IL-4 and IL-5 in maternal mice, similar to previous studies. However, an experimental study on bronchoalveolar lavage specimens taken from BALB/C mice (6-8 wk old) found an increase in IL-4 levels, but not in IL-5 levels. In BALB/C neonates following low dose mite allergen exposure, IL-4 and IL-5 levels were also significantly increased and in agreement with a previous study. Such evidence supports the idea that exposure to low dose mite allergens during pregnancy can induce the expression of Th2 cytokines in both mothers and neonates. We also noted that TGF-β1 expression was significantly increased in maternal mice and neonates at low and high doses of mite allergens compared to controls. The significantly increased TGF-β1 levels in BALB/C neonates were in agreement with a study by Victor et al. They concluded that exposure to low dose mite allergens induced higher levels of TGF-β1 in BALB/C neonates from mothers exposed to the same allergen during pregnancy than in those not exposed. Also, TGF-β1 was significantly increased at low and high doses of mite allergens compared to controls. A recent study reported that the transfer of maternal cytokines could affect the interaction of immature Treg cells in neonates, thereby affecting the function and maturation process of Treg cells themselves. Cytokines derived from atopic mothers (IFN-γ, IL-13, and IL-10) can affect the maturation of Treg cells in the fetal thymus gland. Furthermore, the cytokines TGF-β1 and IL-2 may benefit the maturation process of neonatal Treg cells in mice, but TGF-β1 is not required for the maturation process. Overall, they observed that the increased expression of TGF-β1 in pregnant BALB/C mice exposed to mite allergens had almost no effect on the maturation process of Treg cells in their neonates. This finding is relevant to our results, in which both low and high dose neonatal groups had significantly higher TGF-β1. Hence, this finding suggests that regardless of high or low doses of mite allergen, the activation of Treg cells in the allergy control process is not altered.

In the maternal mice group, there were significant increases in serum IgE expression at a low and high dose of mite allergens compared to the control. The increase in serum IgE levels is in line with previous studies that showed that applying low dose intranasal house dust mite allergens (35 μg) in BALB/C mice aged 8-10 weeks for 2 weeks will increase serum IgE levels. Exposure to house dust mite allergens at high doses (500 μg) via an ocular route in BALB/C mice caused a significant increase in serum IgE levels in maternal mice. Contrary to our findings of no significant increase in IgE in neonates exposed to low dose mite allergen compared to controls, a study revealed that intranasally administered low-dose mite allergen (10 μg) in BALB/C neonatal mice significantly increased serum IgE levels. Furthermore, another study reported that subcutaneous administration of low dose mite allergen (10 μg) to female A/Sn mice who were then mated with male C57BL/6 mice and whose offspring were re-exposed to mite allergen, led to inhibition of IgE formation in their offspring. Our results showed that IgE levels in maternal mice after exposure to mite allergens, both low and high doses, were higher than IgE levels in neonates.

Our results suggest that with regards to mite allergen exposure in BALB/C mice, the regulatory function of Treg is dominated by TGFβ in both maternal mice and neonates, with low and high doses. In a low dose, Th2 cells regulated IL-5 levels against the low and high dose allergens, but Th1 expressed cytokines during exposure only to low dose allergens. We found that exposure to mite allergens could provoke the regulatory functions of Th1, Th2, and Treg cells to activate their cytokines, with the exception of IL-10. In general, BALB/C mice exposure to mite allergens during pregnancy may induce tolerance in neonatal mice. Compared to the control group, the IgG subclasses were significantly regulated after low dose exposure to mite allergens. While the maternal group had increased IgE levels after low and high dose exposures, neonates did not show a significant change.

Further study in BALB/C mice with allergens other than mites is needed to evaluate tolerance.
in neonatal mice. In addition, a more in-depth study regarding postpartum immunoglobulin-class-switching in BALB/C mice should be performed to clarify the chance of tolerance in neonatal mice.

Conflict of Interest

None declared.

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