

## ORIGINAL ARTICLE

# Reovirus type-2 infection in newborn DBA/1J mice reduces the development of late allergic asthma

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## Summary

The aim of the present study was to determine whether or not the development of a helper T (Th) 1 response induced by Reovirus type-2 (Reo-2) infection would protect against the development of Th2-mediated late allergic asthma. This hypothesis was examined by infecting one day old neonatal DB A/1J mice with Reo-2 in an ovalbumin (OVA)-induced late asthma model. Compared with the controls (either infected or uninfected mice with or without OVA sensitization and/or OVA challenge), Reo-2 infection lessened the magnitude of the subsequent allergic Th2-mediated late asthma. In infected mice with allergic late asthma, there was decreased infiltration of interleukin (IL)-4<sup>+</sup>, IL-5<sup>+</sup>, IL-13<sup>+</sup> and very late antigen (VLA)-4<sup>+</sup> lymphocytes, and eotaxin-2<sup>+</sup> and VLA-4<sup>+</sup> eosinophils, in both bronchial and bronchiolar lesions. Also the expression of vascular cell adhesion molecule (VCAM)-1 and eotaxin-2 on vascular endothelial cells was reduced. Moreover, the systemic production of IL-4, IL-5, tumour necrosis factor- $\alpha$  and OVA-specific IgE was reduced, whereas systemic IFN- $\gamma$  production was increased. In addition, there was no increase in IFN- $\alpha$  production. Thus the present study suggests that systemic Reo-2 infection at birth may reduce the development of subsequent late allergic asthma by the induction of a Th1 response. Therefore the potential suppressive mechanism(s) that might be induced by Reo-2 infection in newborn mice and their effects on the development of late allergic asthma are discussed.

## Keywords

allergic asthma, eosinophil, IFN- $\alpha$ , late type, mouse model, Reo-2, Th1, Th2, TNF- $\alpha$

There are several factors, including genetic predispositions and environmental factors, which affect the development of allergic airway inflammation. Airway allergy in humans and animal models is mediated by T helper (Th) 2 immune responses to inhaled environmental allergens in sensitized individuals. This involves a two-phase allergic reaction: early and/or late phases (Skad *et al.* 1999; Hayashi 2011, 2012). In the early phase, IgE-sensitized mast cells degranulate and release mediators such as histamine and leukotriene, whereas the late phase of the allergic response mainly involves infiltration of eosinophils and Th2 type lymphocytes producing interleukin (IL)-4, IL-5 and IL-13 including IL-17 (Cosmi *et al.* 2011) and tumour necrosis factor (TNF)- $\alpha$  (Babu *et al.* 2011) without participation of mast cells and IgE.

It has been hypothesized that the increased prevalence of allergic diseases in developed countries may be associated

with the decline in some infections shifting the relative predominance between Th2 and Th1 responses. (Strachan 1989; Von Hertzen & Haahtela 2004). In addition, lower prevalence of asthma, hay fever and eczema symptoms in autoimmune diseases such as type 1 diabetes (T1D) patients compared with age-matched controls has been reported (Meerwaldt *et al.* 2002), whereas pre-existing asthma seems to protect against the development of autoimmune disorders to varying degrees in both men and women (Tirosh *et al.* 2006). This suggests that there may be induction of mutual reciprocal Th1/Th2 inhibitory effects (Mosmann & Coffman 1989).

Reovirus (Reo; serotypes 1, 2 and 3) is an RNA virus classified in the *Reoviridae* and included in the genus *Orthoreovirus*. All mammals serve as hosts for Reo infection, but the disease is limited to very young people and

manifests as a mild gastroenteritis (Onodera *et al.* 1990; Tyler *et al.* 1995; Danthi *et al.* 2006). However, Reo-2 can induce transient impaired glucose tolerance with mild insulinitis peaking at approximately 10 days after infection. In this model the virus was inoculated into one day old newborn genetically autoimmune sensitive DBA/1J mice (H2<sup>q</sup> haplotype: first chromosome VAS 1 is responsible for autoimmunity; Holmdahl *et al.* 1990). Most mice recovered from the disease within three weeks after infection (Onodera *et al.* 1990; Hayashi *et al.* 1998, 2001). This may be due to the fact that a sufficient number of  $\beta$ -cells were left intact after the destruction, and proliferation accompanied by disappearance of infiltrated cells may occur (Onodera *et al.* 1990).

We have reported previously that Reo-2 infection can induce transient expression of IFN- $\gamma$  mRNA, but not IL-4 mRNA, from splenic cells both shortly after the infection and during the developmental phase of insulinitis when there has been elimination of virus from the mice (Hayashi *et al.* 1998, 2001). This suggests that infected mice may develop two different types of Th1 responses. In the virus multiplication phase, it has been reported that reoviruses infected cells induce high IFN- $\alpha/\beta$  (Steele & Hauser 2005; Errington *et al.* 2008). Also Toll-like receptor-3 on dendritic cells stimulated with reoviruses may induce IFN- $\alpha/\beta$ , leading to Th1 responses (Errington *et al.* 2008). Later, in addition, Th1 cells will be induced as a consequence of the development of autoimmune insulinitis (Hayashi *et al.* 1998, 2001).

At present the cumulative effects of virus infection and the following autoimmune T1D on the development of airway allergy are not known. To clarify this the effects of Reo-2 infection on the development of late allergic asthma were examined during the recovery phase of Reo-2-triggered insulinitis.

## Methods

### Animals

One day old DBA/1J newborn mice of either sex (Japan Charles River Co., Kanagawa, Japan) were used (total number used,  $n = 58$ ) as described previously (Hayashi *et al.* 1998, 2001). They were kept at  $25 \pm 2^\circ\text{C}$  room temperature,  $55 \pm 10\%$  humidity, and at 12-h light/dark cycle (lightening time 08:00–20:00) in metal cages sterilized by dry heating ( $180^\circ\text{C}$ , 30 min), and animals were given autoclaved ( $120^\circ\text{C}$ , 30 min) pellets (CA-1, Clea Japan INC., Tokyo, Japan) and water freely. The number of mice used in each experimental group is given in parenthesis of experimental protocol. The animal experiments were approved by the Research Ethics Board of the Faculty of the Agriculture, Yamaguchi University.

### Virus

The BN-77 strain of Reo-2 was isolated from a cow with diarrhoea and passaged several times in primary bovine kidney cell culture (Kurogi *et al.* 1980), and grown on the HmLu-1 (hamster lung fibroblast recloned in our laboratory)

cell line as described previously (Onodera *et al.* 1990; Hayashi *et al.* 1998, 2001).

### Experimental protocols and sampling

As shown in Figure 1, mice were divided into four groups as follows: Reo-2- infected mice were sensitized and challenged with OVA (group 1;  $n = 12$ ), uninfected mice were sensitized and challenged with OVA (group 2;  $n = 11$ ), Reo-2- infected mice were not sensitized and challenged with OVA (group 3;  $n = 10$ ), and uninfected mice were neither sensitized nor challenged with OVA (group 4;  $n = 9$ ). One day old mice (body weight: approximately 1 g) were infected intraperitoneally (i.p.) with  $5.5 \times 10^5$  plaque-forming units of Reo-2 in 0.05 ml of Eagle's minimal essential medium (MFM; Nissui, Tokyo, Japan) on what was designated 'day -28' (groups 1 and 3). The sensitization and challenge procedures were performed by the methods described previously (Hayashi *et al.* 2003). Mice (groups 1 and 2) were sensitized i.p. with 10  $\mu\text{g}$  of OVA (Grade V; Sigma-Aldrich Co., St Louis, MO, USA) in 1.2 mg aluminum hydroxide gel (Alum; SERVA, Heidelberg, Germany) adjuvant (OVA/Alum), which was suspended in phosphate-buffered saline (PBS; pH 7.4), on day 0 and boosted on day 10. Four days after the second sensitization, the mice were challenged intranasally (i.n.) with 200  $\mu\text{g}$  of OVA in 50  $\mu\text{l}$  of PBS (both right and left nasal cavities by a micropipette alternately) under anaesthesia by injection with 0.5 ml mixture of ketamine and xylazine i.p. Control groups (groups 2–4) were administered with PBS instead of either OVA or Reo-2. Four days after the challenge, blood, left lungs, pancreases and spleens were obtained from all groups. Pancreases were also sampled on day -18 to examine the presence of insulinitis.

### Leucocyte number in blood

The absolute number of leucocytes/ $\mu\text{l}$  was counted on day 0 and 18 ( $n = 8$ –12 in each group). The percentage of eosinophils, neutrophils, lymphocytes and monocytes was calculated

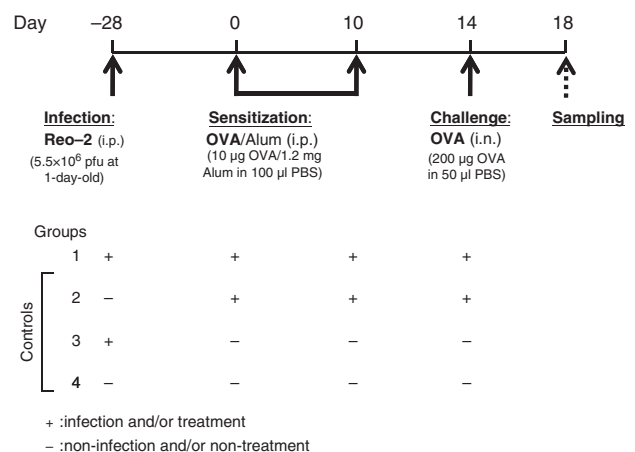


**Figure 1** Experimental protocol in Reo-2 infection and induction of late allergic asthma. See detail in 'Methods'.

from a blood smear stained with May-Grünwald-Giemsa and the total number of each leucocyte was calculated.

#### *Histological evaluation of pancreas*

Pancreatic tissues on day -18 and on day 18 ( $n = 4$  in each group) were fixed in 10% neutral-buffered formalin (pH 7.0) and embedded in paraffin wax. Sections 4  $\mu\text{m}$  in thickness were stained with haematoxylin and eosin (HE). For each mouse, the incidence (%) of insulitis (inflammatory cell infiltration into pancreatic islets) including peri-insulitis in the islets was evaluated, and an 'islet score index' was calculated by a method described previously (Hayashi *et al.* 1998). The severity of the cellular infiltration was evaluated by light microscopy, and an insulitis score was given to each islet on a 0–4 scale (0 = no insulitis or peri-insulitis; 1 = insulitis < 25% of islet area; 2 = insulitis 25–50% of islet area; 3 = insulitis > 50% of islet area). The grade of insulitis/mouse was expressed as the average score [score index (S.I.) = total score/number of islets]. An average of 10 different pancreatic islets was examined.

#### *Histological evaluation of lung lesion*

Left lung samples were fixed in 10% neutral-buffered formalin (pH 7.0) and embedded in paraffin wax ( $n = 8$ –12 in each group). Sections 4  $\mu\text{m}$  thick were stained with HE, alcian blue pH 2.5 and periodic acid-Schiff's (PAS) for goblet cells and/or connective tissue-type mast cell and methylene blue for mucosal type mast cell (Bui *et al.* 2010). The severity of lesions in each transversely sectioned bronchus-bronchiole was evaluated by light microscopy and scored as follows (Hayashi *et al.* 2003): no lesions, 0; mucus or desquamated epithelial cells in the lumen, 1; a few goblet cells in the epithelial layer, 1; many such cells, 2; thickened basement membrane, 1; mild infiltration of eosinophils and lymphocytes in mucosa, 1; severe infiltration of those cells in the mucosa, 2; hypertrophy of smooth muscle cells in mucosa, 1. In each mouse, the S.I. was calculated by dividing the total number of bronchi-bronchioles examined. The average of S.I. was then determined for each group. The number of eosinophils, lymphocytes, neutrophils, macrophages, plasma cells and mast cells in the bronchial-bronchiolar mucosa was determined by eyepiece micrometer on five different bronchi-bronchioles at random in each left lung. Each result was averaged and totalled in each mouse, and thereafter the mean  $\pm$  SEM of each experiment was calculated.

#### *Immunohistochemistry*

Deparaffinized sections of lung from all groups ( $n = 5$  in each group) were treated with microwave oven (500 W for 5 min) in 10 mM citrate buffer (pH 6.0). Endogenous peroxidase activity was inactivated in 3% hydrogen peroxide in methanol for 10 min. Sections were treated with 5% bovine serum albumin (BSA; Sigma-Aldrich) in PBS and normal goat serum for 30 min to block non-specific binding of antibody.

Additionally, sections were treated with avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA, USA). Sections were treated with primary antibodies as follows: rat anti-mouse IL-4 monoclonal antibody (mAb) (Pharmingen, San Diego, CA, USA) diluted in 1/10; rat anti-mouse IL-5 mAb (Southern Biotechnology, Birmingham, AL, USA) diluted in 1/10; goat anti-mouse IL-13 polyclonal antibody (pAb) (R&D system, Inc., Minneapolis, MN, USA) diluted in 1/20; rat anti-mouse IFN- $\gamma$  mAb (Upstate Biotechnology, Lake Placid, NY, USA) diluted in 1/20; rat anti-mouse VLA-4 mAb (Antigenix, Franklin Square, NY, USA) diluted in 1/5; rat anti-mouse VCAM-1 mAb (Antigenix, Huntington Station, NY, USA) diluted in 1/5 and goat anti-eotaxin-2 polyclonal antibody (pAb) (Santa Cruz Biotechnology, <http://www.scbt.com>) diluted in 1/50. Thereafter, sections were treated with secondary antibodies, biotinylated rabbit anti-rat IgG (Vector Laboratories) or biotinylated rabbit anti-goat IgG (Dako Cytomation, Carpinteria, CA, USA) depending on the immunized animals of primary antibodies for one h. Sections were treated with peroxidase-labelled streptavidin (Invitrogen Co., Camarillo, CA, USA) for 10 min and incubated with 3,3'-diaminobenzidine substrate (Roch Diagnostic, GmbH, Penzberg, Germany) for 10 min. Sections were counterstained with methyl green for 5 min at room temperature except for primary antibodies (4 °C), between each step, and were washed three times with PBS. The reaction without primary antibody served as negative control.

#### *Isolation of splenocytes*

Spleens ( $n = 5$  in each group) were removed aseptically and placed in phosphate-buffered-balanced salt solution (PBBS; pH 7.4). Single cell suspensions were made by teasing spleens apart with scissors and passing through a disposable syringe with a 21G needle (Terumo Co., Tokyo, Japan). Cell suspensions were collected in sterile conical tubes (Fisher Sci., Pittsburgh, PA, USA), and thereafter red blood cells were hemolysed by Tris-buffered  $\text{NH}_4\text{Cl}$  solution and the splenocytes washed in PBBS followed by centrifugation at 800  $g$  for 10 min at 4 °C. The total number of splenic cells was counted using a haemocytometer and diluted in MEM with kanamycin (100  $\mu\text{g}/\text{ml}$ ) to a density of  $1 \times 10^6$  cells/ml. Viability of cells was more than 98% by trypan blue dye exclusion test. Splenocytes ( $2 \times 10^5/200 \mu\text{l}$  MEM with 5% heat-inactivated foetal calf serum (Gibco., Grand Island, NY, USA) were cultured for 48 h at 37 °C in 5%  $\text{CO}_2$  in the presence of 5.5  $\mu\text{g}/\text{ml}$  of concanavalin A (Con A; ICN Biomedical Inc., Aurora, OH, USA) in 96-well plates (Coster, Cambridge, MA, USA), and supernatants were obtained and stored at -80 °C until use.

#### *IL-4, IL-5 and IFN- $\gamma$ in supernatants from cultured splenic cells, and OVA-specific IgE, TNF- $\alpha$ and IFN- $\alpha$ in plasma by ELISA*

Concentrations of IL-4 (Bender MedSystems GmbH, Vienna, Austria), IL-5 (R&D system, Inc.) and IFN- $\gamma$  (Bender

MedSystems GmbH) in supernatants from cultured splenocytes, and values for OVA-specific IgE (DS pharma Co., Ltd, Suita, Osaka, Japan), TNF- $\alpha$  (R&D system, Inc.) and IFN- $\alpha$  (PBL Interferon Source, Piscataway, NJ, USA) in plasma were measured by mouse ELISA kits according to the manufacturer's instructions. Detectable amounts of IL-4, IL-5, IFN- $\gamma$ , OVA-specific IgE, TNF- $\alpha$  and IFN- $\alpha$  are 2.0 pg/ml, 3.3 pg/ml, 5.3 pg/ml, 2.7 ng/ml, 5.1 pg/ml or 12.5 pg/ml respectively. In case of cytokines could not be detected, their concentration was estimated as 0.

### Statistical analysis

The means  $\pm$  SEM were calculated and analyzed by one-way ANOVA test and then by Student's *t*-test (two-tailed). The accepted level of significance was  $P < 0.05$ . The data are expressed as the mean of sample examined mice  $\pm$  SEM.

## Results

### Number of leucocytes in blood

As shown in Figure 2, on day 18, but not on day 0, there was a significant increase in the number of total leucocytes (a), eosinophils (b), lymphocytes (c) and monocytes (e) ( $P < 0.05$  or  $P < 0.01$ ) in group 2 compared with the other three groups. Although the number of neutrophils (d) in the groups 1 and 2 increased compared with the groups 3 and 4 ( $P < 0.01$ ), there was no significant difference in the number of neutrophils between the group 1 and the group 2.

### Pancreatic islet lesions

As shown in Figure 3, there was mild cellular infiltration in and/or around pancreatic islets showing cell destruction, although those changes varied among islets and mice 10 days after Reo-2 infection on day -18 in the groups 1 and 3. Some animals developed severe insulitis (a and b). Reacting cells consisted of mostly lymphocytes mixed with some neutrophils, eosinophils, macrophages and plasma cells. Cellular infiltration with oedema and hyperaemia in the interstitial connective tissue, and necrosis of some exocrine pancreatic tissues were also seen. S.I. and % were 1.03 and 63.3% in the group 1 and those were 1.00 and 53.4% in the group 3, 10 days after infection (on day -18), whereas those were not seen in both groups on day 18 (c and d). On the other hand, there was no insulitis in the groups 2 and 4 (e and f) on day -18 and on day 18 (data not shown).

### Bronchial-bronchiolar lesions

As shown in Figure 4, bronchial-bronchiolar lesions in the group 1 were less severe (a and b) compared with the group 2 (c and d). In the group 2, there was goblet cell metaplasia and/or hyperplasia with mucous hypersecretion (f), whereas

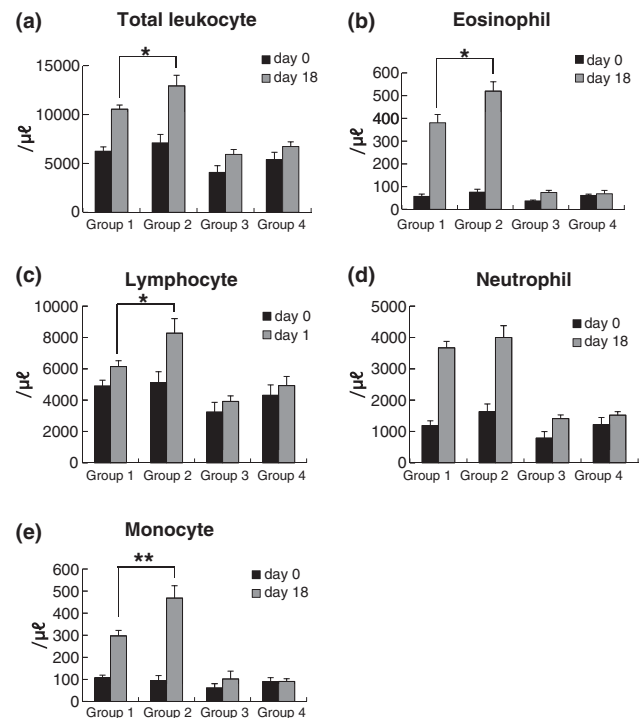


**Figure 2** Number of each leucocyte (a–e) in blood (/μl) in each group on days 0 and 18. Each value represents the mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ .

those changes were minor in the group 1 in general (e). There was severe infiltration of lymphocytes and eosinophils with degranulation, and the mucous membrane was oedematous in the inflamed bronchi-bronchioles. In those lesions, destroyed and desquamated epithelial cells were present in the bronchial-bronchiolar lumen and hypertrophic smooth muscle cells were observed in some cases (d) in group 2.

A number of infiltrating eosinophils and lymphocytes were identified in and/or around blood vessels in groups 1 and 2. There were a few mucosal type, but not connective tissue type, mast cells in the bronchial wall in group 1 (g) and in group 2 (h). There were no bronchial-bronchiolar lesions with eosinophilic infiltration in groups 3 and 4 (data not shown).

### S.I. (severity of lesions) of bronchial-bronchiolar lesions and characterization of inflammatory cell

As shown in Figure 5, the S.I. of bronchial-bronchiolar lesions in the group 2 increased (S.I.;  $1.9 \pm 0.5$ ), whereas bronchial-bronchiolar lesions were mild in the group 1 (S.I.;  $0.8 \pm 0.1$ ) ( $P < 0.05$ ) (a). There was a slight infiltration of lymphocytes, neutrophils and macrophages within the normal range in bronchial-bronchiolar lesions in groups 3 and 4.

The numbers of eosinophils and lymphocytes infiltrating the bronchi-bronchioles increased significantly in group 2 compared with group 1 ( $P < 0.01$ ) (b). There was no significant increase in the number of neutrophils, plasma cells, macrophages and mast cells in all groups.



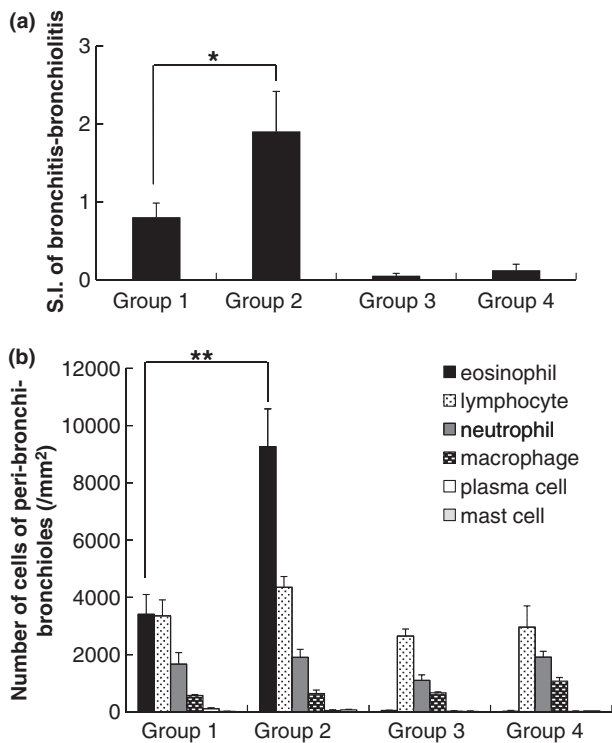


**Figure 5** S.I. of bronchial-bronchiolar lesions (a). Number of inflammatory cells at the site of bronchi-bronchioles ( $/\text{mm}^2$ ) (b). Each value represents the mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ .

#### Immunohistochemistry of bronchi-bronchioles

As shown in Figure 6, a small number of IL-4<sup>+</sup> (a), IL-5<sup>+</sup> and IL-13<sup>+</sup> lymphocytes and some IFN- $\gamma$ <sup>+</sup> lymphocytes were found at bronchi-bronchiolar mucosa in group 1. On the other hand, many IL-4<sup>+</sup> (b), IL-5<sup>+</sup> and IL-13<sup>+</sup> lymphocytes were observed at the bronchial-bronchiolar mucosa with some IFN- $\gamma$ <sup>+</sup> lymphocytes in group 2. Bronchial mucosal epithelium, endothelial cells and eosinophils in the bronchial-bronchiolar walls including pulmonary interstitial tissues strongly expressed eotaxin-2 (e) in group 2. These cells were fewer in number and weakly expressed eotaxin-2 in group 1(d). In addition, lymphocytes and eosinophils were positive for VLA-4 (h), and vascular endothelial cells were positive for VCAM-1 (k), but expression was weak (g and j) in group 1. There was no expression of IL-4, IL-5, IL-13, IFN- $\gamma$ , VLA-4, VCAM-1 and eotaxin-2 in lungs of groups 3 (c, f, i and l) and 4 (data not shown).

#### Production of IL-4, IL-5 and IFN- $\gamma$ in supernatants from cultured splenocytes stimulated with Con A including values of OVA-specific IgE, IFN- $\alpha$ and TNF- $\alpha$ in plasma

As shown in Figure 7, IL-4(a) and IL-5 (b) values in group 1 decreased compared with group 2 ( $P < 0.05$ ). In contrast, there was a marked increase in IFN- $\gamma$  values in group 1 (c) compared with group 2. In addition, IFN- $\gamma$  values in the control group 3 increased compared with the control group

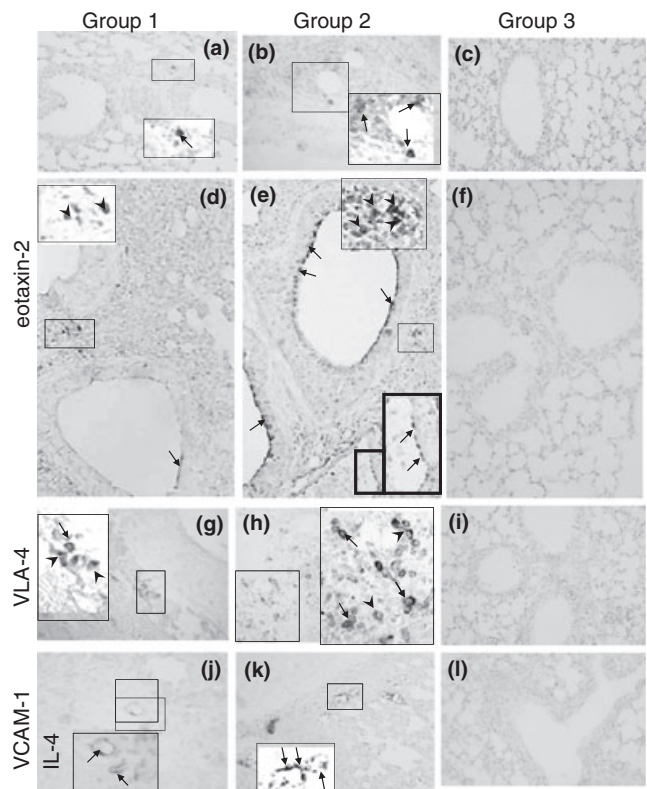


**Figure 6** Immunohistochemistry of bronchial-bronchiolar lesions. Arrows in inserts indicate IL-4<sup>+</sup> (enlargement of rectangles in a and b) lymphocytes. Arrow heads in insert (enlargement of thin rectangles in d and e) show eotaxin-2<sup>+</sup> eosinophils. Arrows in e indicate eotaxin-2<sup>+</sup> bronchial mucosal epithelium, and arrows in insert of e (enlargement of a bold rectangle of e) show eotaxin-2<sup>+</sup> vascular endothelial cells. Arrows of insert in g and h (enlargement of rectangles in g and h respectively) show VLA-4<sup>+</sup> lymphocytes and arrow heads show eosinophils (rectangles in g and h). Arrows of insert (enlargement of rectangle in j) show weak expression of VCAM-1 on endothelium, whereas intense expression of VCAM-1 (arrows of rectangle in k) is visible. Positive reaction; black Groups 1 (a, d, g and j), 2 (b, e, h and k) and 3 (c, d, i and l). a-c; IL-4, d-f; eotaxin-2, g-i; VLA-4, j-l; VCAM-1. a-l;  $\times 200$ , insert of a, b, d, e, g, h, j and k;  $\times 400$ .

4 ( $P < 0.05$ ), and there was no difference in IFN- $\gamma$  values between group 1 and group 3 (c). In groups 1 and 2, compared with groups 3 and 4, OVA-specific IgE (d) and TNF- $\alpha$  (e) values increased. In addition, each value in group 1 was significantly lower than that in group 2 ( $P < 0.05$ ). There was no significant increase in IFN- $\alpha$  value in group 1 compared with the controls (f).

#### Discussion

The present study demonstrated the reduction in the severity of Th2 type late allergic asthma, characterized by the infiltration of lymphocytes and eosinophils, in DBA/1J mice infected with Reo-2 at birth. It is possible that this is because systemic OVA-specific IgE, IL-4 and IL-5 values were low, whereas systemic IFN- $\gamma$  values were high in the

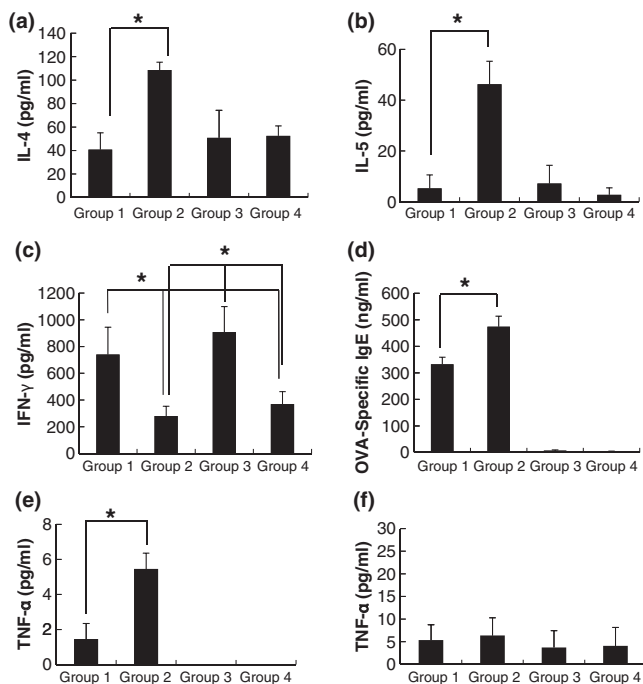


**Figure 7** Values of IL-4 (a), IL-5 (b) and IFN- $\gamma$  (c) in supernatants of cultured splenic cells stimulated with Con A, and OVA-specific IgE (d), TNF- $\alpha$  (e) and IFN- $\alpha$  (f) in plasma. Note the significantly decreased IL-4, IL-5, OVA-specific IgE and TNF- $\alpha$  values, while IFN- $\gamma$  values were increased (the group 1 *vs.* group 2, and the group 3 *vs.* the group 4 respectively). There was no increased IFN- $\alpha$  production in four groups. Each value represents the mean  $\pm$  SEM. \* $P < 0.05$ .

Reo-2 infected group (group 1) compared with the control groups. The mechanisms of the reduced late allergic asthma reaction with low eosinophilic reaction may be induced by mutual Th1/Th2 inhibitory effects (Mosmann & Coffman 1989). Interestingly, high IFN- $\gamma$  production from cultured splenic cells stimulated with Con A was observed not only in group 1 but also in group 3, suggesting that the existence of an antigen-unrelated memory pool of Th1 cells and/or increased Th1 dominant cell population among Th cells, including a balance shift from Th2 cells, to Th1 cells, may be induced in the Reo-2-infected group. Allergen non-specific suppressive effects by Th1 cells in Reo-2-infected mice seem likely, because treatment with environmental factors (such as bacterial DNA and certain oligodeoxynucleotides containing CpG motifs, a 6-base DNA motifs consisting of an unmethylated CpG dinucleotide flanked by two 5'purines and two 3'pyrimidines) can have stimulatory effects on dendritic cells via Toll-like receptors, resulting in production of IFN- $\alpha$ . This stimulates production of IFN- $\gamma$  from Th1 cells, and thus Reo-2-infected mice develop more severe insulinitis compared with that seen in Reo-2-infected mice that had only been treated with virus (Hayashi *et al.* 2002). Moreover, it has been shown that viruses can lead to activation of non-specific memory T cells (Seline *et al.* 1994). Further study is needed to clarify this point.

Alternatively Reo-2 infected mice with late asthma (group 1) which also develop Th1 responses, did not show increased IFN- $\alpha$  production (Errington *et al.* 2008), compared with controls. Thus the explanation for suppressive effects of Reo-2 infection on asthma, may not be related to IFN- $\alpha$  at the effector phase inducing increased may not be related to IFN- $\gamma$  production directly.

Based upon the present immunological and immunohistochemical results, a possible explanation for the inhibitory effects of Reo-2 infection could be as follows. In the initiation phase, Th2 cell dominant reactions occur after interaction between antigen presenting cells and Th0 cells (Hayashi 2011, 2012). Thereafter, IL-5 production from OVA-sensitized Th2 cells interacting with OVA may induce eosinopoiesis in bone marrow (Durham *et al.* 1992), leading to blood eosinophilia in the effector phase. Alternatively, some studies on asthmatic patients have revealed an increased TNF- $\alpha$  production in bronchoalveolar lavage fluid (Keatings *et al.* 1997; Yao *et al.* 2009). TNF- $\alpha$ , which may be produced by Th2 cells being responsible for the development of airway allergy (Ito *et al.* 2005; Babu *et al.* 2011), may be reduced by Th1 cytokines especially IFN- $\gamma$ . TNF- $\alpha$  and also Th2 cytokines (e.g. IL-4 and IL-13) are able to induce expression of adhesion molecules such as VCAM-1 on pulmonary vascular endothelium (Lassalle *et al.* 1993; Yamamoto *et al.* 1998; Wills-Karp & Karp 2004; Hayashi & Fujii 2008). This may lead to attachment of lymphocytes and eosinophils expressing the  $\beta$ 1-integrin VLA-4, a ligand for VCAM-1 (Okigami *et al.* 2007). Eotaxin-2 may also play an important role in the accumulation of eosinophils and infiltration of those cells at the site of bronchi-bronchioles (Yao *et al.* 2009). As demonstrated here in the group 2, pulmonary vascular endothelial cells and bronchi-bronchioles epithelial cells expressed eotaxin-2 intensely, which may be induced by IL-4 (Zimmermann *et al.* 2000) and IL-13 (Li *et al.* 1999), and eotaxin-2 may act as chemoattractants for eosinophils, but not for lymphocytes (Ponath *et al.* 1996). Thereafter IL-4 from Th2 cells may activates eosinophils and lead to the production of eotaxin-2 and IL-13 from Th2 cells, leading to hyperplasia/metaplasia of goblet cells (Miyahara *et al.* 2006; Hayashi 2011a,b). Finally, harmful substances (e.g. major basic protein, reactive oxygen species) from activated eosinophils may damage epithelial cells (Tagari *et al.* 1992; Honda & Chihara 1999). This seems likely because degranulation of eosinophils was often observed. In these processes mast cells may not contribute to the development of lesions. These steps may be impaired by systemic, but not local, Reo-2 infection, leading to the reduction in the development of asthma.

It has been reported recently that Reo-1 prevented the development of allergy induced by peanut antigens in young mice (Feczek *et al.* 2010). Similarly, Reo-2 infection during early life may ameliorate the development of asthma at a later age by dual effects on Th1 cells even after the stage of no viral footprint and the recovery from the disease (insulinitis). Reoviruses are widely distributed in human (more than 60%) and in general reovirus is non-pathogenetic (Onodera *et al.* 1990; Selb & Webr 1994; Zurney *et al.* 2009).

However, whether these viruses play a similar role in human asthma remains to be established.

## Acknowledgements

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## Conflict of interest

The authors declare that we have no conflict of interest.

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