Allergology International 65 (2016) 44–51

Contents lists available at ScienceDirect

Allergology International

journal homepage: http://www.elsevier.com/locate/alit

Original article

Presensitization to Ascaris antigens promotes induction of mite-specific IgE upon mite antigen inhalation in mice

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ARTICLE INFO

Article history:
Received 26 September 2014
Received in revised form
2 July 2015
Accepted 12 July 2015
Available online 12 August 2015

Keywords:
Antibody
Ascaris
Cross-reactivity
House dust mite
Th2

Abbreviations:
Ab, antibody; Ag, antigen; APC, antigen presenting cell; CGS, CanGetSignal; DF, *Dermatophagoides farinae*; DP, *Dermatophagoides pteronyssinus*; HDM, house dust mite; i.n., intranasal, intranasally; i.p., intraperitoneal, intraperitoneally; mAb, monoclonal antibody

ABSTRACT

Background: Patients with house dust mite (HDM) allergy or Ascariasis produce serum IgE specific to the antigens of HDM or nematode Ascaris, respectively. Although human IgE cross-reactivity has been reported between HDM and Ascaris antigens, it remains unclear whether it contributes to the pathogenesis of allergic diseases. We herein investigated the induction of cross-reactive antibodies and T cells in mice and effects of airway exposure to HDM antigens after preimmunization with Ascaris antigens.
Methods: Mice were intraperitoneally immunized with HDM or Ascaris antigens with Alum, followed by the intranasal administration of HDM antigens. Serum antigen-specific IgE and IgG were measured by ELISA. Cytokine release in splenocytes from Ascaris-immunized mice upon *in vitro* restimulation with HDM antigens were measured by ELISA.
Results: Immunization with Ascaris or HDM antigens induced cross-reactive IgG1. Splenocytes from Ascaris-immunized mice released IL-5 and IL-13 in response to the restimulation with HDM antigens. Subsequent airway exposure to HDM antigens promoted the induction of HDM-specific IgE and upregulation of HDM-specific IgG1 in Ascaris-immunized mice, whereas these responses were not detected or smaller without the Ascaris presensitization.
Conclusions: We demonstrated that the immunization of naïve mice with Ascaris antigens induced production of antibodies and differentiation of Th2 cells, which were cross-reactive to HDM antigens, and accelerated induction of serum HDM-specific IgE upon subsequent airway exposure to HDM antigens in mice. These results suggest that sensitization to HDM towards IgE-mediated allergic diseases is faster in individuals with a previous history of Ascaris infection than in those without presensitization to Ascaris.
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Introduction

House dust mites (HDMs) are a major causative factor for allergic diseases. In underdeveloped countries, infections with intestinal parasites such as Ascaris are important health problems, with co-exposure to HDM antigens (Ags) being reported. It currently remains unclear whether Ascaris infection causes or protects against allergic diseases. Previous epidemiological studies suggested the promotion of allergic diseases by helminth infection[1–8] while others indicated the protective role of helminth infection against allergies.[9–12] IgE from allergic patients is known to cross-react with Ascaris Ags and HDM allergens,[13,14] and Nakazawa *et al.*[15] recently reported that the immunization of rabbits with Ascaris Ags induced IgG antibodies (Abs) with reactivity to HDM Ags. These findings suggest that infection with Ascaris influences the natural history of allergic diseases via cross-reactivity between Ascaris and HDM Ags.

Animal models in which sensitization processes can be controlled and monitored are considered useful in the study of the molecular and cellular mechanisms as well as roles of the induction of cross-reactive Abs and T cells in the pathogenesis of allergic diseases. In the present study, we investigated whether sensitization to Ascaris Ags influenced allergic diseases via cross-reactivity with HDM Ags by using a murine model as an initial, simple approach, *i.e.*,

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Peer review under responsibility of Japanese Society of Allergology.

http://dx.doi.org/10.1016/j.alit.2015.07.003
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examining cross-reactivity of HDM and Ascaris Ags with the antibodies and T cells raised by the immunization of naïve mice as well as the effects of presensitization to Ascaris Ags on the induction of HDM-specific IgE upon subsequent airway exposure to HDM Ags.

Methods

Ascaris and HDM extracts

Freeze-dried extracts of *Ascaris suum* (Ascaris), *Dermatophagoides farinae* (DF), and *Dermatophagoides pteronyssinus* (DP) (LSL, Tokyo, Japan)[15] were reconstituted according to the manufacturer's instructions and were diluted with saline to a concentration of 2.5 mg/ml.

Treatment of mice

Six-to eight-week old female C3H/HeN mice were purchased from Charles River Japan, Inc. (Yokohama, Japan). Mice were maintained in a specific pathogen-free animal facility at Juntendo University and used in accordance with the guideline of the Institutional Committee on Animal Experiments. Ags were diluted with phosphate-buffered saline (PBS) and gently mixed with Alum (Imject Alum; Pierce Biotechnology Inc., Rockford, IL, USA) for 30 min at room temperature and then further diluted to appropriate volumes with saline. Mice were given 4 weekly intraperitoneal (i.p.) injections of 4 μg/head of each Ag. Sera collected 1 week after the last i.p. injection were stored at −20 °C before assays were conducted. In some experiments, beginning on the day after serum collection, mice were intranasally (i.n.) administrated DF (10 μg/40 μl/head) 3 times per week for two weeks (a total of 6 times). Sera collected 2 days after the last i.n. administration were stored at −20 °C before assays were conducted.

Immobilization of Ags and blocking in ELISA

Ags (DF, DP, and Ascaris extracts) (20 μg/ml, 50 μl/well) were diluted with PBS, and 96-well microtiter plates (MaxiSorp; Nunc, Roskilde, Denmark) were incubated with the monoclonal Ab or Ags overnight at 4 °C. After 2 washes with PBS containing 0.05% (v/v) Tween 20 (PBST), the plates were incubated with BlockAce (MEG-MILK Snow Brand, Sapporo, Japan) diluted with pure water (1/4

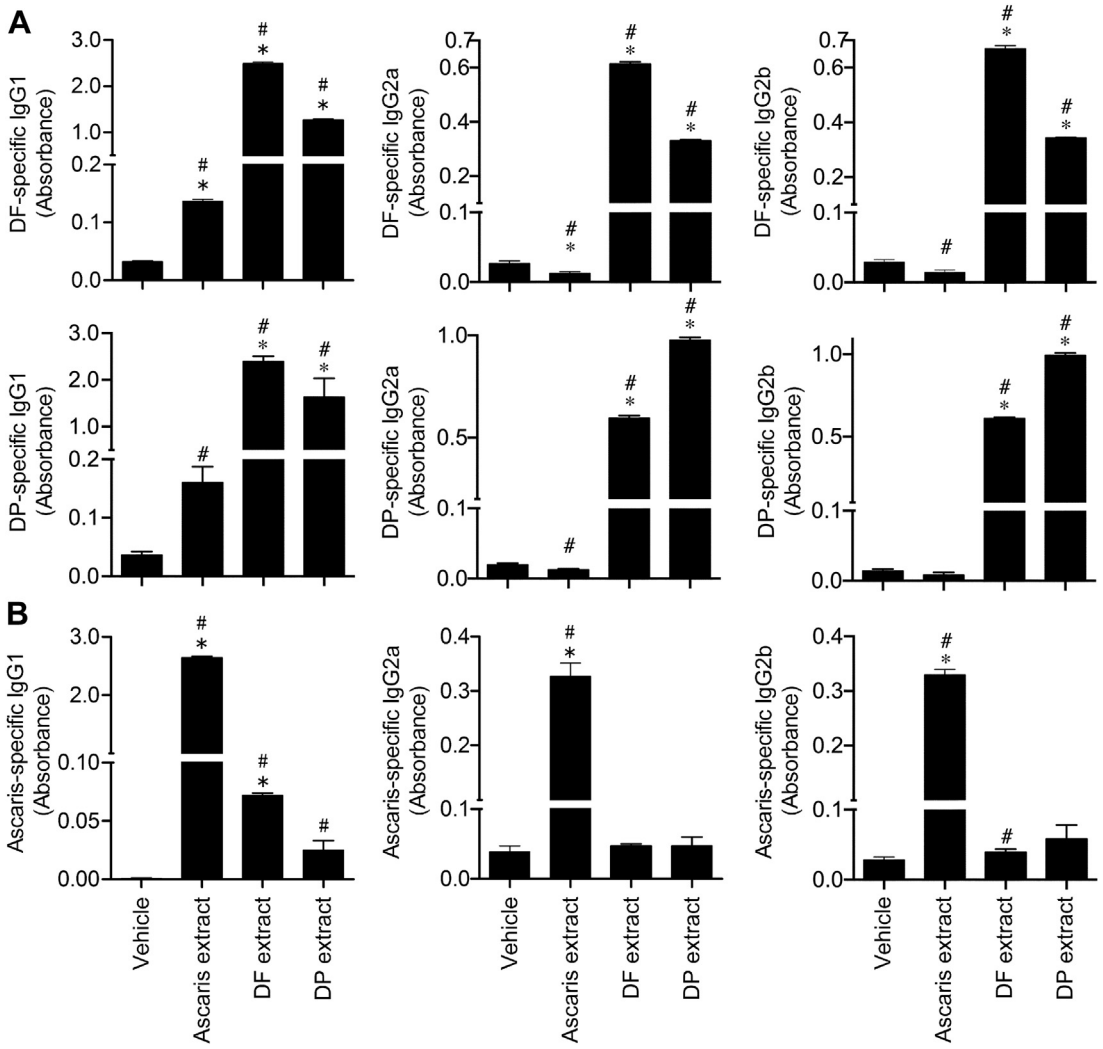

Fig. 1. Induction of Ag-specific IgGs in mice intraperitoneally immunized with Ascaris or HDM extract. HDM (**A**) and Ascaris (**B**)-specific IgG1, IgG2a, and IgG2b in mixtures of sera pooled from 5 mice in each group were analyzed by ELISA. The serum dilution factor was 800. The data shown represent the means + SD of the values for three wells. *$p < 0.05$ vs *Vehicle* by a one-way ANOVA followed by the Tukey *post hoc* test. #$p < 0.05$ vs *Vehicle* by the Student's *t*-test. Data are representative of three independent experiments with similar results.

dilution, 200 μl/well) for 1.5 h at 37 °C. After 2 washes with PBST, the plates were incubated with diluted sera.

ELISA for serum Ag-specific Abs

In order to detect Ag-specific Abs, sera diluted with Solution 1 of CanGetSignal (CGS1) (Toyobo, Osaka, Japan) were added to the wells of plates.[16,17] Serum dilutions were 1/200, 1/800, 1/800, and 1/800 to detect Ag-specific IgE, IgG1, IgG2a, and IgG2b, respectively. Wells were incubated with sera for 1.5 h at 37 °C for the detection of Ag-specific IgG1, IgG2a, and IgG2b. Wells were incubated with sera overnight at 4 °C after a 1.5-hr incubation at 37 °C for the detection of Ag-specific IgE. After five washes with PBST, horseradish peroxidase (HRP)-conjugated anti-mouse IgE monoclonal Ab (clone LO-ME-2; Technopharm Biotechnology, Paris, France) diluted with solution 2 of CanGetSignal (CGS2) (Toyobo) was added to the plates (1/10,000 dilution, 50 μl/well). After a 2-hr incubation at room temperature and 5 washes with PBST, color was developed for 20 min using tetramethyl benzidine (BD-OptEIA kit; BD Biosciences, San Jose, CA, USA) (100 μl/well), and the reaction was stopped by adding 2 N sulfuric acid (50 μl/well). Absorbance at 450 nm, from which that at 570 nm was subtracted, was used as the signal for Ag-specific Abs. Ag-specific IgGs were detected as well as Ag-specific IgE with the modification that incubations with the sera and those with HRP-conjugated anti-mouse IgG1 monoclonal Ab (clone X56; BD Biosciences), HRP-conjugated anti-mouse IgG2a monoclonal Ab (clone R19-15; BD Biosciences), and HRP-conjugated rat anti-mouse IgG2b Ab (Zymed, San Francisco, CA, USA) were for 1.5 h at 37 °C. We measured the induction of Ag-specific Abs by comparing absorbance in ELISA with a control group.

Inhibition ELISA for Ag-specific Abs

Sera were mixed with inhibitors (DF, DP, Ascaris extract), diluted with CGS1 (dilution factors at 300, 3,000, and 30,000), pre-incubated for 30 min at room temperature, and then added to plates (50 μl/well), which were coated with Ag solutions (*Ascaris*, DF, or DP extract) and blocked. The plates were incubated for 1.5 h at 37 °C. After 5 washes with PBST, the plates were incubated with HRP-conjugated anti-mouse IgG1 mAb diluted with CGS2 for 1.5 h at 37 °C (50 μl/well). The binding of Ab to solid phase Ags was visualized and measured. The percentage of inhibition was

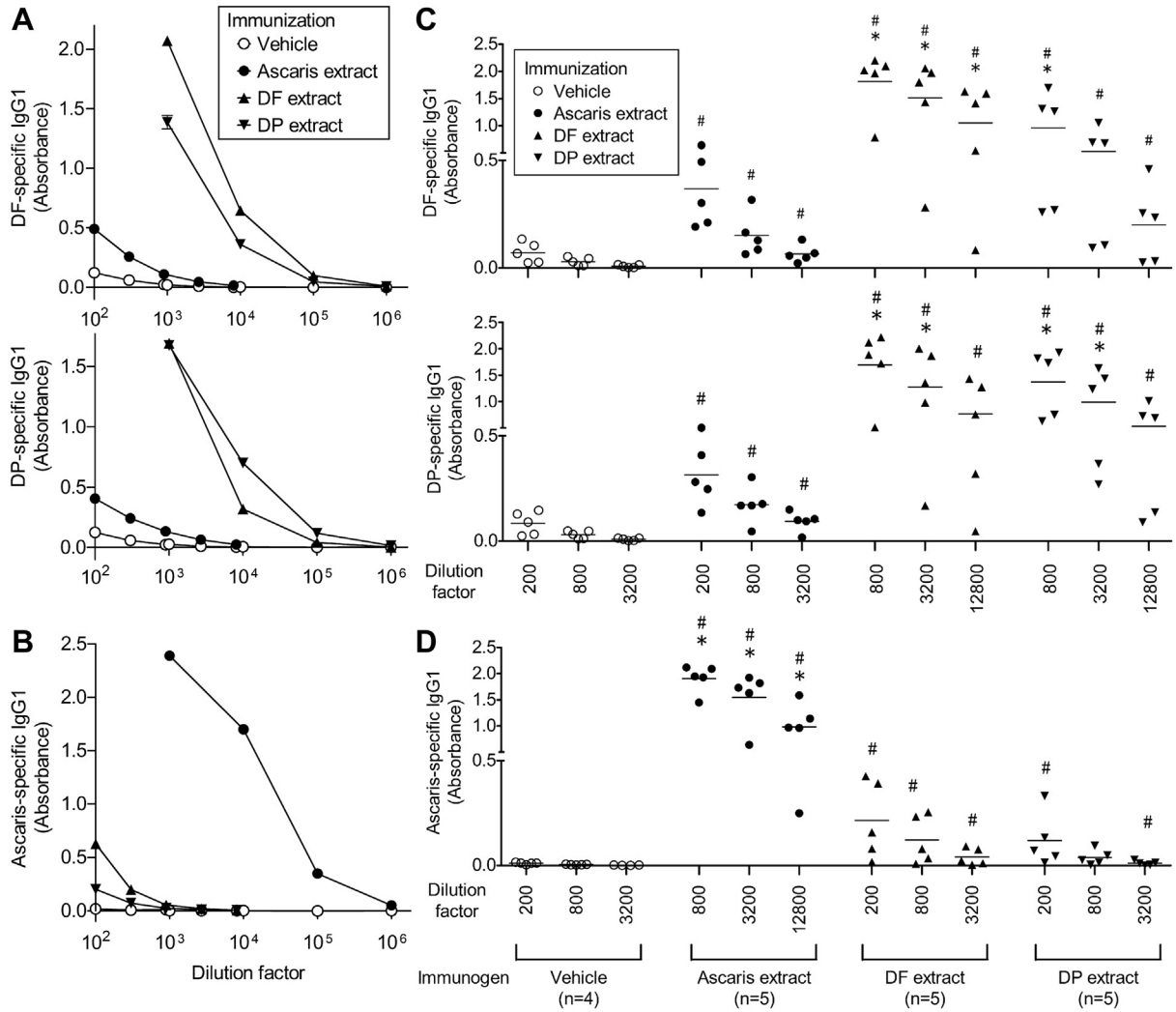

Fig. 2. Induction of IgG1 reactive to HDM and Ascaris Ags in mice intraperitoneally immunized with Ascaris or HDM extract. Titration curves for DF- and DP-specific IgG1 (**A**, **C**) and Ascaris-specific IgG1 (**B**, **D**) analyzed by ELISA using mixtures of sera pooled from five mice in each group (**A**, **B**) or using individual sera (**C**, **D**). **A**, **B**: The data shown represent the means ± SD of the values for three wells. **C**, **D**: Data are values for 5 mice per group and bars indicate means. *$p < 0.05$ vs *Vehicle* by a one-way ANOVA followed by the Tukey *post hoc* test. #$p < 0.05$ vs *Vehicle* by the Mann–Whitney *U* test. Data are representative of three independent experiments with similar results.

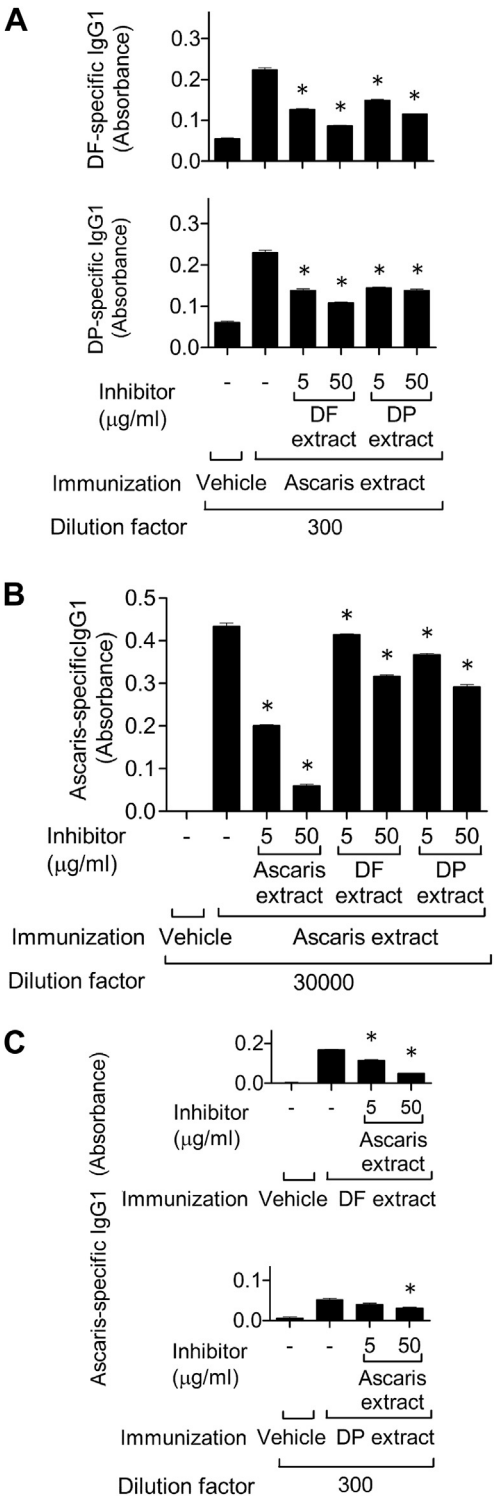

Fig. 3. Cross-reactivity confirmed by inhibition ELISA. The cross-reactivity of IgG1 reactive to HDM (**A**) and Ascaris Ags (**B**, **C**) induced in mice intraperitoneally immunized with Ascaris (**A**, **B**) and HDM Ags (**C**) was confirmed by inhibition ELISA using mixtures of sera pooled from five mice in each group. Serum dilution factors were 300 (**A**, **C**) and 30,000 (**B**). The data shown represent the means + SD of the values for six (sera from Ascaris-immunized mice with no inhibitor) or three wells (the others). $^{*}p < 0.05$ vs *Vehicle* by a one-way ANOVA followed by the Tukey *post hoc* test. Data are representative of three independent experiments with similar results.

expressed as a reduction in absorbance in each sample to that when no inhibitors were added.

Splenocyte responses

Mice were given 4 weekly i.p. injections of Ascaris extract or vehicle with Alum. One week after the last i.p. injection, mice were i.n. administrated PBS 3 times per week for two weeks (a total of 6 times). Spleen cells were prepared according to the methods described previously[18] with minor modifications. Briefly, on the day after the last PBS i.n. administration, spleens were asceptically removed. Cells suspension were prepared by gently teasing apart the tissue in RPMI1640 medium (Sigma–Aldrich, Tokyo, Japan) supplemented with 2 mM L-glutamine, 10% (v/v) heat-inactivated fetal bovine serum, 10 mM HEPES, 0.05 mM 2-mercaptoethanol, and antibiotics. Following homogenization by passage through EASYstrainer (70-μm pore size, Greiner Bio-One, Tokyo, Japan) and centrifugation at 500 ×*g* for 5 min at 4 °C, cells were resuspended in 1 ml of ACK buffer at room temperature for 5 min to deplete red blood cells. After washing with the medium, cells were stimulated with Ags in 96-well, round-bottom, tissue culture plates (Corning Life Sciences, Corning. NY, USA) (5×10^5/200 μl/well). To determine cytokine production, culture supernatants were collected at 96 h and subjected to ELISA using kits (DuoSet; R&D Systems, Minneapolis, MN, USA).

Statistical analysis

The Student's *t*-test (two-tailed) or one-way ANOVA followed by the Tukey *post hoc* test, and the Mann–Whitney *U* test were used to evaluate the significance of differences. A value of $p < 0.05$ was regarded as significant.

Results

Induction of Ab specific to HDM Ags by i.p. immunization of mice with Ascaris extract

HDM-specific IgG1, IgG2a, and IgG2b were detected in the sera of mice i.p. immunized with HDM extracts, and lower, but significant levels of HDM-reactive IgG1 were detected in the sera of mice immunized with Ascaris extract (Fig. 1A and 2A, C). The results of the titration of HDM-specific IgG1 revealed that the immunization of mice with Ascaris extract induced HDM-reactive IgG1 with approximately 1/200–1/100 efficiencies of that with the immunization with HDM extracts (Fig. 2A). Significant levels of IgE specific to HDM or Ascaris Ags were not detectable with reproducibility in the sera of mice i.p. immunized with the HDM extracts.

Induction of Ab specific to Ascaris Ags by i.p. immunization of mice with HDM extracts

Ascaris-specific IgG1, IgG2a, and IgG2b were detected in mice immunized with Ascaris extract, and lower, but significant levels of Ascaris-reactive IgG1 was detected in the sera of mice i.p. immunized with HDM extracts (Fig. 1B and 2B, D). The results of the titration of Ascaris-specific IgG1 revealed that the immunization of mice with HDM extracts induced Ascaris-reactive IgG1 with approximately 1/1,000 (DF) and 1/5,000 (DP) efficiencies of that with the immunization with Ascaris extract (Fig. 2B). Significant levels of IgE specific to Ascaris or HDM Ags were not detectable with reproducibility in the sera of mice i.p. immunized with Ascaris extract.

Cross-reactivity confirmed by inhibition ELISA

Inhibition ELISA was performed to examine IgG1 cross-reactivity between HDM and Ascaris Ags (Fig. 3, Supplementary Fig. 1). HDM extracts inhibited the binding of IgG1 from Ascaris-immunized mice to the solid phase DF (81% and 64% reductions by 50 μg/ml DF and DP extracts, respectively), DP (72% and 54% reductions by 50 μg/ml DF and DP extracts, respectively) (Fig. 3A), or Ascaris Ags (27% and 33% reductions by 50 μg/ml DF and DP extracts, respectively) (Fig. 3B).

Similarly, Ascaris extract inhibited the binding of IgG1 from HDM-immunized mice to the solid phase Ascaris Ags (72% and 44% reductions in DF- and DP-immunized mice, respectively, by 50 μg/ml Ascaris extract) (Fig. 3C). Ascaris extract did not inhibit the binding of IgG1 from HDM-immunized mice to the solid phase HDM Ags (Supplementary Fig. 1).

Restimulation with DF extract of spleen cells from mice i.p. immunized with Ascaris extract induced release of IL-5 and IL-13

Spleen cells were prepared three weeks after the last i.p. immunization with Ascaris extract as described in the Methods section and were stimulated with DF or Ascaris extract *in vitro*. Spleen cells from Ascaris-immunized mice responded to stimulation with Ascaris extract to release considerable amounts of Th2 cytokines, IL-4 (average: 888 pg/ml), IL-5 (5,770 pg/ml) and IL-13 (average: 12,700 pg/ml), (Fig. 4A–C). They responded to stimulation with DF extract to release much smaller but significant amounts of IL-5 (43.0 pg/ml) and IL-13 (67.8 pg/ml), which was not observed in spleen cells from vehicle-immunized mice (Fig. 4B, C).

Stimulation with Ascaris or DF extract did not significantly affect the release of IFN-γ (Fig. 4D) and that with DF extract induced release of IL-17A (Fig. 4E) in vehicle-immunized or Ascaris-immunized mice.

Airway exposure to DF extract after i.p. immunization with Ascaris Ags increased HDM-specific IgG1 and induced HDM-specific IgE

The effects of the i.n. administration of DF extract after the i.p. preimmunization with Ascaris Ags on Ab production was examined (Fig. 5). HDM-specific IgG1 levels after the i.n. administration of DF extract to Ascaris-immunized mice were significantly higher than those before the i.n. administration (Fig. 5A). HDM-specific IgE was not detectable before, but was induced after the i.n. administration of DF extract to Ascaris-immunized mice (Fig. 5B). HDM-specific IgG1 and IgE were not induced in unimmunized control mice even after the i.n. administration of DF extract under this experimental setting (Fig. 5, *Vehicle*).

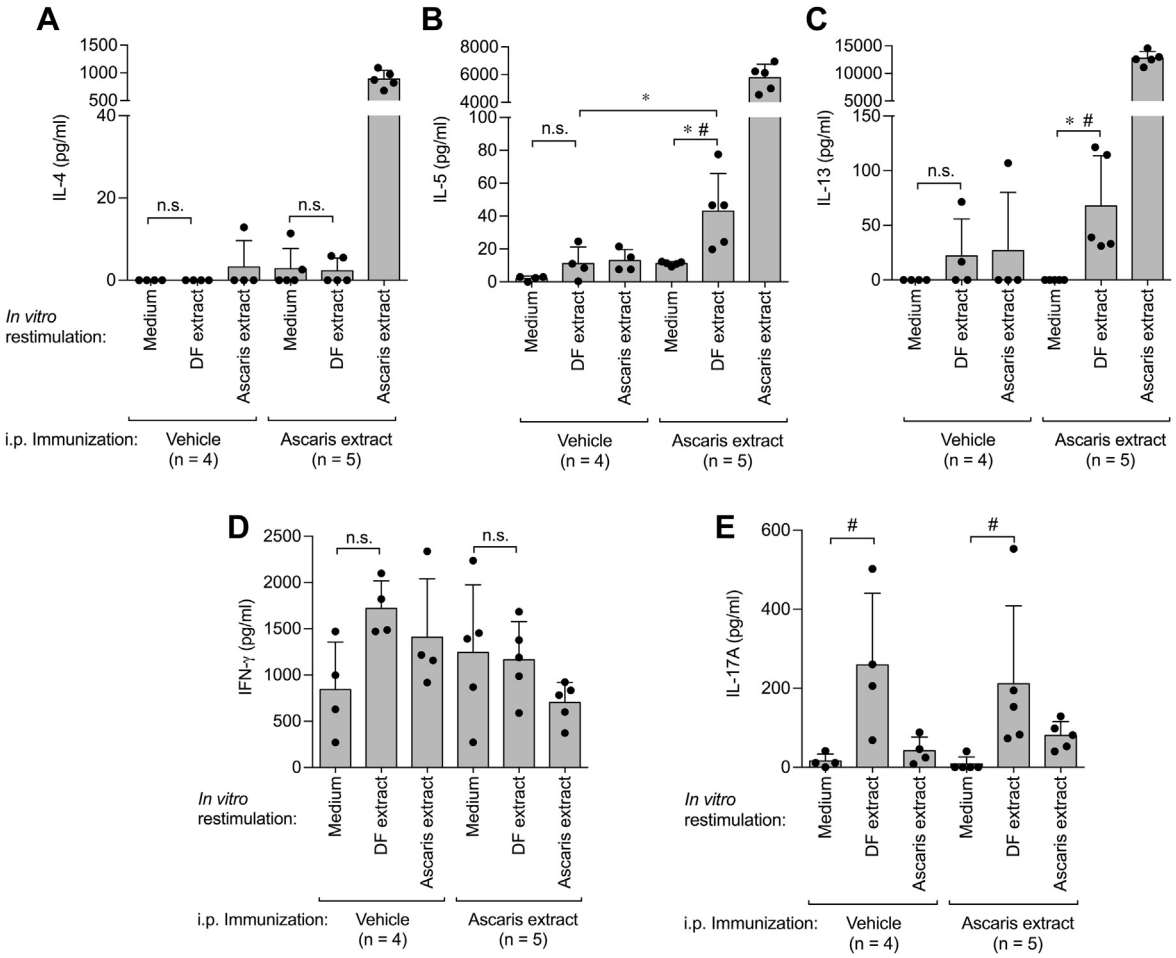

Fig. 4. Restimulation with DF extract of spleen cells from mice i.p. immunized with Ascaris extract induced release of IL-5 and IL-13. Spleen cells were prepared three weeks after the last i.p. immunization with vehicle or Ascaris extract as described in the Methods section and were cultured with medium alone, DF extract (100 μg/ml), or Ascaris extract (100 μg/ml) *in vitro*. To determine cytokine production, culture supernatants were collected at 96 h and subjected to ELISA. Data are values for 4 or 5 mice per group and bars represent the means + SD of the values. $^{*}p < 0.05$ by a one-way ANOVA of four groups cultured with medium alone or DF extract followed by the Tukey *post hoc* test. $\#p < 0.05$ by the Mann–Whitney *U* test. Data are representative of two independent experiments with similar results. *n.s.*, no statistically significant difference.

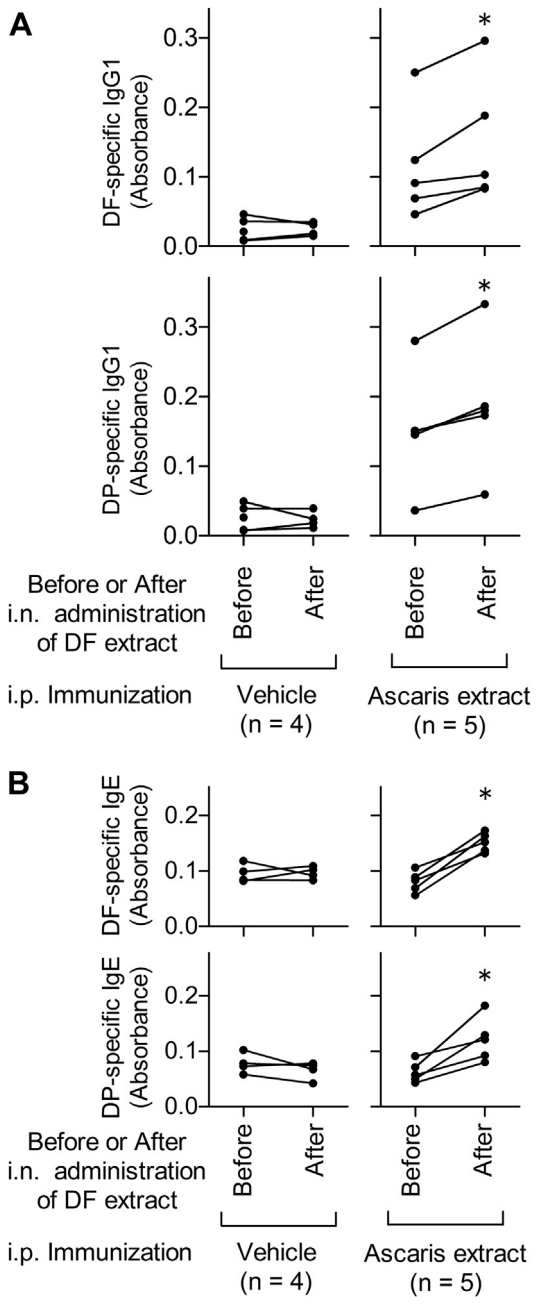

Fig. 5. Upregulation of HDM-specific IgG1 and induction of HDM-specific IgE by intranasal administration of DF extract after intraperitoneal preimmunization with Ascaris Ags. Serum DF- and DP-specific IgG1 (**A**) and IgE (**B**) before and after the intranasal administration of DF extract to mice with (*Ascaris extract*) or without (*Vehicle*) preimmunization with Ascaris Ags were analyzed by ELISA. Individual sera were used for the assays. Serum dilution factor: 800 (**A**) and 200 (**B**). Data are values for 4 or 5 mice per group. $^{*}p < 0.05$ vs *Before* the intranasal administration of DF extract by the Student's *t*-test (paired). Data are representative of two independent experiments with similar results.

Discussion

Although human IgE cross-reactivity has been reported between HDM and nematode Ascaris Ags, it has yet to be determined whether it contributes to the pathogenesis of allergic diseases. In the present study, we addressed this issue by using a murine model as an initial and simple approach. We showed that the immunization of naïve mice with HDM and Ascaris Ags induced cross-reactive antibodies to each Ag, respectively, at efficiencies that were detectable as the induction of Ag-specific IgG1 (Fig. 1–3). The absence of the detectable inhibition of binding of IgG1 from HDM-immunized mice to solid phase HDM Ags by the addition of Ascaris extract as the inhibitor (Supplementary Fig. 1) may have been attributed to the Ascaris-reactive IgG1 fraction in HDM-immunized sera being too small (Fig. 2B, D) because immunization with HDM Ags may induce cross-reactive IgG1 at lower efficiencies (Fig. 2B, D, triangles) than that with Ascaris Ags (Fig. 2A, C, closed circles). The induction of release of IL-5 and IL-13 by *in vitro* restimulation with DF Ags in spleen cells from mice immunized with Ascaris Ags suggests differentiation of Th2 cells cross-reactive to DF and Ascaris Ags in the Ascaris-immunized mice (Fig. 4B, C), although induction of IL-4 release upon restimulation with DF extract were hardly detectable by ELISA (Fig. 4A). The DF extract showed activity to stimulate cells contained in spleen cells to produce IL-17A independent of immunization with Ascaris Ags (Fig. 4E). The induction of HDM-specific IgE and upregulation of HDM-specific IgG1 (Fig. 5) were facilitated by the inhalation of HDM Ags by Ascaris-preimmunized mice, but not in mice without this preimmunization.

Although the mechanisms underlying the induction of HDM-specific IgE in Ascaris-preimmunized, DF-inhaled mice have not yet been elucidated in detail, we proposed a hypothesis in Fig. 6. In the initial step, the Ascaris preimmunization induces the differentiation of Th2 cells specific to the T-cell epitopes of Ascaris Ags, which contain a population reactive to the T-cell epitopes of DF Ags with considerable homology to Ascaris Ags (Fig. 6A). In the second step, Th2 cells cross-reactive to HDM and Ascaris Ags produce Th2 cytokines and induce an IgE class-switch in B cells, which recognize B-cell epitopes on HDM Ags containing the cross-reactive T-cell epitopes, upon airway exposure to DF Ags (Fig. 6B). In the final step, the Th2 cytokine milieu facilitates the differentiation of Th2 cells specific to HDM Ags with no or low homology with Ascaris Ags, which induces the production of IgE specific to HDM Ags with no or low homology with Ascaris Ags (Fig. 6C). HDM-derived adjuvants[19] and epithelial Th2-inducing cytokines such as thymic stromal lymphopoietin, IL-33, and IL-25[20–23], which could be released in airway epithelia upon exposure to HDM extract, may also contribute to the promotion of Th2 differentiation and IgE production. In the present study, we demonstrated that the immunization with Ascaris Ags induces cross-reactive immune responses at the B-cell (Fig. 1–3) and T-cell levels (Fig. 4) and accelerates induction or upregulation of HDM-specific IgE upon subsequent airway exposure to HDM Ags (Fig. 5), however, acceleration of Th2 cell differentiation in the draining lymph nodes or local airway tissues is yet to be examined in Ascaris-preimmunized, HDM-inhaled mice.

The results obtained in the present study suggest that cross-reactive immune responses at humoral and/or cellular levels against Ascaris and HDM Ags promote induction or upregulation of IgE specific to inhaled HDM in mice presensitized to Ascaris, thereby indicating that sensitization to HDM towards IgE-mediated allergic diseases is faster in individuals with earlier Ascaris infection than in those without a previous history of Ascaris infection. Although DF-specific IgE was detectable in Ascaris-preimmunized, DF-inhaled mice, but not in mice without the Ascaris preimmunization (Fig. 5B), whether this immunological modulation leads to relevant allergic reactions remains unknown, and we cannot exclude the possibility that presensitization to helminth protects against allergic responses in different experimental settings via the induction of regulatory cells or production of blocking IgG Abs.[24] Nevertheless, animal models in which sensitization processes can be controlled and monitored are considered useful in the study of the molecular and cellular mechanisms as well as roles of the

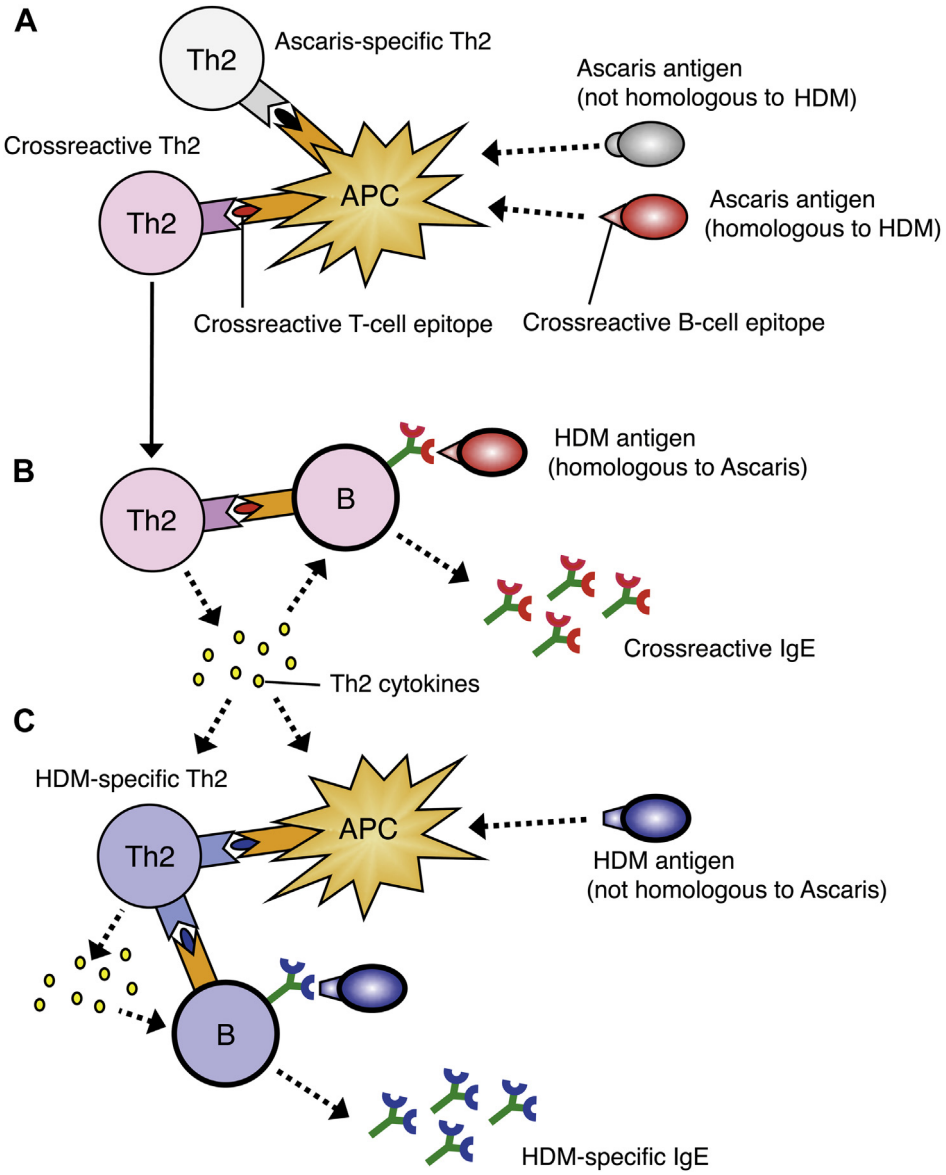

Fig. 6. Hypothetical mechanisms behind the induction of HDM-specific IgE in Ascaris-preimmunized, HDM-inhaled mice. (**A**). Ascaris immunization induces the differentiation of Ascaris-specific Th2 cells, which contain a population reactive to the T-cell epitopes of HDM Ags homologous to Ascaris Ags. (**B**). Th2 cells cross-reactive to HDM and Ascaris Ags produce Th2 cytokines and induce an IgE class-switch in B cells, which recognize B-cell epitopes on HDM Ags containing the cross-reactive T-cell epitopes, upon airway exposure to HDM Ags. (**C**). The Th2 cytokine milieu facilitates the differentiation of Th2 cells specific to non-homologous HDM Ags, which induces the production of IgE specific to non-homologous HDM Ags. HDM-derived adjuvants and epithelial Th2-inducing cytokines, which could be released in airway epithelia upon exposure to HDM, may also contribute to the promotion of Th2 differentiation and IgE production. *APC*, antigen presenting cell.

induction of cross-reactive Abs and Th cells. A recent study involving another murine model demonstrated that the infection of mice with an intestinal nematode *Heligmosomoides polygyrus* induced IgE against some allergen components from HDM, cockroaches, and pollen, and the infected mice developed positive skin reactions upon injection of the allergens.[25]

Various components of allergen sources or house dust, including enzymes, TLR ligands, and lipids, have been reported to act as Th2 adjuvants in the promotion of allergic sensitization.[19,20,26–32] The present study suggested another possible mechanism in an animal model that is dependent on the characteristics of Ags for the promotion of allergic sensitization to HDM in Ascaris-sensitized individuals, which is based on the cross-reactivity of HDM and Ascaris Ags.

Acknowledgments

We thank Michiyo Matsumoto for her secretarial assistance and Hiroshi Kawai, Toyoko Hidano, and Takatoshi Kuhara for animal care. This study was supported by Ministry of Education, Culture, Sports, Science and Technology (MEXT)-supported Program for Strategic Research Foundation at Private Universities (S1101010).

Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

MS: experimental design, data acquisition, analysis, interpretation, drafting, and revising; MH and SK: data acquisition; SI, TN, HH, KA, HO, and KO: conception; TT: conception, experimental design, interpretation, drafting, and revising.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.alit.2015.07.003.

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