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Notch Ligand Delta-Like 4 Regulates Development and Pathogenesis of Allergic Airway Responses by Modulating IL-2 Production and Th2 Immunity

Sihyug Jang, Matthew Schaller, Aaron A. Berlin, and Nicholas W. Lukacs

Activation of the canonical Notch pathways has been implicated in Th cell differentiation, but the role of specific Notch ligands in Th2-mediated allergic airway responses has not been completely elucidated. In this study, we show that delta-like ligand 4 (Dll4) was upregulated on dendritic cells in response to cockroach allergen. Blocking Dll4 in vivo during either the primary or secondary response enhanced allergen-induced pathogenic consequences including airway hyperresponsiveness and mucus production via increased Th2 cytokines. In vitro assays demonstrated that Dll4 regulates IL-2 in T cells from established Th2 responses as well as during primary stimulation. Notably, Dll4 blockade during the primary, but not the secondary, response increased IL-2 levels in lung and lymph node of allergic mice. The in vivo neutralization of Dll4 was associated with increased expansion and decreased apoptosis during the primary allergen sensitization. Moreover, Dll4-mediated Notch activation of T cells during primary stimulation in vitro increased apoptosis during the contraction/resting phase of the response, which could be rescued by exogenous IL-2. Consistent with the role for Dll4-mediated IL-2 regulation in overall T cell function, the frequency of IL-4-producing cells was also significantly altered by Dll4 both in vivo and in vitro. These data demonstrate a regulatory role of Dll4 both in initial Th2 differentiation and in Th2 cytokine production in established allergic responses. The Journal of Immunology, 2010, 185: 5835–5844.
Materials and Methods

Mice

BALB/c and 4get mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal work was performed in accordance with the University of Michigan Committee on Use and Care of Animals policy.

Airway response

Airway hyperreactivity was assessed as previously described using direct ventilation methodology and airway resistance measurements (31). Briefly, mice were anesthetized with sodium pentobarbital, intubated via cannula- tion of the trachea, and ventilated with a ventilator (0.3-ml tidal volume; 120 breaths/min). Airway hyperresponsiveness (AHR) was measured using a Buxco (Wilmington, NC) mouse plethysmograph and software for calculation of the measurements. After baseline measurements, mice were injected i.v. with 7.5 µg methacholine (Sigma-Aldrich, St. Louis, MO), and the peak airway resistance was recorded as a measure of AHR.

Cockroach Ag model

Cockroach Ag (CRA) sensitization was performed as previously described (32, 33). Briefly, female mice, 6–8 wk of age, were sensitized with a 1:1 mixture of cockroach allergen extract (CRA, Hollister-Stier, Spokane, WA) and IFA (Sigma-Aldrich), both s.c. and i.p. on day 0. This cockroach al- lergen is a skin test/immunotherapy-grade preparation that has very little endotoxin contamination (<10 ng/ml). At day 14, mice were locally sensitized by intranasal challenge of CRA followed by intratracheal challenges on day 19.

Generation of bone marrow-derived dendritic cells

For generation of bone marrow-derived dendritic cells (BMDCs), after depletion of erythrocytes with lysis buffer, bone marrow cells were seeded in T-150 tissue culture flasks at 5 × 10^6 cells/ml in RPMI 1640-based complete medium with GM-CSF 20 ng/ml (R&D Systems, Minneapolis, MN). On day 3, GM-CSF was supplemented into cultured cells again. Six days later, loosely adherent cells were collected and incubated with anti-CD11c coupled to magnetic beads for positive selection of conventional DCs from the GM-CSF cultures using a magnetic column (Miltenyi Biotec, Auburn, CA).

Anti-Dll4 Ab and in vivo administration

Rabbit anti-murine dll4 Abs were prepared by multiple-site immunization of New Zealand White rabbits with recombinant murine dll4 (R&D Systems, Rochester, MN) and specificity verified as previously described from our laboratory (26). Briefly, polyclonal Abs were titered by direct ELISA against dll4 coated onto 96-well plates and titered at 10^-7 with no cross-reactivity to the other Notch ligands. Ab specificity was verified by immunofluorescent staining and flow cytometry of Notch ligand-expressing OP9 stably transfected with the five different Notch ligands dll1, dll3, dll4, and lagi. 1,2. Only dll4-expressing cell lines were positive for staining using our polyclonal Ab. In vivo administration used 0.5 ml serum from preimmunized orDll4-immunized rabbits as previously described (26) at the time points indicated.

In vitro CD4+ T cell culture

CD4+ splenic T cells were purified by negative selection using CD4+ T Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's recommendations. Purified CD4+ cells were activated with plate-bound anti-CD3 and anti-CD28 Abs (2 µg/ml). Plate-bound recombinant Dll4 was used at a final concentration of 2.5 µg/ml. Th2 conditions included IL-4 (10 ng/ml) and anti-IFN-γ (10 µg/ml). Recombinant proteins (Dll4, IL-4, and IL-2) were purchased from R&D Systems (Minneapolis, MN). Anti-IFN-γ, anti-CD3, and anti-CD28 Abs were purchased from eBioscience (San Diego, CA).

Protein assays

Single-cell suspensions of lung-draining lymph nodes were plated at a concentration of 5 × 10^6 cells/ml onto a 96-well plate and restimulated with CRAs (3 µg/ml) for 48 h, and supernatant was harvested for cytokine determination. Cytokines were quantified using a Bio-Plex bead-based (Luminex) cytokine assay purchased from Bio-Rad Laboratories (Hercules, CA). For detection of IL-2, ELISA (eBioscience) was used according to the manufacturer’s recommendations.

Histology and RT-PCR

Right lobes from infected mice were removed, fixed in 10% formalin, and stained with periodic acid-Schiff (PAS) to detect mucus production. Total RNA was isolated from lower-left lobes of lungs using TRIzol (Invitrogen, Carlsbad, CA). Real-time PCR was performed on cDNA using primers. Primers and probes used for the detection of mRNA in lung samples were determined using predeveloped primer/probe sets (PE Biosystems, Foster City, CA). Murine GAPDH (PE Biosystems) was used as an internal control for quantification of the total amount of mRNA used in the reaction. Results are normalized to GAPDH expression and are presented as the folds increase in mRNA expression over the naive mouse group. The SYBR primer sets for Dll1, Dll4, Jagged1, and Jagged2 were purchased from Sigma- Aldrich and were described previously (19). For comparisons, DCs without outliers were assigned an arbitrary value of 1.

Flow cytometry

Lungs and lymph nodes were harvested from mice. They were digested with collagenase and dispersed to obtain single-cell suspensions. RBCs were lysed and the remaining cells spun down and resuspended in PBS containing 1% BSA. Cells were Fc-blocked for 10 min. Cells were stained with the mAbs anti-Dll4 (HMD4-1) from Biologend (San Diego, CA), anti-CD45 (30-F11), CD4 (RM4-5), CD8 (53-6.7), CD69 (H.2F3), and CD11c (N418), CD11b (M170), and PDCA (eBio129c) (all from eBioscience) and analyzed by LSR II flow cytometer (Becton-Dickinson, Franklin Lakes, NJ) using FlowJo software (Tree Star, Ashland, OR). The absolute number of each cell type was determined by multiplying the percentage by total cell number isolated from each organ. For apoptosis staining of T cells, cells were collected on the indicated day and stained with annexin V and 7-AAD (eBioscience). CFSE staining was used by standard protocol with 9 µM incubated with 4 × 10^6 cells/ml.

Statistics

Statistical significance was determined by one-way ANOVA with a Newman–Keuls posttest. Significant differences were regarded as p < 0.05.

Results

Cockroach allergen induces Dll4 in DCs and regulates Th2 cytokine production

To investigate whether the expression of Notch ligands is induced in DCs in response to cockroach allergen, CD11c+ BMDCs were stimulated with cockroach allergen, and the expression of Notch ligands was analyzed by real-time PCR. Cockroach allergen induced the expression of several Notch ligands including Dll1, Dll4, and Jagged1, but the expression of Dll4 was increased to a much higher extent compared with that of the other ligands (Fig. 1A). In addition, we have also used flow cytometric analysis to determine whether DCs in the lungs of allergen-challenged mice (24 h post-allergen challenge) express Dll4. The data in Fig. 1B indicate that when we specifically examine the CD11b+CD11c+ and CD11c+PDCA+ cell populations, there is a significant increase in Dll4 expression based on mean fluorescence intensity compared with that of cells from lungs of naive mice with the most profound effect observed on the CD11b+CD11c+ subset. Recently, it has been shown that Notch signaling modulates T cell cytokine production. To determine whether Dll4 alters the capacity of T cells for production of Th2 cytokines, lymph node cells were isolated from allergen-challenged mice and restimulated with CD3/CD28 either in the presence or absence of Dll4 ligand-coated plates. As shown in Fig. 1C, Th2 cytokines IL-4, IL-5, and IL-13 were significantly reduced in the presence of Dll4, confirming earlier reports that demonstrate regulation of Th2 cytokines by Dll4 (26). Notably, in the current studies IL-2 production was also decreased in the presence of Dll4 (Fig. 1C). Also of note, when we examined IFN-γ production, it was also reduced in the cells from this Th2-skewed environment. We next examined the effect of Dll4 on the capacity of CD4+ T cells to produce cytokines in Th2-biased conditions using a primary skewing culture. Similar to what was observed in lymph node cells from allergic mice, Th2 cytokines and IL-2 were significantly reduced in the presence of Dll4 under Th2-skewed conditions (Fig. 1D). Thus, Dll4 appears to modulate
Th2 cytokines established under allergen-induced responses as well as in primary Th2 skewing conditions.

To address whether the changes that we observed in cells from animals fully sensitized and skewed toward a Th2 allergic response manifested themselves early during the sensitization process, immunized mice given anti-Dll4 Ab prior to sensitization were assessed for Th2 cytokine levels at day 7 postimmunization. To perform these studies, splenocytes were isolated and rechallenged with allergen in vitro, and cytokines were measured from supernatants of the in vitro cultures (Fig. 1). The data indicate that in the anti-Dll4–treated group, there was a significant increase in Th2 cytokines, including IL-4, IL-5, and IL-13, with no activation of IFN-γ, which is typical with sensitization with this allergen preparation. In addition to the Th2 cytokine regulation, when IL-2 production was examined at this early time point, the anti-Dll4–treated animals had a significant increase in allergen-specific IL-2 production. Thus, these data support an early alteration in the skewing of T cells in vivo and further linked a relationship with IL-2.

Anti-Dll4 Ab treatment during the primary response exacerbates allergic lung disease

Because Dll4 suppressed Th2 cytokine production, the role of Dll4 in the development of allergic disease was investigated using an established model of cockroach allergen-induced airway disease. To address whether Dll4 has a role in the development of allergic AHR during the primary response, mice were injected with anti-Dll4 Abs 2.5 h prior to primary cockroach allergen sensitization. Two weeks after immunization, the mice were locally sensitized with cockroach allergen through an intranasal route. Five days later, the mice were challenged with cockroach allergen through an intratracheal route, and 24 h later, lungs and lymph nodes were harvested for histology and cytokine expression. Treatment with anti-Dll4 Abs only prior to primary sensitization resulted in a significantly enhanced AHR compared with that of the control Ab-treated group (Fig. 2A). Moreover, histological examination of lungs showed increased mucus production in anti-Dll4 Ab-treated mice compared with that of control Ab-treated mice (Fig. 2B). Examination of the mucus-associated genes, gob5 and muc5ac, in the lungs from anti-Dll4–treated mice demonstrated a substantial increase in expression of those genes compared with that of the control Ab-treated mice (Fig. 2C). To assess the immunologic mechanism for anti-Dll4 Ab-induced exacerbation of allergy, we examined the Th2 cytokines in draining lymph nodes. Lymph node cells were restimulated with cockroach allergen for 48 h, and supernatant was harvested for cytokine analyses. Th2 cytokines, IL-5 and IL-13, from cockroach allergen-stimulated T cells were significantly increased in anti-Dll4 Ab-treated mice compared with that in control Ab-treated mice (Fig. 2D). These data suggest that aggravated clinical disease in anti-Dll4 Ab-treated mice is associated with enhancement of type 2 cytokines.

Anti-Dll4 Ab treatment during the secondary response increases severity of allergic lung disease

Next, to investigate whether Dll4 alters development of allergic response during the secondary response, the anti-Dll4 or control Ab
was injected i.p. 2.5 h prior to the final allergen challenge. Similar to what was observed in the mice treated with anti-Dll4 Abs only during the primary response, increased airway hyperactivity (Fig. 3A) and mucus overproduction histologically (Fig. 3B) and enhancement of muc5ac and gob5 gene expression (Fig. 3C) in the lungs from anti-Dll4 Ab-treated mice were observed compared with that in control Ab-treated mice. Allergen restimulated lymph nodes from anti-Dll4 Ab-treated mice had increased IL-4, IL-5, and IL-13 cytokines from cockroach allergen-specific T cells compared with that in control Ab-treated mice (Fig. 3D). Overall, data thus far suggested that Dll4 modulates type 2 immunity during allergenic responses, with subsequent regulation of pathophysiologic changes in the lung during the allergic reaction.

**FIGURE 3.** Blockade of Dll4 signaling during secondary response enhances cockroach allergen-induced AHR and mucus production in the lung. A, Airway responses were measured in control Abs and anti-Dll4 Ab-treated mice after one dose of methacholine. Data are represented as mean airway resistance in H2O/ml/s ± SEM. B, Lungs were taken 1 d after allergen challenge and were stained with PAS (original magnification ×100). C, One day after allergen challenge, lungs were isolated and assayed for gob5 and muc5ac expression by performing real-time PCR. D, Analysis of cytokines from allergen-restimulated lung-draining lymph nodes assessed by Bio-Plex assay. Data represent mean ± SEM from five mice/group, and the experiment was repeated with five mice/group and demonstrated a similar response. *p < 0.05.

Dll4 signaling blockade during both primary and secondary responses alters the number of activated T cells in lungs and lymph nodes

To characterize further the effect of the Dll4 blockade, T cells in lungs and draining lymph nodes of mice 1 d after final allergen challenge were analyzed by flow cytometry. Regardless of timing of Dll4 blockade, significantly more CD4+ and CD8+ T cells expressing the early activation marker CD69 were found in the lungs from mice treated with anti-Dll4 Abs than in lungs from mice treated with control Abs (Fig. 4). We also quantified the number of T cell subsets in the draining lymph nodes of anti-Dll4 Ab-treated mice. Similar to lungs, lymph node FACS data showed that there was a tendency of an increase of activated T cell subsets in anti-Dll4 Ab-treated mice compared with the control group, but...
FIGURE 4. The absence of Dll4 alters the number of activated T cells in the lungs and lymph nodes of allergen-challenged mice. Flow cytometry was performed using lung and lymph node digests taken from 1 d after allergen challenge. A, The total number of CD69+ T cell subsets in the lungs and lymph nodes from mice blocked Dll4 signaling during primary response. B, The total number of CD69+ T cell subsets in the lungs and lymph nodes from mice blocked Dll4 signaling during secondary response. Data represents mean ± SEM from five mice/group. *p < 0.05; **p < 0.01.

this increase did not reach statistical significance except with CD8+CD69+ T cells from mice treated with Dll4 during the secondary response (Fig. 4B). These data further imply that anti-Dll4 blockade notably alters the immune environment of the lung and the number of activated lymphocytes.

To address specifically whether Dll4 altered Th2 cytokine-producing T cell subsets, we used 4get mice in which IL-4 mRNA expression is “reported” by enhanced GFP through a bicistronic reporter gene construct. This reporter construct, although not fully representative of IL-4 levels, identifies IL-4–competent cells that are transcriptionally poised for IL-4 and other Th2 cytokine production and therefore can be used as a readout of cells that have transitioned toward Th2 cell differentiation (34). We performed flow cytometry analysis on lung and lymph node cells to investigate IL-4–expressing Th2 cells in the mice treated with either control Abs or anti-Dll4 Abs as indicated by the flow cytometry (Fig. 5). There were significantly more CD4+ T/IL-4–expressing (GFP+) cells in the lungs (Fig. 5A) and lymph nodes (Fig. 5B) of anti-Dll4 Ab-treated 4get mice compared with that in control Ab-treated mice regardless of Dll4 blockade timing, primary or secondary (Fig. 5). Very few CD8+/IL-4–expressing cells were detected indicating that CD4+ T cells are the main source of IL-4. These data confirm that anti-Dll4 Ab treatment enhances Th2 immunity by increasing the frequency of Th2 type cells during allergic AHR.

Dll4 regulates IL-2 production, T cell apoptosis, and Th2 cell frequency

The mechanism of Dll4 function on the frequency of IL-2–producing cells may result from more cell proliferation and/or enhancement of Th2 differentiation. IL-2 has been shown to be a T cell growth factor and more recently demonstrated to enhance directly Th2 differentiation. Notably, as shown in Fig. 1, Dll4 suppressed IL-2 production from T cells. Therefore, we analyzed the level of IL-2 in the lung and in restimulated lymph node cells with the hypothesis that Dll4 may be altering the responses through IL-2. Of note, we observed an increase of IL-2 levels, which had been previously treated with Dll4 ligand (Fig. 7A, 7B). To determine whether the Dll4-mediated decrease of IL-2 during
rescue the rest phase-associated apoptosis, the frequency of IL-4–producing cells at day 5 of culture (Fig. 7D, 7E). Thus, these final studies suggest that the defect in Th2 cell development may include a consequence of Dll4 regulation of IL-2 production.

Discussion

Although Notch signals have been shown to be important in early T cell progenitor development (35–37), increasing evidence suggests that Notch signaling is required for Th cell activation and differentiation. However, a conflict of data on the specific role of the Notch system during T cell activation appears to depend upon the ligand used and the immune conditions being examined (19, 21, 22, 25, 26, 29, 38–40). In vivo or in vitro treatment with a γ-secretase inhibitor, an enzyme regulating signaling through all Notch receptors, can lead to inhibition of Th1 response through the blockade of T-bet expression (20). More specific to Th2 biology, the genetic ablation of Notch signaling in T cells results in a reduction in Th2 but not Th1 responses (22–24). In an OVA-induced murine model of allergic lung disease, γ-secretase inhibitor administration was shown to inhibit asthma-like phenotypes, which was accompanied by an increase of Th1 cytokines and decrease of Th2 cytokines (28), supporting the genetic ablation studies. In contrast, the current research indicated that Dll4 blockade aggravated the pathological features of allergic lung disease, including AHR and mucus production, no matter at what phase of disease the blocking Ab was administered. Moreover, lymph node cytokine data showed that Dll4 blockade increased Th2 cytokine production, which is a key factor for development of allergic diseases. Consistent with cytokine data, neutralization of Dll4 signaling during both the primary and secondary response increased the frequency of IL-4+/CD4+ T cells in the lung and lymph nodes of 4get mice and also altered IL-4+ T cell development in primary activation using in vitro studies. Thus, these latter data appear to be contrary to previous findings that Notch1/Notch2-deficient T cells and dominant-negative MAML transgenic T cells have impairment in Th2 cell differentiation (22, 24). However, due to the complexity of potential Notch receptor–ligand interactions as well as the presence of multiple receptors and ligands on cells, it would be reasonable to hypothesize that Notch signaling would result in a different outcome in different settings (38). This may be a consequence of differential Notch ligand signaling and/or use of different receptors (38, 41–44). Alternatively, it may also be possible that Notch can elicit non-canonical pathways that are independent of MAML/RBPJk transcriptional regulation (39).

Our data suggest that Dll4 Notch signaling suppresses Th2 immune responses as previously indicated in independent studies (25, 26, 29). Another interesting finding in these studies was that Dll4 affects both naive T cell differentiation into Th2 cells as well as already differentiated Th2 cell responses. Although the exact mechanism by which Dll4 inhibits Th2 cell responses remains to be elucidated, the data from the current set of studies along with previous findings suggest that the regulation results from two possible consequences. The first consequence of Dll4 signaling relates to its ability to regulate IL-2 mRNA and protein production and alter initial expansion of the allergen-specific T cells as well as their survival, but which would also reduce their development into Th2 cytokine-producing effector cells. These latter mechanisms of IL-2–induced Th2 cell differentiation have previously been established, and it may be this early event during T cell differentiation that Dll4 is regulating (45–48). However, the observation of decreased IL-2 was not observed by Sun et al. (25) when they used Dll4-transduced DCs from IL-12−/− mice to stimulate OVA TCR transgenic T cells followed by an ionomycin/PMA rechallenge to examine IL-2 frequency by flow cytometry.
whereas the current studies examined IL-2 mRNA and protein. It may be that Dll4 needs to be present upon restimulation to maintain the altered IL-2 phenotype, as the former study did not provide Dll4 during the subsequent PMA restimulation. The second consequence of Dll4 activation would relate to the ability of Dll4 to alter Th2 cytokine production in previously skewed T cells as a result from a direct effect on important transcription factors such as GATA-3, as previously demonstrated using ectopic expression of Dll1 and Dll4 in IL-12/2 DCs (25). The T cells that are already skewed in the challenged mice would be less dependent upon IL-2, which is needed for their development and for stabilization of IL-4 gene expression (46), and more dependent upon the regulation of critical transcription factors, such as GATA-3. Notably, one target of Notch activation in T cell development is PU.1 (49–51), which has a regulatory role in Th2 cell differentiation (46, 47). More recently, Dll4 has been shown to enhance RORγt as well as IL-17 when cells are skewed toward a Th17 phenotype (54). While we did not observe any increase of Foxp3+ cells by flow cytometric analysis in our in vitro analyses (data not shown), it has also been demonstrated that Notch signaling and specific ligands can promote regulatory T cell development under TGF-β–mediated skewing conditions (55–57). Thus, the resulting immune regulation induced by Dll4 likely depends upon the immune environment in which the ligand functions, the state of the activated T cell (primary versus secondary), and the context of activation.

The expression of Dll proteins by DCs is induced by several stimuli that depend upon MyD88-associated TLR signals, including bacterial, viral, and fungal pathogenic stimuli (25, 26, 54, 58, 59). Notably, in contrast with OVA, which does not activate DCs via a MyD88 pathway, it has been shown that German cockroach frass contains a TLR2 agonist that regulates the intensity of the immune response (60, 61). Furthermore, TLR2 agonists can directly facilitate induction of the Dll4, but not Jagged1, on DCs (62, 63). The data in the current studies demonstrate that CRA induced significant levels of Dll4 mRNA expression in vitro and Dll4 protein on DCs in lungs of allergic mice. The suppressive effect of allergen-induced Dll4 on Th2 immunity may be part of a negative feedback regulatory mechanism that limits the exaggerated Th2 immune responses in the host and therefore modulates...
detrimental immune activation pathways. Of note, a recent study found that regulatory T cells expressed up to 20-fold more Dll4 than did effector T cells and may contribute to the regulation in allergic airway disease (64). Perhaps the responses observed in the current studies may be related to similar signals induced by Dll4. Subsequent studies will further examine these signaling pathways.

Most novel in these studies, however, is the fact that Dll4 regulates IL-2 and subsequent T cell survival during the activation of T cells leading to T cell apoptosis during the rest or contraction stage. These latter observations appeared to be related to the reduced IL-2 production from Dll4-pretreated T cells, as the cells could be rescued during the contraction phase by addition of exogenous IL-2. This latter mechanism was manifested in the proliferative and apoptotic responses that were altered by the presence and/or neutralization of Dll4, and therefore, the Th2 development may be a reflection of allergen-specific T cell expansion and survival. Although most studies have identified that Notch-mediated pathways have led to T cell survival especially during thymic development and in transformed T cell populations (16, 65–69), there also appears to be activation of regulatory pathways (70). Examination of the IL-2 promoter in silico did not identify a consensus binding site for conical CSL (RBPJk), the transcriptional binding partner for the intracellular domain of Notch, and therefore the regulation is likely indirect. More specifically, studies have shown effects of Notch signaling with up-
regulation of GRAIL (gene related to anergy in lymphocytes) in CD4+ T cells, with effects on other regulatory E3 ubiquitin ligases, such as Cbl-b and Itch (71). Furthermore, it was identified that deltex1, a downstream target of Notch activation, leads to development of anergic T cells related to increased expression of Cbl-b via a NFAT-mediated activation pathway (72). As Cbl-b is a known regulator of T cell activation (73, 75), it may be these latter pathways that have a most significant role on IL-2 regulation that alter T cell development mediated by DI4.

This study presents data that suggest that DI4 has a relevant role for Th2 cell regulation and further highlights the complexity of Notch ligand-associated activation during development of immune responses. This mechanism may be informative in terms of new therapeutic opportunities that could arise from the manipulation of Notch pathway signaling and/or specific ligand blockade in developing and established allergic diseases.

Disclosures

The authors have no financial conflicts of interest.

References


