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**Induction and Maintenance of Airway Responsiveness to Allergen Challenge Are Determined at the Age of Initial Sensitization**

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Age is an important factor in determining the quantity and quality of immune responses when challenged with allergen. In a model of allergen-induced airway hyperresponsiveness and inflammation, where the sensitization phase and challenge phases can be dissociated in time, we examined the impact of age on these two phases. Sensitization of young mice (1–20 wk), but not older animals (30–40 wk), led to the development of airway hyperresponsiveness, airway eosinophilia, Th2 cytokine responses, and allergen-specific IgE, regardless of the age when the challenge phase was conducted. Thus, age at the time of initial sensitization was shown to be the critical factor dictating the nature of the response to later allergen challenge, as older mice remained responsive to allergen challenge if sensitized at a young age. These effects were shown to be mediated by lung T cells from sensitized young mice. Moreover, the failure of old sensitized mice to mediate these effects was shown not to be the result of active suppression of the responses. These data define the importance of age at initial allergen exposure in dictating subsequent responses in the lung when exposed to allergen and may help to define why asthma, even in adults, is most often initiated in early childhood. *The Journal of Immunology*, 2004, 173: 1298–1306.

Asthma is characterized as an inflammatory disease of the airways, with significant accumulation of eosinophils, lymphocytes, and other cell types (1). It is increasingly apparent that the disease features in children differ from those in adults (2). Further, the interplay between the environment and the developing immune system may have more profound effects than when these interactions occur in the context of a developed immune response (3, 4). Differences in the degree of atopy or IgE levels, the type and extent of inflammatory response, the incidence of viral respiratory tract infection, and structural changes all serve to distinguish childhood and adult populations. Importantly, a large percentage of adult asthmatics can trace the origins of their asthma to their childhood years, at a time when allergen was first encountered (5).

A number of studies have suggested that immune responses may be more vigorous during the earlier years and that Th2-like responses may dominate during this period (6, 7). Given the importance of early allergen exposure in the development and persistence of asthma, this might suggest that the younger host may not only develop a more polarized T cell response, but that memory T cell responses are generated more vigorously at earlier ages. In the mouse there is support for these ideas based on the demonstration of defective generation of memory T cells in older mice (8) and that naive CD4 T cell function also dramatically declines with age (9). The responses to various stimuli (10, 11) and the rate of repair (12–14) may also show a degree of age dependency.

We hypothesized that the induction and maintenance of allergic airway hyperresponsiveness (AHR) are related to the age at initial allergic sensitization. These differences could, if translated to human asthma, significantly affect our thinking about intervention in and particularly prevention of asthma. Critical issues lie not only in comparing allergen exposure at early time points vs later in life, but to define what extent does the age at initial exposure influence responses to subsequent exposures at a later date.

In the present study we examined the age-dependent consequences of allergen sensitization and challenge. Mice were sensitized and challenged at different ages, and the extent of airway inflammation and AHR were examined. The responses in young mice far exceeded those in older mice; in fact, older mice appeared to be refractory to allergic sensitization and from developing an allergic inflammatory response in the lung or AHR. Further analysis revealed that this was not due to the development of negative regulatory cells or to an incapacity to respond to airway challenge. Instead, the response appeared to be dictated by the age at the time of initial allergen sensitization, without concomitant exposure in the lungs.

**Materials and Methods**

**Animals**

Pathogen-free, female BALB/c BYJ mice of 1–40 wk were bred at National Jewish Medical and Research Center and maintained on an OVA-free diet. All studies were conducted under institutional-approved guidelines.

**Sensitization and challenge**

Sensitization to OVA was conducted with two i.p. injections of 20 μg of OVA (grade V; Sigma-Aldrich, St. Louis, MO) emulsified in 2.25 mg of

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alum hydroxide (AlumInjject; Pierce, Rockford, IL) in a total volume of 100 μl conducted 14 days apart. Aerosol challenges were conducted for 20 min on 3 consecutive days with 1% OVA in PBS using an ultrasonic nebulizer (AeroSonic ultrasonic nebulizer; DeVilbiss, Somerset, PA). Mice were initially sensitized at 1, 4, 8, 20, 30, and 40 wk of age. The corresponding challenges were initiated at 5, 8, 12, 24, 34, and 44 wk of age, respectively, unless otherwise stated. The first day of challenge always began 4 wk after the initial sensitization injection. Endotoxin levels in the OVA solution were assayed and were shown to be below 12.5 endotoxin U/mg protein.

**Cell preparation and culture**

Lung T cells were isolated by collagenase digestion of the lungs and were enriched using nylon wool columns (15). Briefly, lungs were perfused via the right ventricle with warm (37°C) calcium- and magnesium-free HBSS containing 10% heat-inactivated FCS (Gemini, Woodland, CA), 0.6 mM EDTA, 100 U/ml penicillin, and 100 μg/ml streptomycin. Lungs were removed and minced into small pieces (300 μm). Minced tissue, suspended in 5 ml of HBSS containing 175 U/ml collagenase (type IA; Sigma-Aldrich), 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin, was incubated for 60 min in an orbital shaker at 37°C. The digested lungs were sheared with a 20-gauge needle and filtered through 45- and 15-μm pore size filters. Total lung cells, after lysis of RBC with 10 ml of RBC lysis buffer (Sigma-Aldrich) at 37°C for 15 min, were counted (Counter Count; Coulter, Hialeah, FL). Lung mononuclear cells were separated by Ficoll-Hypaque gradient centrifugation (Lymphocyte Separation Medium; Organon Teknika, Durham, NC). Cells were washed, counted, and resuspended in RPMI 1640 (Cellgro; Mediatech, Herndon, VA) tissue culture medium containing FCS (10%), 1-glutamine (5 mM), 2-ME (2 mM), HEPES buffer (15 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml; all from Invitrogen Life Technologies, Grand Island, NY). The cell suspension was further enriched for T cells by passage through a nylon wool column, resulting in a population of cells that contained 99% CD3⁺ T cells. Examination of CD4⁺ and CD8⁺ T cell numbers before adoptive transfer showed little difference between sensitized young/challenged young vs sensitized old/challenged old mice. In addition, CD4⁺ and CD8⁺ T cell numbers were similar in sensitized young/challenged old and sensitized old/challenged old mice.

**Adoptive transfer**

For adoptive transfer, 5 × 10⁶ cells were injected i.v. into each recipient mouse. Immediately after adoptive transfer, nonsensitized recipient mice received aerosol allergen challenges (or PBS) for 20 min on 6 consecutive days; previously sensitized recipient mice received 3 consecutive days of aerosolized 1% OVA in PBS (or PBS alone).

**Measurement of airway responsiveness**

Measurement of cytokines in BAL fluid

Cytokine levels in the BAL fluid were measured by ELISA using commercial kits for IL-4, IL-5, IL-10, and IFN-γ (BD Pharmingen, San Diego, CA). ELISAs were performed according to the manufacturer’s directions. The limits of detection were 4 pg/ml for IL-4 and IL-5, and 10 pg/ml for IL-10 and IFN-γ.

**Statistical analysis**

ANOVA was used to determine the levels of difference between all groups. Comparisons for all pairs were performed by Tukey-Kramer highest significance difference test. The p value for significance was set at 0.05. Values for all measurements were expressed as the mean ± SEM.

**Results**

**Induction of allergic airway responsiveness is related to age at initial sensitization**

Age at sensitization is associated with AHR and airway inflammation. To initially address the issue of how age may regulate the development of allergic responses in the lung, mice were sensitized twice over a 2-wk period, beginning at different ages, and then challenged to allergen via the airways 2 wk later. Airway function to inhaled MCh was measured in two independent ways as described in Materials and Methods: by invasive plethysmography, monitoring RL and Cdyn, or by whole body plethysmography monitoring Penh in spontaneously breathing, nonanesthetized mice.

As shown in Fig. 1, following changes in RL (Fig. 1A) or Cdyn (Fig. 1B) in response to inhaled MCh, there was an association between airway responsiveness to allergen challenge and age at the time of initial sensitization. Mice initially sensitized at 1, 4, and 8 wk of age developed increased RL (Fig. 1A) and decreased Cdyn (Fig. 1B) throughout the MCh dose-response curve. The response in 20-wk-old mice was intermediate, whereas 40-wk-old mice failed to develop significant alterations in lung resistance or dynamic compliance at any MCh concentration. Because changes in RL may reflect alterations in large or central airway function, whereas Cdyn may be linked to smaller or peripheral airway function (19), the age-dependent changes were evident at both levels of the airways. In the absence of sensitization, allergen challenge alone resulted in small increases in RL or decreases in Cdyn after MCh inhalation at all ages.

Lung function was also monitored in a noninvasive manner using whole body plethysmography. Virtually identical patterns of responsiveness to inhaled MCh were seen when Penh was monitored directly in conscious, spontaneously breathing animals (data not shown). The same age-dependent pattern was maintained from the younger to the older mouse.

After sensitization and challenge of BALB/c mice at 8 wk of age, a marked increase in BAL eosinophilia was detected, peaking 48 h after the last challenge (20). BAL fluid was obtained to assess the extent of infiltration in mice sensitized at different ages. As shown in Fig. 1C, younger mice developed increases in total cell numbers and marked increases in BAL eosinophil numbers, whereas the changes in 20-wk-old mice were less dramatic, and the numbers were lowest in 40-wk-old mice. Increases in lymphocyte numbers showed a similar pattern. BAL from mice challenged alone at any age essentially contained macrophages (>97% of total cells).

**Age-associated changes in cytokine levels**

After allergen sensitization and challenge of BALB/c mice, increases in BAL levels of Th2 cytokines, IL-4 and IL-5, and a reduction in the levels of IL-10 and IFN-γ have been demonstrated (21, 22). When BAL cytokine levels were assessed in the different groups of mice, this pattern of cytokine responses also appeared to be age associated (Fig. 2). This was most obvious with levels of IL-4. Challenged-only mice at the various ages had an IL-4 level of 17 ± 3 pg/ml.
Mice sensitized at the younger ages had the highest levels of IL-4, which progressively decreased with increasing age at sensitization. These levels more than doubled in sensitized and challenged mice at all except the oldest groups. Challenged-only mice at the different ages had an IL-5 level of 417 pg/ml, and all except the oldest group had a significant increase in BAL IL-5 levels after sensitization and challenge. IL-10 levels in challenged-only mice were 1210 ± 230 pg/ml over the entire age span. These levels were significantly reduced after sensitization and challenge in all groups except for the oldest mice. A similar pattern was observed when IFN-γ levels were compared; levels of IFN-γ were reduced at all ages compared with challenged-only mice. Levels of IFN-γ were highest in the sensitized and challenged older mice.

Age-associated changes in allergen-specific Ab levels. Allergen sensitization and challenge are also associated with increases in total IgE and allergen-specific IgE and IgG1 in serum. In nonsensitized, but challenged, mice, total IgE levels were similar at all ages (38 ± 6 ng/ml). In these mice, no OVA-specific IgE, IgG1, or IgG2a could be detected. Sensitization and challenge of mice at the different ages showed a pattern consistent with the results of Th2 cytokine production (Fig. 3). That is, younger mice tended to develop greater levels of OVA-specific IgE and IgG1 (as well as total IgE) compared with the oldest group of mice. In contrast, OVA-specific IgG2a levels were highest in this oldest group, perhaps in keeping with the higher IFN-γ levels found in these mice. Importantly, it suggests that the oldest mice were not unresponsive to allergen sensitization and challenge, but responded in a distinct way.

Influence of age on proliferative responses of lung mononuclear cells. It has previously been shown that cells from younger mice proliferate at a higher rate in response to Ag than cells from older mice (23). As the allergic (Th2) responses in the older mice appeared to be diminished quantitatively and qualitatively from those in the younger mice, we examined allergen-induced proliferative responses, measured as [3H]thymidine incorporation in mononuclear cells isolated from the lungs of mice at the different ages and cultured for 5 days in the presence or the absence of OVA. When
lung mononuclear cells from sensitized and challenged animals were cultured with OVA, the proliferative responses were diminished in the older animals compared with the younger mice (Fig. 4). A similar pattern was observed when cells from sensitized and challenged mice were cultured in medium alone in the absence of OVA. Nevertheless, OVA-induced proliferation was detected in the oldest mice, further establishing that the failure to develop AHR or lung inflammation could not simply be attributed to allergen unresponsiveness.

**Maintenance of airway responsiveness is dependent on the age at initial sensitization.**

*Age at sensitization, but not age at challenge, dictates airway responsiveness.* To this point, the data indicated that sensitized younger mice were capable of developing a more vigorous Th2 response and AHR when exposed to allergen challenge 4 wk later via the airways. These data could not distinguish whether the effects were determined at the time of initial sensitization or reflected differences in allergen recognition in the lung at the time of challenge.

To determine whether age at the time of initial sensitization was the critical factor, we focused on mice initially sensitized at two distinct ages. Mice were initially sensitized at either 8 or 30 wk of age, but the challenges were conducted in both groups of mice at the same age, beginning at 34 wk. As shown in Fig. 5A, mice initially sensitized at 30 wk of age developed low levels of AHR when challenged at 34 wk, 2 wk after the last sensitization. However, despite challenge at the same age (34 wk), mice initially sensitized at 8 wk of age still developed significant AHR, similar to completion of sensitization and challenge at a younger age (Fig. 1A). As a control for the time frame used, mice sensitized at 30 wk and challenged 26 wk later showed no significant alteration in airway function (data not shown). These data suggest that not only induction, but maintenance of airway responsiveness, are dictated by the age at initial sensitization. The age at challenge does not appear to influence this responsiveness. Moreover, the findings indicate that the failure to normally develop AHR in mice sensitized at 30 wk and challenged at 34 wk is not due to intrinsic alterations in the airway response to inhaled MCh, because mice of this age, but sensitized at 8 wk of age, remained fully responsive. When C57BL/6 mice were examined in an identical fashion, similar results were observed (data not shown), indicating that these results were not limited to a single strain of mouse.

When BAL fluid was examined in these same animals, the results for airway function were paralleled by the BAL eosinophil numbers. Fig. 5B illustrates that late challenge in mice sensitized at a young age did not affect the marked airway eosinophilia that develops in mice sensitized and challenged at an early age. Mice challenged at 34 wk, but sensitized at 30 wk, showed a much lower increase in BAL eosinophilia.

**T cell-mediated airway responsiveness is dependent on age at initial sensitization.**

**Functional capacity of transferred T cells from mice at different ages into recipients of different ages.** Based on the results presented, the failure of sensitized/challenged older mice to respond appeared not to be intrinsic to the airways themselves, because challenge of older mice sensitized at a young age did not affect the marked airway eosinophilia that develops in mice sensitized and challenged at an early age. One possibility is that the animals fail to generate memory T cells, so that allergen challenge via the airways elicits little, if any response, in particular Th2 responses. A second possibility, based on the cytokine data, is that sensitization of older mice triggers the development of regulatory
T cell responses with increased IL-10 production, and these cells negatively regulate the development of AHR and lung eosinophilia (24, 25). In an attempt to examine this potential for functional differences among T cells after sensitization at different ages, a series of transfer experiments were conducted.

When isolated lung T cells from mice sensitized and challenged at the different ages were transferred into naive recipients, functional differences were detected. Transfer of lung T cells from sensitized young (challenged old) mice into naive young mice before allergen challenge (at 12 wk) conferred the ability to develop AHR (Fig. 6A) and lung eosinophilia (Fig. 6B) when challenged with allergen. Transfer of lung T cells from sensitized old (challenged old) mice into naive young mice before challenge (at 12 wk) failed to induce AHR (Fig. 6A) or airway eosinophilia (Fig. 6B). As a corollary, transfer of lung T cells from sensitized young (challenged old) mice into naive old recipients before challenge (at 34 wk) did lead to the development of AHR (Fig. 7A) and eosinophilia (Fig. 7B), in contrast to the failure of lung T cells from sensitized old (challenged old) mice to do so. This response in the old recipients to T cells from sensitized young donors further supports the integrity of the immune/inflammatory and airway responses in older mice, when appropriate signals were delivered from sensitized young T cells. Together, these data are in keeping with the conclusion that T cells from sensitized older mice fail to convey a memory response to naive mice before airway allergen challenge.

To address whether the isolated lung T cells from sensitized old/challenged old mice were capable of suppressing AHR and eosinophilia, lung T cells were transferred into sensitized recipients before allergen challenge. As shown in Fig. 8, transfer of lung T cells from sensitized old (challenged old) mice into sensitized young recipients still resulted in the full development of AHR (Fig. 8A) and airway eosinophilia (Fig. 8B) when challenged 4 wk later, indicating that T cells from sensitized old (challenged old) mice do not actively suppress the responses, but fail to support their development.

Discussion
Our understanding of the pathogenesis of allergic asthma or allergen-induced AHR and lung eosinophilia continues to evolve. Central to current thinking is that αβ+ CD4+ memory T cells are essential; they initiate and mediate the responses to allergen challenge of sensitized mice through the release of a number of important Th2 cytokines and chemokines (22). Few studies have examined the development of allergen-induced AHR and eosinophilic inflammation in the context of age at the time of first exposure to allergen. The onset of asthma in young children is much more frequent than in older individuals and importantly, when initiated in childhood, persists into adulthood (5). To some extent this has been linked to a Th2 cell polarization in the very young, although there are conflicting data on the existence of a predisposing Th2 imbalance in the young (26) or that asthma is simply a Th1/Th2 imbalance (27). Cord blood CD3+CD4+ T cells may be hypermethylated at CpG and non-CpG sites within the IFN-γ promoter, accounting for lower IFN-γ production in neonates (28).

In the present study we examined the consequences of age at the time of sensitization on the airway response to subsequent airway challenge with allergen. These studies clearly showed that the age...
at the time of initial sensitization to allergen dictates the final outcome; the age at the time of airway exposure to allergen (challenge) was not particularly important to the nature or extent of the response. Thus, sensitized young mice developed marked increases in airway responsiveness to inhaled MCh and a much more prominent airway eosinophilia than did the sensitized older mice when challenged with allergen. In parallel, younger mice appeared to have higher IL-4 levels (and IL-5, to a lesser extent), Ag-specific serum IgE levels, and low IL-10 and IFN-γ production. The association of IL-4 (IL-5), IgE, and lung eosinophilia with age at initial sensitization suggested that sensitization at a young age resulted in a Th2-predominant response. In a number of species, these Th2-like responses have been associated with allergen-induced AHR (29). In contrast to the younger mice, older mice failed to develop an obvious Th2-like response; IL-4, IgE, and airway eosinophilia were not prominent. On the contrary, IL-10 and IFN-γ levels were higher, as were serum levels of Ag-specific IgG2a. However, a vigorous Th1-predominant response was not observed in these older mice, perhaps more in keeping with an overall decrease in T cell-mediated immunity as opposed to strong immune deviation. This was supported by studies of lung T cell proliferation in which Ag-specific proliferative responses were examined and shown to be lower than in the younger mice, confirming the results of other studies in mice (23).

In a similar study, when 4- and 13-wk-old rats were compared, the younger animals had more pronounced airway functional alterations and inflammatory changes, supporting increased susceptibility at a younger age (10). In other reports, aged dogs, rats, rabbits, and mice showed a significant impairment in the generation of Th2-type allergic responses (11, 30–34), as demonstrated in this study. As shown in the present study, it is important to emphasize that the older mice remained responsive to the signals developed as a consequence of earlier sensitization. Thus, mice sensitized at 8 wk of age, but not challenged until 34 wk, still developed the full alterations in lung function to inhaled MCh and the marked accumulation of eosinophils in the airways. This further indicated that target cell responses triggered by inhalational allergen challenge in the lungs of older mice were not simply lost with age, but that maintenance of allergic airway responsiveness was also dependent on age at initial sensitization.

CD4+ T cells appear essential for the development of AHR and lung eosinophilia (1, 29, 35). In fact, we have shown that depletion of CD4+ T cells during the sensitization phase (but not the
challenge phase) prevents the development of AHR and lung eosinophilic inflammation (36). Thus, the allergen sensitization phase appears critical for the generation of memory CD4+ T cells capable of eliciting a polarized Th2 response on subsequent allergen exposure; we found that this response to allergen inhalation in previously sensitized mice can persist for at least 1 year in BALB/c mice (our unpublished observation). This idea was supported by the results of adoptive transfer experiments. In this study T cells isolated from the lungs of sensitized young/challenged young mice conferred the ability of both naive young and naive old recipient mice to develop AHR and airway eosinophilia when challenged with allergen. Adoptive transfer of T cells isolated from the lungs of sensitized young/challenged old mice were similarly effective, in contrast to T cells from sensitized old/challenged old mice. Defective generation of CD4+ T cells in older mice has been reported in different model systems (8, 9, 23). Haynes et al. (37) concluded that the age of the CD4 T cells at the time of first encounter with Ag is a critical factor in determining whether memory responses develop. Our data support this conclusion, with confirmation in an in vivo setting, examining the functional consequences of allergen exposure in the airways on airway allergic responses. Interestingly, IL-2 may overcome the deficiencies in generating memory T cells from aged CD4+ T cells, at least in vitro (9).

IL-10 can regulate Th2 responses, AHR, and allergic inflammation (21, 38, 39). The failure of older mice to generate functional memory T cells could, at least in part, have been the result of induction of IL-10-secreting, regulatory (CD4+/CD25+) T cells in the lung. These cells could control inflammatory responses, including CD4 T cell-mediated inflammatory responses in the lung (40–43). In vivo, these CD4+ regulatory T cells can inhibit Th2-specific responses (24, 44). Somewhat to the contrary, CD4+/CD25+ T cells have also been implicated in the development of allergic lung inflammation (45).

We showed that in the BAL fluid of sensitized and challenged older mice, increased levels of IL-10 could be detected. To determine whether lung T cells from older mice could suppress the development of allergic lung inflammation and AHR, we transferred lung T cells from sensitized old/challenged old mice into sensitized young recipients. Transfer of these lung T cells did not modify the response of the recipients to subsequent allergen challenge. Similarly, transfer of these cells into recipients before sensitization did not modify the response to subsequent sensitization and challenge (data not shown). These data suggest that active suppression is probably not the mechanism accounting for the failure to generate memory responses in mice sensitized at an older age. Cumulatively, the transfer studies all indicate that age per se is not the limiting factor, because naive older mice retained the full potential to respond under the correct conditions and did not appear to induce suppression.
FIGURE 8. Effect of adoptive transfer of lung T cells on airway function (A) and inflammation (B) in sensitized young or old recipients. Donor lung T cells were obtained from mice sensitized at 8 wk and challenged at 34 wk (SRC34) or from mice sensitized at 30 wk and challenged at 34 wk (S30C34). Recipient mice were sensitized at 8 wk (S8) and received 5 × 10^6 cells 4 wk later, just before three daily inhalational challenges with allergen or PBS. n = 8 in each group. *, p < 0.05.

In vivo animal models provide opportunities to address issues that are impossible to study in humans, especially during the newborn period. In contrast, extrapolation from animal studies to human asthma is difficult. Nonetheless, it is now recognized that the majority of asthmatics can trace the onset of their disease to the first years of life. The results described in this study define how the age at initial allergen sensitization and the long-lasting effects on lung T cells can impact and determine the response to later allergen exposure. The present study adds to the growing recognition that early life exposures to allergen have a profound impact on the later consequences of allergen exposure and underscores the need to intervene as early as possible if we are to impact the course or natural history of asthma.

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