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Allergy and asthma

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Initiation and regulation of allergic inflammation is influenced by many factors, including cell type, membrane receptors, and mediators generated. Furthermore, the altered response of targeted tissues (ie, airway smooth muscle) becomes important to the eventual expression of asthma. Finally, the genetic regulation and association of genetic polymorphisms has enhanced our understanding of host susceptibility. In this review key findings published in 2004 issues of the *Journal of Allergy and Clinical Immunology* are highlighted to demonstrate recent advances in these areas. (J Allergy Clin Immunol 2005;115:953-9.)

Key words: *Eosinophils, mast cells, airway smooth muscle, cysteinyl leukotrienes, asthma*

The allergic processes involved in asthma and other inflammatory cells represent a complex interaction between resident and recruited cells at sites of injury (ie, the nose, the lung, and vascular tissue). Over the years, new segments, or chapters, are added to our ever-expanding macrocosm and microcosm of these events. In reading the *Journal of Allergy and Clinical Immunology (JACI)* on a monthly basis, the reader gets a piecemeal view of advances. At the end of a year, it is insightful to review the new published information and try to put these individual observations into context with what we know from the past and integrate the new pieces of the puzzle with one another. In the following section, we begin with the 2004 published findings on individual cells and then expand this discussion to a disease process: asthma.

Abbreviations used

BAL: Bronchoalveolar lavage
CTLA: Cytotoxic T-lymphocyte antigen
EDN: Eosinophil-derived neutrophil
eNO: Exhaled nitric oxide
EP: E prostanoid
ITIM: Tyrosine-based inhibitory motif
MIP: Macrophage inflammatory protein
NO: Nitric oxide
OX40L: OX40 ligand
PG: Prostaglandin
SNP: Single nucleotide polymorphism
TLR: Toll-like receptor
VEGF: Vascular endothelial growth factor

EOSINOPHIL BIOLOGY

If the years 2002 and 2003 signaled the death of the eosinophil in asthma, then 2004 was a comeback year. The exact role of the eosinophil in asthma and other allergic diseases remains somewhat controversial, but numerous articles in the *JACI* and elsewhere continue to provide interesting information on eosinophil biology and its potential relevance to diseases such as asthma.

Given the selective expression of the chemokine receptor CCR3 on eosinophils, Radinger et al,[1] using the BALB/c ovalbumin sensitization and challenge model, performed experiments to determine the importance of airway-expressed CCR3-active chemokines in eosinophil recruitment. Their study used separate antibodies to eotaxin 1 and eotaxin 2 administered alone or in combination to the airway or systemically to test the hypothesis that these chemokines are important for recruitment of both mature and newly produced eosinophils in the bone marrow. To accomplish the latter, they used a clever technique to label these newly produced cells via incorporation of a thymidine analog, which also served to help in monitoring for residual CD34 expression on these new eosinophils. Their data showed that both mature and newly released eosinophils are recruited after ovalbumin challenge but that intrapulmonary generation of these chemokines was particularly important for eosinophil recruitment.

Duez et al[2] tracked eosinophils in the blood, lung, bronchoalveolar lavage (BAL) fluid, and thoracic lymph nodes after allergen sensitization and challenge in mouse

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models. Not surprisingly, they found eosinophil counts increased in all compartments. Most remarkable, however, was that eosinophils quickly accumulated in the thoracic lymph nodes and that phenotypically they were different from eosinophils in other compartments. These lymph node eosinophils closely resembled dendritic cells in their phenotype in that most of these eosinophils expressed MHC class II, CD80, and CD86. How these alterations occur will require further study, but this strongly suggests, on the basis of both phenotype and location, that eosinophils might leave the lung to function as antigen-presenting cells.

In a September 2004 issue of *Science*, back-to-back articles appeared by 2 laboratories using different genetically altered ''eosinophil knockout mice.'' One model was specifically designed to deliver a suicide signal only in eosinophils,[3] whereas the other model took advantage of an unexpected observation that a deletion in the GATA-3 promoter site resulted in an eosinophil-free mouse.[4] In the eosinophil suicide model convincing data were presented that eosinophils were absolutely necessary for the pathophysiologic consequences of the asthma-like phenotype in the mouse. Amazingly, this was quite different from the other model, in which eosinophils seemed to play little, if any, role. One of the few things that the 2 models appear to agree on, however, is that the eosinophil might play a role in several aspects of airway remodeling. This is somewhat reminiscent of recent work showing that anti-IL-5 administration in human subjects reduces eosinophils and the deposition of several tissue matrix proteins.[5] Two other articles recently appeared involving compassionate use of anti-IL-5 antibody to treat hypereosinophilic syndrome, including one in the *JACI*.[6,7] These reports found strikingly beneficial effects of administration of the anti-IL-5 antibody mepolizumab in patients with cutaneous, esophageal, and other organ involvement. Improvements were seen in symptoms, as well as local and systemic eosinophilia. A potential note of caution, however, comes from a different study using a different anti-IL-5 antibody, SCH55700, in which rebound eosinophilia was seen after discontinuation of the treatment.[8] Nevertheless, GlaxoSmithKline, the maker of mepolizumab, has initiated a multicenter clinical trial (see www.clinicaltrials.gov/ct/show/NCT00086658?order=1) in an attempt to get approval for anti-IL-5 therapy in hypereosinophilic syndrome as a steroid-sparing agent.

One additional article focused on a novel aspect of eosinophilia. Sedgwick et al[9] found almost twice as much intracellular eosinophil-derived neurotoxin (EDN) in eosinophils from untreated allergic asthmatic subjects compared with that in eosinophils from healthy control subjects, subjects with allergic rhinitis, or others. In fact, these concentrations were similar to those found in airway eosinophils isolated from BAL fluid 48 hours after segmental allergen challenge. These eosinophils also released greater quantities of EDN during degranulation in response to cytokines such as IL-5 or GM-CSF. Particularly interesting was the fact that eosinophils from allergic asthmatic subjects taking inhaled corticosteroids had EDN levels similar to those of normal eosinophils, suggesting that peripheral blood eosinophils from subjects with untreated asthma have a greater EDN inflammatory capacity. Their work also suggests that EDN content per eosinophil could be a useful surrogate marker of asthma control, inhaled corticosteroid medication compliance, or both.

MAST CELLS AND BASOPHILS

Since its US Food and Drug Administration approval and introduction to the market in September 2003, omalizumab (Xolair; Novartis, Genentech Pharmaceuticals) provides the latest option for management of moderate-to-severe persistent asthma. In addition to its direct ability to block IgE attachment to FcεRI, studies uncovered a remarkable ability of IgE to regulate surface expression of its own receptor.[10] This initial report suggested the effect on circulating basophils was rapid and complete within 2 weeks, but circulating basophils have a much shorter half-life than tissue mast cells. Subsequent studies in human subjects extended this phenomenon to dendritic cells,[11] but *in vivo* data on human mast cell FcεRI have been lacking. Two articles in the *JACI* addressed this issue by studying the kinetics of omalizumab effects on mast cell phenotype. Beck et al[12] found marked reductions in skin mast cell FcεRI levels and skin test responses to dust mite antigen at 70 days and longer but not at 7 days. Unfortunately, no further time points between these were studied. In contrast, the density of skin mast cells in the skin biopsy specimens remained unchanged. Lin et al[13] confirmed previous results showing downregulation of FcεRI on circulating basophils that was maximal 2 weeks into therapy. The magnitude of the effect in the Lin article was less than that of the studies by MacGlashan et al,[10] probably because of less aggressive dosing with omalizumab. What was particularly novel about the Lin article[13] was that it showed about a 20% reduction of nasal challenge responses at 7 to 14 days of omalizumab therapy and about a 50% reduction at days 35 to 42. However, compared with the placebo-treated group, omalizumab did not appear to have as clear of an effect until days 35 to 42. Whether further reductions in responses would have occurred with continued dosing was not studied. A further note is that neither the study by Beck et al[12] (''high-dose'' omalizumab) nor the study by Lin et al[13] (standard omalizumab dosing) showed complete elimination of allergen challenge responses. This leaves open the possibility that although the allergic responses are reduced, they perhaps could be eliminated with even more aggressive omalizumab dosing.

Given the proof of concept regarding omalizumab and the importance of IgE in asthma and allergic responses, interest has intensified in targeting not only IgE but also the IgE receptor and its signaling cascade. This could herald a new generation of so-called mast cell–stabilizing drugs. For example, previous work suggested a critical role for the Syk tyrosine kinase in IgE receptor signaling,[14] and clinical trials with Syk inhibitors are already underway.[15]

MacGlashan and Miura[16] showed that prolonged stimulation (>18 hours) of FcεRI on human basophils resulted in marked reductions in Syk levels, probably because of ubiquitylation and degradation. The authors proposed that such alterations might lead to basophil desensitization and could be involved in clinically beneficial procedures, such as rush immunotherapy and drug desensitizations.

Zhang et al[17] attempted to antagonize the tyrosine-based activating motif–mediated signaling through FcεRI by generating a unique reagent that would simultaneously engage an antagonistic surface receptor containing a cytoplasmic tyrosine-based inhibitory motif (ITIM) that instead of recruiting tyrosine kinases recruits phosphatases. The reagent is a human IgG Fcγ-Fcε bifunctional fusion protein called GE2, and it appears to be capable of simultaneously engaging both the tyrosine-based activating motif–containing high-affinity IgE receptor and the ITIM containing surface structure FcγRII (CD32). In this article they show that GE2 markedly blocked antigen-driven basophil histamine release, passive cutaneous anaphylaxis in mice, and skin test responses in allergic monkeys. This probably occurs through the FcγRIIb ITIM-containing isoform of CD32. Regardless of its exact mechanism of action, the GE2 reagent appears to have intriguing antidegranulation properties both *in vitro* and *in vivo*.

Tryptase, thought to be a selective marker of mast cell activation *in vitro* and *in vivo*, has proved clinically useful in plasma-based assays to track acute mast cell activation (release of mature β-tryptase) and total body mast cell burden (total α- and β-tryptase) in conditions such as mastocytosis and more recently in certain forms of idiopathic hypereosinophilic syndrome.[18] Jogie-Brahim et al[19] report that tryptase is not truly mast cell specific by showing nearly all preparations of basophils contain tryptase, albeit only about 1% of the quantity found in mast cells. The tryptase present in basophils is both mature and enzymatically active. Given the sensitivity of the plasma tryptase assay, however, it seems prudent to continue to clinically interpret this test as a way to exclusively monitor mast cell events. We are still in search of a clinically useful basophil activation assay. One candidate is basogranulin, a basophil-specific protein released during degranulation.[20] Following another approach, Saini et al[21] found that nasal challenge of allergic subjects resulted in transient activation of circulating basophils for most individuals, as detected on the basis of increased FcεRIβ mRNA and protein, as well as enhanced spontaneous IL-13 secretion. Perhaps with these and other assays we will one day be able to distinguish between anaphylactic events that are driven primarily by mast cells or basophils.

CONTRIBUTION OF SMOOTH MUSCLE CELLS TO AIRWAY INFLAMMATION IN ASTHMA

A model of the contribution of smooth muscle cells to airway inflammation in asthma is shown in Fig 1. Airway

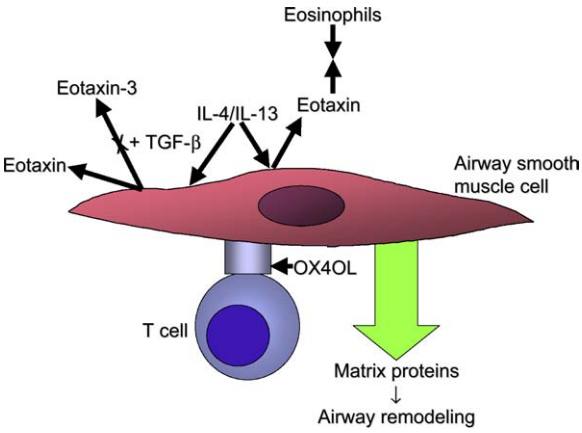

FIG 1. Airway smooth muscle generation of inflammation.

inflammation is a characteristic feature of asthma and likely contributes to both airway obstruction and airway hyperresponsiveness. Although many factors contribute to airway inflammation, studies to date have largely focused on the effect of cells recruited to the airway and their activation and release of inflammatory mediators to cause pulmonary dysfunction. During the past year, the *JACI* has featured a number of articles on how and under what conditions airway smooth muscle might be not only a target for airway inflammation but also a potential and important contributor to this process.

Burgess et al[22] obtained airway smooth muscle cells from asthmatic and nonasthmatic subjects to study whether OX40 ligand (OX40L), a molecule involved in T-cell activation, was present on airway smooth muscle cell surfaces. Normally, $CD4^+$ T cells stimulate activated antigen-presenting cells to express OX40L, which is a member of the TNF-α receptor family. T cells can also express OX40, the receptor for OX40L, and through this interaction cause bidirectional signaling. The investigators were also able to detect OX40L on airway cells of both healthy and asthmatic subjects. When these airway smooth muscle cells were activated by an appropriate ligand, release of IL-6, but not other cytokines, occurred. This study showed that airway smooth muscle cells have surface receptors similar to those of other inflammatory cells and can be activated to release inflammatory mediators to further bronchial inflammation and airway dysfunction.

In another study from the same group, Johnson et al[23] compared the profile of extracellular matrix proteins produced by asthmatic and nonasthmatic airway smooth muscle cells to determine whether these cells could also contribute to airway remodeling. When airway smooth muscle cells were cultured for 7 days in the presence of 10% serum, both nonasthmatic and asthmatic airway smooth muscle cells produced varying amounts of 15 extracellular matrix proteins evaluated. Interestingly, there was an increase of perlecan and collagen I production by cells from asthmatic subjects, whereas laminin α1 and collagen IV production were decreased. Chondroitin sulfate was detectable only in cells from the nonasthmatic

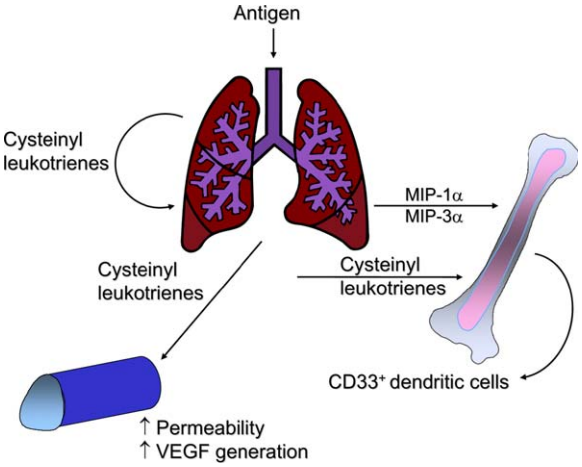

FIG 2. The role of cysteinyl leukotrienes in the generation of allergic inflammation.

subjects. Furthermore, proteins generated by the asthmatic airway smooth muscle cells enhanced smooth muscle cell proliferation. Because these extracellular matrix proteins can be generated by airway smooth muscle cells in patients with asthma, a selective profile of matrix proteins has the potential to cause cell proliferation and contribute to airway remodeling.

To further explore the involvement of airway smooth muscle in the regulation of inflammation, Zuyderduyn et al[24] from Leiden evaluated the effects of T_H2 cytokines and TGF-β on the expression of eotaxin, eotaxin 2, and eotaxin 3. Using cell lines, the investigators incubated human airway smooth muscle cells with IL-4, IL-13, and IL-9. Both IL-4 and IL-13 induced eotaxin release from airway smooth muscle cells. TGF-β, by itself, did not induce eotaxin release. However, when cells were incubated with TGF-β and the T_H2 cytokines IL-4 and IL-13 in combination, TGF-β enhanced eotaxin production but inhibited the T_H2 cytokine generation of eotaxin 3. These studies are further evidence that airway smooth muscle cells can function as inflammatory cells. Interestingly, their contribution to this inflammatory process is complex and potentially altered in asthma.

Prostanoids are COX metabolites and include prostaglandin (PG) D_2, PGE_2, $PGF_{2\alpha}$, and PGI_2. Of these, PGE_2 is produced in inflammatory reactions and can regulate affected tissues through activation of 4 distinct cell-surface receptors. Ying et al[25] first determined the expression of these PGE_2 receptor subtypes on cells obtained from sputum samples after inhaled antigen challenge. Sputum cells of all phenotypes expressed all 4 E prostanoid (EP) receptors, with an increased percentage of macrophages expressing EP_2 and EP_4. In contrast to sputum, only a small percentage of peripheral blood eosinophils expressed each receptor, but this expression was increased after incubation with either LPS or IL-5. The next step will be to define the role, regulation, and contribution of these receptor subtypes in asthma.

Airway smooth muscle can also express these same prostanoid receptors. Burgess et al[26] evaluated the regulation of proliferation exerted by PGE_2 on asthmatic airway smooth muscle cells. They initially speculated that there might be a deficiency of receptors for this agonist on asthmatic airway smooth muscle cells because PGE_2 inhibits muscle cell proliferation. Both EP_2 and EP_3 were detected on asthmatic and nonasthmatic airway smooth muscle cells, with significantly more receptors found on the asthmatic cells. In addition, airway smooth muscle cell proliferation in asthmatic subjects was more sensitive to inhibition by PGE_2. Therefore on the basis of these observations, airway smooth muscle hyperplasia in asthma is unlikely to be explained by abnormalities either in the expression or responsiveness of EP receptors.

CYSTEINYL LEUKOTRIENES

A model of the role of cysteinyl leukotrienes in the generation of allergic inflammation is shown in Fig 2. To further understand the complexity and contribution of cysteinyl leukotrienes to processes of airway inflammation in asthma, Parameswaran et al[27] at McMaster University evaluated the effect of the leukotriene receptor antagonist pranlukast on allergen-induced changes in circulating dendritic cells in patients with mild asthma. Blood samples were obtained before and 3 and 24 hours after antigen challenge to evaluate the proportion of myeloid ($CD33^+$) and plasmacytoid ($CD123^+$) dendritic cells among all PBMCs. In addition, the fraction of dendritic cells expressing cysteinyl leukotriene type 1 receptors was also determined. After antigen challenge, the number of circulating $CD33^+$ cells decreased at 3 hours, and this response was blocked by pranlukast. The decrease in circulating $CD33^+$ cells was believed to represent the consequence of their recruitment to the airway. No change in the $CD123^+$ cells occurred. Myeloid dendritic cells are believed to be responsible for antigen presentation, and their presence can further airway inflammation. Pranlukast also decreased the sputum concentration of macrophage inflammatory protein (MIP) 1α and MIP-3α that normally follows antigen challenge but not RANTES. These data raise the possibility that the generation of cysteinyl leukotrienes contributes to airway inflammation through the recruitment of myeloid dendritic cells to the airway to promote antigen presentation and the generation of chemokines. These effects can be blocked by leukotriene receptor antagonists.

Using a BALB/c mouse model of allergic inflammation and asthma, Lee et al[28] evaluated the effects of cysteinyl leukotriene receptor antagonists on bronchial inflammation, including changes in vascular permeability and the generation of vascular endothelial growth factor (VEGF), as a method to further the understanding of leukotrienes in airway inflammatory events. In the presence of 2 leukotriene receptor antagonists, montelukast and pranlukast, the inflammatory cell response to inhaled antigen was reduced. In addition, increases of IL-4 and IL-5 normally seen after antigen were reduced by these antagonists. Plasma extravasation and increases in VEGF occur with

allergen-induced airway inflammation but were also blocked by these antagonists. Furthermore, by using immunohistochemical techniques, VEGF was found to be located primarily in inflammatory cells around the bronchioles after antigen challenge. These data support the concept that antigen-induced inflammation generates cysteinyl leukotrienes that can contribute to enhanced vascular permeability and the generation of VEGF, which might influence airway remodeling.

NITRIC OXIDE

Nitric oxide (NO) generation is associated with ongoing airway inflammation. Measurements of exhaled nitric oxide (eNO) have begun to serve as a biomarker for the presence of airway inflammation, and a reduced eNO level is an indicator of positive responses to anti-inflammatory medications, such as corticosteroids. Mahut et al[29] evaluated levels of eNO in 28 children with refractory asthma, which they defined as persistent airflow limitation, or exacerbations, despite the use of high-dose inhaled corticosteroids. They also measured 2 components of eNO–alveolar NO and conducting airway NO. Bronchoscopy with BAL and biopsy was performed in these children to measure the cytokines IL-4, IFN-γ, and TGF-β, along with matrix metalloproteinase 9 and tissue inhibitor matrix protein 1. As expected, NO values were associated with the intensity of inflammation and the T_H1/T_H2 imbalance. More interestingly, alveolar NO concentrations correlated with TGF-β levels. In contrast, conducting airway NO output correlated with reticular basement membrane thickness, as well as the ratio of tissue inhibitor matrix protein 1 to matrix metalloproteinase 9 in the BAL fluid. These data indicate the benefit of measuring eNO in different airway compartments and the possibility that the generation of NO in these various compartments might contribute to distinct key features of asthma inflammation and remodeling. NO can also have an important role in regulating inflammatory responses. Sanders et al[30] had shown that NO inhibits respiratory virus growth and, through this action, might be a factor regulating virus replication *in vivo*. To assess this possibility, 6 individuals were experimentally infected with rhinovirus 16, and eNO was measured from both the nose and lower airway. A brisk increase in eNO levels occurred early in the cold. The symptom scores on day 4 of the cold were found to be inversely correlated with the increase in nasal eNO levels. Although studied in a limited number of subjects, these findings suggest that the generation of eNO during a cold might be a factor in viral clearance. Whether there are defects in this process in asthma exacerbations with colds has not been studied.

T CELLS IN ASTHMA

Using a rodent model, Isogai et al[31] evaluated the hypothesis that T cells might migrate from the airways to the bone marrow to stimulate cells at this location and thus perpetuate the inflammatory response. Using the Brown Norway rat, the investigators introduced purified fluorescein-labeled $CD4^+$ T cells into the trachea of naive or sensitized animals. Eighteen hours later, the rats were challenged with antigen, and cells were then harvested from the bone marrow, BAL fluid, lungs, lung blood pools, lung-draining lymph nodes, peripheral blood, and spleen. Challenge of sensitized animals alone resulted in an increase in BAL and bone marrow eosinophils. Most importantly, there was an exclusive increase of the fluoresceinated T cells in the bone marrow, strongly suggesting that airway lymphocytes migrate from the lung into the bone marrow after antigen challenge of sensitized animals. The mechanisms regulating cell migration to the lung were not fully defined, but cytokines and chemokines, including IL-16, eotaxin, RANTES, and MIP, are of obvious interest. These investigators limited their evaluation to IL-16 and eotaxin and found both to be increased in the bone marrow after antigen provocation, and thus they may be mediators involved in T-cell trafficking.

Using the same rat model, Isogai et al[32] evaluated the possibility that $CD8^+$ αβ T cells are proinflammatory and participate in allergic airway reactions. Rat recipients of sensitized $CD8^+$ αβ T cells had significant late-phase allergic responses after antigen challenge. Furthermore, these same $CD8^+$ cells were type 2 in character because they expressed IL-4 and IL-5. Thus $CD8^+$ cells can also participate in this allergic inflammatory process, and subsets of these cells might be instrumental in allergic inflammatory responses.

GENETICS

The association of genes and their polymorphisms with features of asthma has been an important advance over the past decade. Last year saw a number of studies that evaluated the associations of various genes with features of asthma. As noted in a number of these articles, replication of earlier reports is essential to confirm these genetic associations. From these studies, it has also become apparent that different populations of patients might have different asthma characteristics, and the association of specific genetic markers might be limited to very specific traits and groups of patients.

Raby et al[33] evaluated the genetic linkage between asthma phenotypes to chromosome 20 p13, the location of a disintegrin and metalloproteinase *(ADAM33)*, which was described as an asthma susceptibility gene by Van Eerdewegh et al.[34] Raby et al[33] performed a family-based study with 652 nuclear families. Seventeen *ADAM33* single nucleotide polymorphisms (SNPs) were genotyped. Because no single SNP had an association with asthma, the possibility exists that the association between *ADAM33* and asthma might be found in only selected populations with very specific characteristics (ie, hyperresponsiveness). Such findings further underscore the need to closely evaluate phenotypic features to genetic association.

Munthe-Kaas et al[35] evaluated the association of the development of allergy and asthma with chromosome 2q33, which contains the candidate gene cytotoxic T-lymphocyte antigen 4 *(CTLA4)*. Seven SNPs were analyzed for associations between total serum IgE, allergen sensitivity, bronchial hyperresponsiveness, asthma, and lung function in 364 asthmatic families. Associations were found among 4 newly discovered SNPs in the *CTLA4* gene and the following features: serum IgE, allergy, asthma, and reduced lung function, but not bronchial hyperresponsiveness. Furthermore, some of the SNP alleles that were found to be positively associated with asthma phenotypes were recently shown to be negatively associated with autoimmune disorders. From these findings, the authors speculated that a role for *CTLA4* polymorphisms exists in determining the T_H1/T_H2 balance and identifying *CTLA4* signaling as a potential target in atopic diseases.

Toll-like receptors (TLRs) are important in innate immunity. To evaluate TLR genotypes to asthma, Eder et al[36] evaluated polymorphisms in genes encoding for TLR that might modulate the asthma-protective effects observed in farming communities. Children living in rural areas of Austria and Germany were recruited and genotyped for SNPs associated with *TLR2/TLR4* genes. Children carrying the T allele in *TLR2* were significantly less likely to have a diagnosis of asthma, current asthma and hay fever symptoms, and atopic sensitization. The association between the *TLR2*/−16934 allele in asthma among children of farmers was independent of atopy. Thus the authors concluded that genetic variations in *TLR2* might be a major determinant in the susceptibility to asthma and allergies in children of farming families.

Finally, Fageras et al[37] investigated the relationships between *TLR4* and *CD14* gene polymorphisms, the LPS responsiveness of PBMCs, and the clinical presence of asthma and allergic rhinoconjunctivitis. Decreased IL-12 and IL-10 production was associated with a *TLR4* polymorphism (Asp299Gly) and independently with asthma, especially atopic asthma. Because the generation of these particular cytokines might be important in downregulating the T_H2 response, a deficiency in this response might explain how innate immune responses could be important determinants of allergy and influence the outcome of intervention studies that use microbial stimuli as immune modulators.

CONCLUSIONS

Our understanding and appreciation of allergic cellular events and their integration into diseases like asthma continues to expand. Although these processes appear complex and sometimes independent when presented as single articles, their contribution to the overall theme of allergic diseases becomes more apparent when looked at over a longer time period, such as the past year. The *JACI* has been pleased to provide its readers with new and key observations that help to fit the puzzle together not only more expansively but also more comprehensively. We all look forward to the next year as new and, in many cases, unexpected observations emerge to continue to move toward a more comprehensive and complete understanding.

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