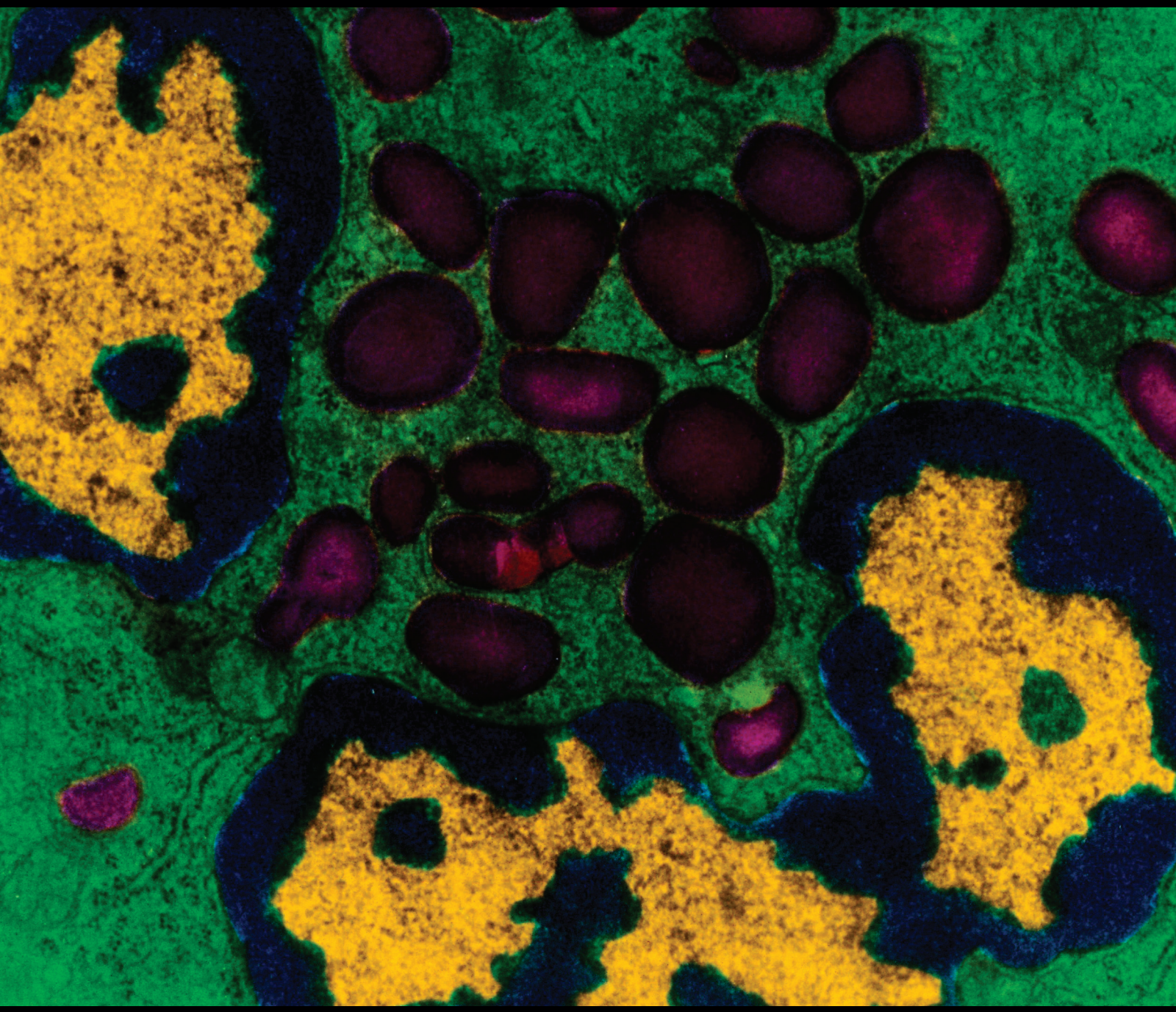
Mediators of Inflammation

Actions of Allergens and Mediators in Allergy

Guest Editors: Shaoheng He, Taro Shirakawa, Huiyun Zhang, and Peisong Gao



Actions of Allergens and Mediators in Allergy

Mediators of Inflammation

Actions of Allergens and Mediators in Allergy

Guest Editors: Shaoheng He, Taro Shirakawa, Huiyun Zhang, and Peisong Gao

Editorial Board

Contents

Actions of Allergens and Mediators in Allergy, Shaoheng He, Huiyun Zhang, and Peisong Gao
Volume 2014, Article ID 207345, 2 pages

ADAM Metallopeptidase Domain 33 (ADAM33): A Promising Target for Asthma, Priya Tripathi, Shally Awasthi, and Peisong Gao
Volume 2014, Article ID 572025, 8 pages

Aryl Hydrocarbon Receptor (AhR) Modulates Cockroach Allergen-Induced Immune Responses through Active TGFβ1 Release, Yufeng Zhou, Sarah Mirza, Ting Xu, Priya Tripathi, Beverly Plunkett, Allen Myers, and Peisong Gao
Volume 2014, Article ID 591479, 13 pages

Reduction of the Number of Major Representative Allergens: From Clinical Testing to 3-Dimensional Structures, Ying He, Xueting Liu, Yuyi Huang, Zehong Zou, Huifang Chen, He Lai, Lida Zhang, Qiurong Wu, Junyan Zhang, Shan Wang, Jianguo Zhang, Ailin Tao, and Baoqing Sun
Volume 2014, Article ID 291618, 11 pages

Importins and Exportins Regulating Allergic Immune Responses, Ankita Aggarwal and Devendra K. Agrawal
Volume 2014, Article ID 476357, 14 pages

Splenic Stromal Cells from Aged Mice Produce Higher Levels of IL-6 Compared to Young Mice, Jihyun Park, Takuya Miyakawa, Aya Shiokawa, Haruyo Nakajima-Adachi, Masaru Tanokura, and Satoshi Hachimura
Volume 2014, Article ID 826987, 9 pages

5-Lipoxygenase-Dependent Recruitment of Neutrophils and Macrophages by Eotaxin-Stimulated Murine Eosinophils, Ricardo Alves Luz, Pedro Xavier-Elsas, Bianca de Luca, Daniela Masid-de-Brito, Priscila Soares Cauduro, Luiz Carlos Gondar Arcanjo, Ana Carolina Cordeiro Faria dos Santos, Ivi Cristina Maria de Oliveira, and Maria Ignez Capella Gaspar-Elsas
Volume 2014, Article ID 102160, 13 pages

Preparation and Identification of Per a 5 as a Novel American Cockroach Allergen, Ji-Fu Wei, Haiwei Yang, Dongning Li, Peisong Gao, and Shaoheng He
Volume 2014, Article ID 591468, 10 pages

Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2014, Article ID 207345, 2 pages
http://dx.doi.org/10.1155/2014/207345

Editorial

Actions of Allergens and Mediators in Allergy

Shaoheng He,[1] Huiyun Zhang,[2] and Peisong Gao[3]

[1] *Allergy and Clinical Immunology Research Centre, Hospital of Liaoning Medical University, No. 2, Section 5, Renmin Street, Guta District, Jinzhou, Liaoning 121001, China*
[2] *Department of Pathophysiology, Hainan Medical College, Haikou, Hainan 571101, China*
[3] *Johns Hopkins Asthma and Allergy Center, Johns Hopkins University School of Medicine, Baltimore, MD 21224, USA*

Correspondence should be addressed to Shaoheng He; shoahenghe@hotmail.com

Received 13 March 2014; Accepted 13 March 2014; Published 13 April 2014

It has long been recognized that allergic inflammation is the fundamental pathological changes of allergy. Soluble allergens, specific IgE (sIgE), and mast cells or basophils are three key factors of allergy, representing causative factors, messenger, and primary effector cells, respectively. Eosinophils and neutrophils are known as secondary effector cells. In recent years, varieties of novel allergens and molecules involved in the allergic inflammation have been discovered, which makes the current understanding of the mechanisms of allergy even more confusing. We still have long way to go before the clear picture of pathogenesis of allergic inflammation can be accomplished, but our continuous and hard effort will help us to finally reach our destination drawing a complete picture of allergy. In this respect, this special issue will add a few new points in the picture of allergic inflammation.

Cockroach allergy has been associated with the development of asthma and recognized as a risk factor for emergency room admission of asthmatic patients, especially among inner city children living in low-income houses infested with cockroaches [1]. In the special issue, American cockroach allergen Per a 5 has been recognized as a minor allergen for American cockroach. Since its secondary protein structure is obtained and recombinant Per a 5 is generated, it will be a useful tool for studying the role of Per a 5 in cockroach allergy. Moreover, it is discovered that aryl hydrocarbon receptor (AhR) expression is higher in airway fibroblasts from asthmatic subjects, particularly when fibroblasts are treated with cockroach extract. An AhR agonist, 2,3,7,8-tetrachlorodibenzo-p-dioxin, can significantly enhance cockroach extract induced TGFβ1 production in fibroblasts. These results suggest the role of AhR in modulating cockroach allergen induced immune responses through controlling the active TGFβ1 release.

Classification of allergens in food plants and pollens is never easy. By using sequence alignment, progressive clustering, and comparison of 3-dimensional structures, Y. He and colleagues classify major allergens into 21 representative allergen groups, which are further divided into seven structural classes, each of which contains similar structural components. The grouping of allergens may help to understand the cross-reactivity between allergens.

Unlike studies on eosinophils and neutrophils alone, the interactions between these two secondary effector cells of allergy are relatively less investigated. Since it is an essential issue for understanding pathogenesis of allergic inflammation, it is examined by R. A. Luz and colleagues. Significant recruitment of eosinophils, neutrophils, and monocytes/macrophage by eotaxin is found in BALB/c mice, which can be abolished by eotaxin neutralization and 5-lipoxygenase-activating protein inhibitor MK886, suggesting that eotaxin induced recruitment of eosinophils is the initial event, and then followed by a 5-lipoxygenase dependent secondary neutrophil accumulation.

Inflamm-aging indicates the chronic inflammatory state resulting from increased secretion of proinflammatory cytokines and mediators such as IL-6 in the elderly. It is found that IL-6 protein production in cultured stromal cells from aged mouse spleen is significantly high compared to young mice upon LPS stimulation, which suggests that stromal cells

may contribute to the chronic inflammatory condition such as allergic inflammation in the elderly.

As reviewed by P. Tripathi and colleagues, over the last few years, a significant progress has been made in understanding the role of a disintegrin and metalloproteinase 33 (ADAM33) in asthma. ADAM33 may play a role in airway remodeling because of its high expression in epithelium, myofibroblasts, and airway smooth muscle cells and its role in promoting angiogenesis and stimulating cell proliferation and differentiation. Molecules involved in allergic inflammation also include importins and exportins. A. Aggarwal and D. K. Agrawal described that the classical nuclear export signals are found on many transcription factors and molecules that are involved in the pathogenesis of allergic diseases. In addition, several immune modulators, including corticosteroids and vitamin D, elicit their cellular responses by regulating the expression and activity of importin molecules. In their review article, they provide also a comprehensive list of importin and exportin molecules and their specific cargo that are shuttled between cytoplasm and the nucleus. The review on the role and regulation of specific importin and exportin involved in the transport of activated transcription factors in allergic diseases will enable us to understand further cell signaling pathways in allergic inflammation.

Shaoheng He
Huiyun Zhang
Peisong Gao

References

[1] L. K. Arruda, L. D. Vailes, V. P. L. Ferriani, A. B. R. Santos, A. Pomés, and M. D. Chapman, "Cockroach allergens and asthma," *Journal of Allergy and Clinical Immunology*, vol. 107, no. 3, pp. 419–428, 2001.

Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2014, Article ID 572025, 8 pages
http://dx.doi.org/10.1155/2014/572025

Review Article

ADAM Metallopeptidase Domain 33 (ADAM33): A Promising Target for Asthma

Priya Tripathi,[1] Shally Awasthi,[2] and Peisong Gao[1]

[1] *Johns Hopkins Asthma and Allergy Center, Johns Hopkins University School of Medicine, 5501 Hopkins Bayview Circle, Room 3B.71, Baltimore, MD 21224, USA*
[2] *Department of Pediatrics, King George's Medical University, Lucknow, India*

Correspondence should be addressed to Peisong Gao; pgao1@jhmi.edu

Received 21 November 2013; Accepted 12 February 2014; Published 10 April 2014

Academic Editor: Shaoheng He

Over the last few years, a significant progress has been made in understanding the role of a disintegrin and metalloproteinase 33 (ADAM33) in asthma. The previous observations for the association with asthma have been replicated in over 33 different population samples worldwide. We and others have performed association analysis and meta-analysis and provided further evidence that several polymorphisms in the ADAM33 are risk factors for asthma, especially in the Asian population. Further, several studies have suggested that alterations in epigenetic marks alter the patterns of DNA methylation of ADAM33 and result in potentially adverse biological effects. Finally, while the biological activities of ADAM33 are as yet unknown, ADAM33 may play a possible role in airway remodeling because of its high expression in epithelium, myo/fibroblasts, and airway smooth muscle cells (ASMCs) and its role in promoting angiogenesis and stimulating cell proliferation and differentiation. Thus, ADAM33 represents a promising target for asthma. However, further investigations are clearly needed to discover functional ADAM33 gene polymorphisms and the role of genetic/epigenetic factors in conferring genetic susceptibility to environmental exposure induced asthma as well as biological function in asthma. This, in turn, will unlock the possibility of ADAM33 as a target for asthma therapy.

1. Introduction

Asthma is a complex inflammatory disorder of airways of lungs resulting in airflow obstruction and bronchial hyperresponsiveness (BHR) to a variety of stimuli and symptoms of wheeze, cough, and breathlessness. It continues to have a severe impact on global public health problem, affecting an estimated 300 million people worldwide [1]. The major obstacle in preventing and treating asthma has been our incomplete understanding of its etiology and biological mechanisms. Recent studies have changed our understanding of asthma from a purely inflammatory disease to a disease in which both inflammatory and structural components are equally involved [2]. Asthma is often associated with structural remodeling of the airways characterized by airway epithelial damage, wall thickening, and subepithelial fibrosis [2, 3]. Although environmental factors are important in the origins and progression of asthma, it is widely recognized that asthma has a strong genetic component and is the result of complex interactions between genes and environment [3–5]. In the last decade, tremendous progress has been made in the genetic study of asthma with many genes identified as asthma-susceptible genes. Of these, a disintegrin and metalloproteinase 33 (ADAM33) gene is the first novel susceptibility gene for asthma and airway hyperresponsiveness (AHR) identified by positional cloning [6] and has been replicated in over 33 different population samples worldwide [7]. We and others have recently performed meta-analysis and provided further evidence that several polymorphisms in the ADAM33 are risk factors for asthma, especially in the Asian population. Although the biological activities of ADAM33 remain unknown, we speculate that ADAM33 might be associated with airway remodeling because of its high expression in airway fibroblasts, myofibroblasts, and smooth muscle cells and its function in protecting the airway from increased repair processes [8]. In this paper, we reviewed the studies on ADAM33, including replication of associations and meta-analysis between ADAM33

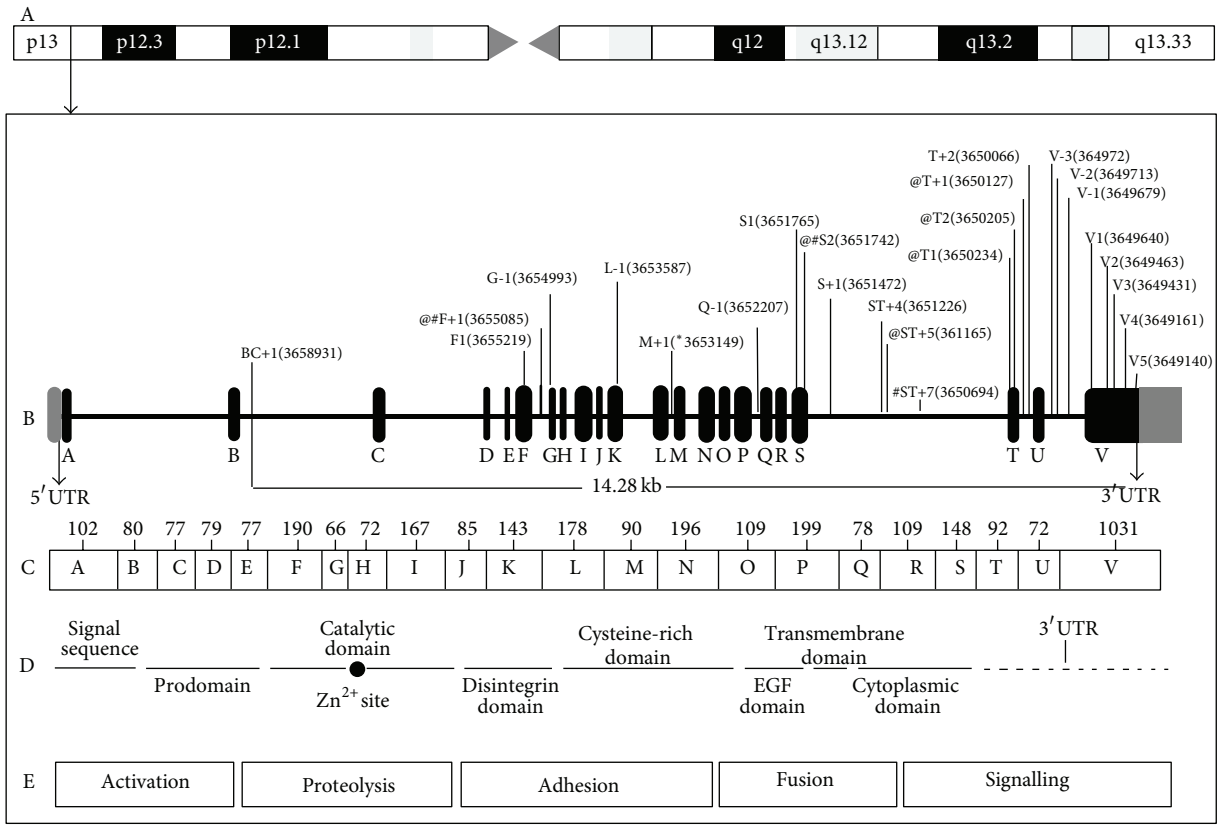


Figure 1: Schematic representation of the ADAM33 gene on chromosome 20. (a) Chromosome 20 showing ADAM33 gene position on 20p13. (b) Region covered by all most common studied polymorphisms by study groups in worldwide population and covered size in Kb. (c) Exons and size in base pairs. (d) Domain structure. (e) Functions of ADAM33 domain. @ represents positive association among Asians; # represents positive association among Caucasians.

polymorphisms from the original studies and asthma and related phenotypes in different populations, particularly in the Asian populations, epigenetic mechanisms for ADAM33 in asthma, and possible biologic link to the pathogenesis of asthma.

2. Association of ADAM33 Gene Polymorphisms with Asthma and Related Phenotypes

The first asthma-susceptibility locus to be identified by positional cloning was reported by Van Eerdewegh et al. A genomewide scan in 480 asthma sibling-pair families from the UK and US revealed an evidence for linkage between asthma and BHR on chromosome 20p13 (Figure 1), where ADAM33 is located and associated with asthma [6]. ADAM33 belongs to members of disintegrin and metalloprotease family that code for zinc-dependent metalloproteases. It is a type I transmembrane zymogen glycoprotein. The ADAM33 protein harbors several domains that include prometalloprotease-like, disintegrin-like, cysteine-rich, epidermal growth factor-like, transmembrane, and cytoplasmic domains facilitating its participation in many cellular processes [9–12]. Its adhesion domain as well as protease domain makes it exclusive among cell surface proteins. The autocatalytic removal of the prodomain is activation signal for ADAM proteins [12]. ADAM33 is a complex molecule whose expression is restricted largely to mesenchymal cells including airway fibroblasts, myofibroblasts, and smooth muscle cells [6, 13]. Figure 1 is the schematic representation of the ADAM33 gene on chromosome 20, including the ADAM33 gene location (A), all most common studied polymorphisms by study groups in worldwide populations (B). ADAM33 includes 22 exons size in base pairs (C) with different genomic domains (D), which contribute to several important biological functions of the ADAM33 gene, including cell activation, proteolysis, adhesion, fusion, and signaling (E).

After Van Eerdewegh et al.'s study, a large number of studies have been carried out. For instance, Howard and colleagues assessed 8 selected ADAM33 SNPs from the original study and provided further evidence of association with asthma in four case-control asthma populations—African American (AA), US Caucasian, US Hispanic, and Dutch Caucasian [14]. While association was identified for at least one SNP in each population, none of SNPs was seen to be associated with asthma phenotype across all those

studied populations. Association with asthma was also found in family-based studies. Werner et al. analyzed 15 highly selected ADAM33 SNPs in 171 German families and found that SNPs F+1, ST+4, and ST+5 were associated with asthma [15]. Several other reports demonstrated that ADAM33 polymorphisms have shown association with decline in FEV1 in asthmatic adults [16] and impaired lung function at ages 3 and 5 years [17], respectively. In contrast, few studies failed to replicate the association including a study in 583 Hispanic (Puerto Rican and Mexican) trios [18] and a study in a large population of North American asthma trios [19]. In Asian populations, the associated ADAM33 SNPs were different. Chi and his colleagues reported that SNP S2 was associated with risk of asthma in the Chinese Han population [20]. However, in the same population, Jie et al. failed to find association for S2 but found significant association for SNPs F+1, T1, and T2 [21]. In a Thai population Thongngarm et al. found that SNP S2 and ST+4 were associated with asthma susceptibility [22]. We conducted a study in an Indian population and assessed association for 14 ADAM33 SNPs (F+1, V4, ST+4, S2, ST+5, V2, T2, T1, BC+1, Q-1, S1, S+1, V-3, and T+1) and found 8 SNPs (F+1, V4, ST+4, S2, ST+5, T2, T1, and S1) in association with asthma [23–26]. In Supplementary Table 1 (see Supplementary Material available online at http://dx.doi.org/10.1155/2014/572025), we listed a total of 26 studies extracted from PubMed of different groups of populations. While studies have suggested that ADAM33 gene polymorphisms are important in conferring susceptibility to asthma, the data are controversial. Moreover, the true causative variant that determines asthma predisposition is still unclear. Furthermore, many of these individual studies had a relatively smaller sample size, which are statistically underpowered. Finally, it is worth noting that environmental exposure may modulate the genetic associations observed in different populations (gene-environment interaction). Indeed, Reijmerink et al. confirmed that interaction of in utero cigarette smoke exposure with ADAM33 results in reduced lung function and the development of BHR [27]. Similarly, we also observed enhanced association of ADAM33 SNP V4 heterozygous with reduced FEV1 among smokers [28].

Very recently, we observed that one of asthma associated ADAM33 ST+5 was significantly associated with heavy traffic, an indication of air pollution, in a total of 386 Indian individuals. The heavy traffic was defined based on the distance from heavy traffic [29]. We have found that the heavy traffic-predicted percentages for the homozygous normal genotype (CC), the heterozygous genotype (TC), and the homozygous mutant genotype (TT) of ST+5 SNP were 4.4, 33.3, and 62.2%, respectively. In contrast, nontraffic area predicted percentages for CC, TC, and TT of ST+5 SNP were 13.5, 42.6, and 43.9%, respectively (OR = 1.934, 95% CI = 1.306–2.863, and $P = 0.001$; Figure 2), indicating that TT genotype may protect against heavy traffic. These findings suggest that exposure to environmental chemicals, together with genetic variations, may contribute to the increased risk of asthma.

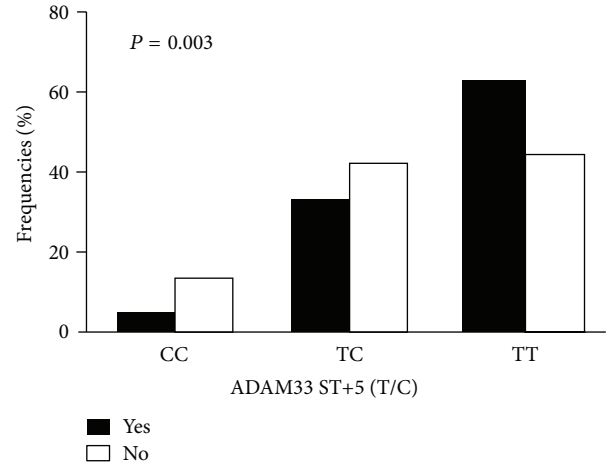


Figure 2: Environmental exposure modulates the association for ADAM33 ST+5 and asthma. x-axis represents ST+5 genotypes; y-axis represents frequencies of genotype in individuals with (yes) or without (no) exposure to heavy traffic.

3. ADAM33 Gene Polymorphisms and Risk of Asthma: A Meta-Analysis

In the association studies, there are possibilities that some positive results might be specious and some negative findings might be a consequence of low statistical power. It could be due to their smaller sample size or methodological liabilities, such as the selection of an appropriate control group. Meta-analysis might be a means of determining reflective results. Conceptually, a meta-analysis uses a statistical approach to combine the results from multiple studies [30]. It has been widely used for genetic association analysis by combining the results from different studies with a relatively larger sample size. Specifically, combining samples from several studies could make greater power than from individual studies or might increase trends for association in small individual studies. Further, meta-analysis might be useful to identify the causative gene polymorphisms with consistency and to quantify with accuracy the genetic risks. Thus, in this review, we summarized the major meta-analyses to see whether these ADAM33 SNPs were associated with asthma in an increased sample size, including our meta-analysis in the Asian population [31].

In our study, we reviewed 13 studies on 12 ADAM33 polymorphisms in Asian populations and quantitatively summarized the association between ADAM33 SNPs (S1, V4, T1, ST+4, T2, F+1, S2, Q-1, T+1, ST+5, V-3, and S+1) and asthma using meta-analysis. A dominant (MM+ML versus LL), recessive (MM versus ML+LL), additive (MM versus LL), and allelic models (M versus L) were used to estimate the association between ADAM33 gene polymorphisms and asthma risk in a total of 6212 individuals consisting of 3,270 patients and 2,922 controls. Significant associations were found for ST+5, S2, and T1 with odds ratios from 1.67 to 4.34 in the overall population [31] (Figure 3). The evidence from the meta-analyses supports the notion of a role for the SNPs ST+5, S2, and T1 in the ADAM33 gene in

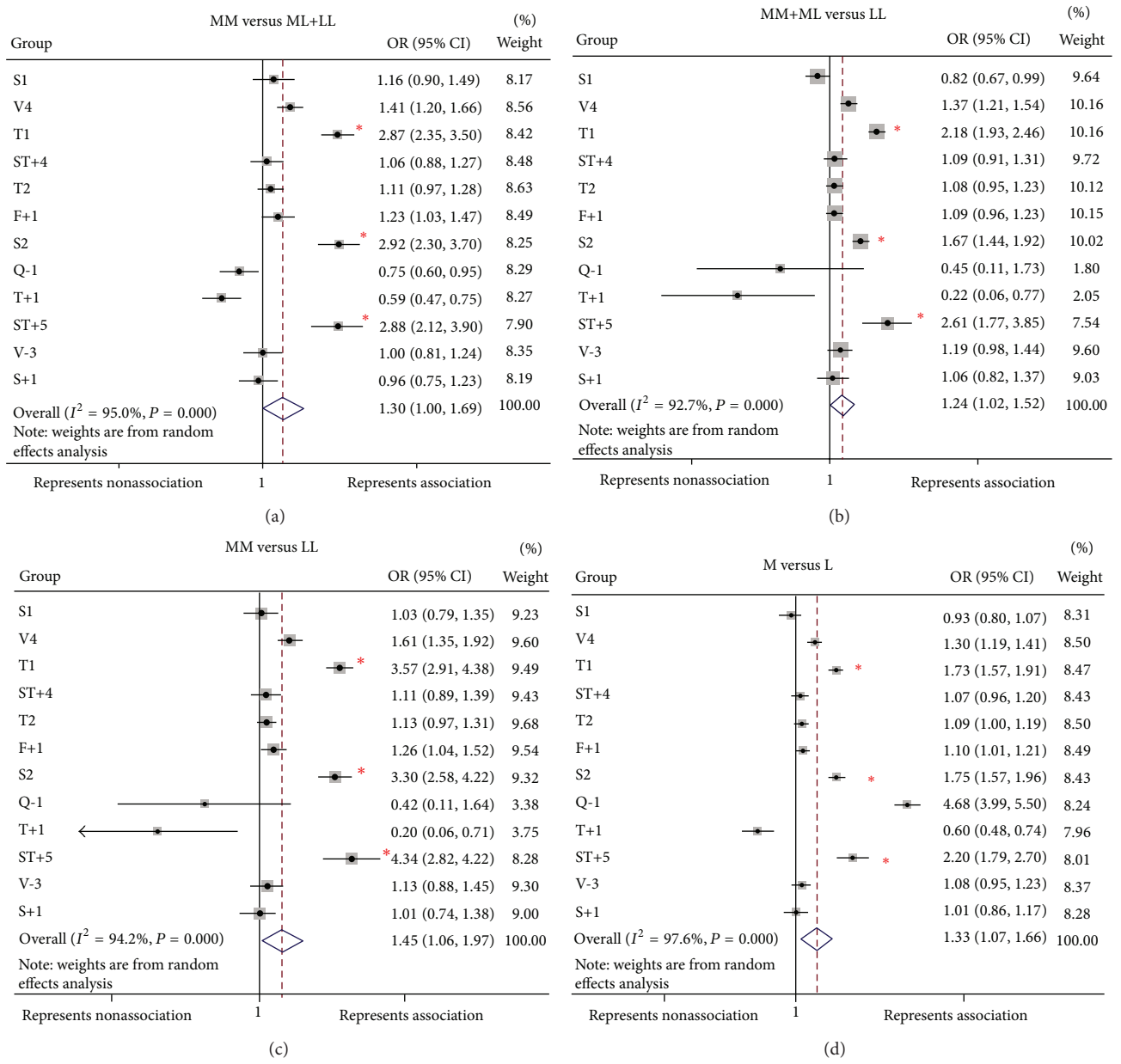


Figure 3: Forest plots from my meta-analysis study on Asian population. (a) MM versus LL+LM, (b) MM+ML versus LL, (c) MM versus LL, and (d) M versus L, where M is mutant allele, L is wild-type allele, LL is homozygous normal genotype, LM is heterozygous genotype, and MM is homozygous mutant genotype. *Significant associations with odds ratios from 1.67 to 4.34 in the overall population under different models.

conferring susceptibility to asthma in the Asian population. Very recently, Liang et al. performed similar meta-analysis to summarize the associations between ADAM33 polymorphisms and asthma risk for a total of 29 case-control studies and 14 SNPs. Although they failed to find association for ADAM33 SNPs S1, V-1, V5, S+1, S2, ST+4, ST+7, ST+5, and Q-1, significant associations were observed for T1, V4, F+1, and T+1 in the overall population. Interestingly, a positive result was found for the T1, V4, F+1, and T2 polymorphisms only in Asia but not in Europe or Latin America. This meta-analysis provides further evidence that the T1, V4, F+1, and T2 in the ADAM33 gene may be the major risk factors for asthma, especially in the Asian population [32]. Another meta-analysis performed by Song et al. included thirteen studies in ten reports for the association between the ADAM33 SNPs and asthma in a total of 12,875 subjects consisting of 4942 patients and 7933 controls. Song et al.'s group was limited to selection of SNPs. This group selected only five ADAM33 SNPs, named as S2, ST+4, F+1, S1, and V4. They found that the ADAM33 SNP S2 confers susceptibility to asthma in Europeans and ST+4 in Asians and adults [33]. Taken together, these meta-analyses suggest that the ADAM33 SNPs

confers susceptibility to asthma. Different ethnic populations showed the associations with different SNPs, which may be in linkage disequilibrium with the same causal variants in ADAM33. Further studies are required to identify functional mutations, which may modulate the ADAM33 function and subsequently development of asthma. Meta-analysis results also highlight the fact that no single SNP is associated with asthma across all the populations studied. This may be due to the compound effects of multiple alleles, multiple genes, and environmental factors. For example, the effect of a given SNP could be restricted to an ethnic group or a population; thus, there may be genetic differences among subtypes of asthma, such as pediatric, adult, and allergic/nonallergic. Secondly, this could be due to the difference in the exposure to allergens, which could vary from one geographical region to another and their interactions with the genetic factor(s) could vary. Thirdly, the linkage disequilibrium patterns that exist between the identified SNP and the undetected causative defect in the gene could differ from one population to another [34]. Thus, through understanding the genetic factors and their interactions with the environmental factors for each population, this would aid in developing effective predictive markers for the multifactorial diseases like asthma.

4. Epigenetic Mechanisms for ADAM33 in Asthma

Several studies have suggested that the pathogenesis of asthma may be affected by epigenetic regulation. So far, there have been 4 studies on the ADAM33 DNA methylation and asthma [35–38]. Yang et al. investigated the methylation status of CpG island within its promoter of ADAM33, and its association with ADAM33 expression. Interestingly, they found that the CpG site in the promoter (−362 to +80) of ADAM33 was hypermethylated in epithelial cells but hypomethylated in ADAM33-expressing fibroblasts [35] and suggested that the methylation status controls ADAM33 expression in a cell-type-specific manner. Studies from the same group reported that no changes were observed in methylation status of the ADAM33 promoter in normal or asthmatic fibroblasts. However, they found that transforming growth factor-beta 2 (TGF-β2) downregulates ADAM33 mRNA expression in normal and asthmatic fibroblasts by causing chromatin condensation around the ADAM33 promoter with deacetylation of histone H3, demethylation of H3 on lysine-4, and hypermethylation of H3 on lysine-9. Findings from this study suggested that TGF-β2 suppresses expression of ADAM33 mRNA expression by chromatin modification, rather than by gene silencing through DNA methylation [36]. This was further supported by a very recent study by investigating the methylation patterns of ADAM33 in adult asthma [37]. They designed a case-control study with 50 asthmatic patients and 50 age- and sex-matched healthy controls to examine the relationship between the CpG methylation of the ADAM33 gene and asthma using bisulfite deoxyribonucleic acid modification and sequencing. A total of 14 CpG sites in exon 9 of the ADAM33 gene were found to be highly methylated (100%) in all individuals, but no clear difference in DNA methylation patterns between asthmatic and controls groups was shown. The negative observation may be due to the limited sample size and confounding factors like environmental exposures. Alterations in epigenetic marks have been associated with exposures relevant to asthma, particularly air pollution and tobacco smoke, as well as asthma phenotypes [38]. We postulate that exposure to environmental chemical or allergens can alter the patterns of DNA methylation of ADAM33, resulting in potentially adverse biological effects such as aberrant gene expression and ADAM33-involved airway remodeling. Further investigations are needed to examine the epigenetic changes of ADAM33 in a larger population with or without the considerations of environmental factors. Thus, epigenetic mechanisms represent a promising direction that might, in part, explain the inheritance and immunobiology of asthma.

5. Biological Link of ADAM33 to Asthma

ADAM33 has been identified as an asthma susceptibility gene; however, the role of ADAM33 in the pathogenesis and progression of asthma remains to be elucidated. ADAM33 is predominantly expressed in cells of mesenchymal origin, mainly as fibroblasts, myofibroblasts, and smooth muscle cells, indicating a possible role in airway remodeling (see Figure 4) [6, 13, 39]. Indeed, increased expression of ADAM33 was detected in human airway from subjects with asthma as compared to that in controls. Further, the increased expression was correlated with asthma severity progressed from mild to severe and with lung function as defined by FEV1% [40, 41]. We investigated the expression of ADAM33 protein in bronchial biopsy tissues from 27 patients with asthma and 7 nonasthmatic controls [42]. We, for the first time, identified increased expression of ADAM33 protein in epithelium, smooth muscle and mesenchymal cells of asthmatic patients when compared to those nonasthmatic controls. Given the small sample size, we failed to find a correlation between ADAM33 expression and severity of asthma. As ADAM33 is predominantly expressed in airway smooth muscle cells (ASMCs), Lin et al. investigated whether ADAM33 protein expression is correlated with ASMC mechanics in an ovalbumin- (OVA-) sensitized rat model. Increased ADAM33 expression was observed in ASMCs from the OVA-sensitized rats when compared with the nonsensitized rats. Importantly, ADAM33 expression was positively correlated with cell stiffness, traction force, and expression of vinculin and Factin, suggesting that ADAM33 is a mediator of ASMC dysfunction in asthma [43]. These increased expression of ADAM33 in epithelium in asthmatic patients and ASMCs in allergen-induced animal models further support the possibility that ADAM33 may play a role in airway remodeling.

Although the mechanisms for the ADAM33 involved remodeling are not clear, it has been suggested that ADAM33 may affect the epithelial-mesenchymal trophic unit (EMTU) [35, 44]. Moreover, the soluble form of ADAM33 causes rapid induction of endothelial cell differentiation in vitro and neovascularization ex vivo and in vivo, suggesting that ADAM33 can promote angiogenesis and lead to airway

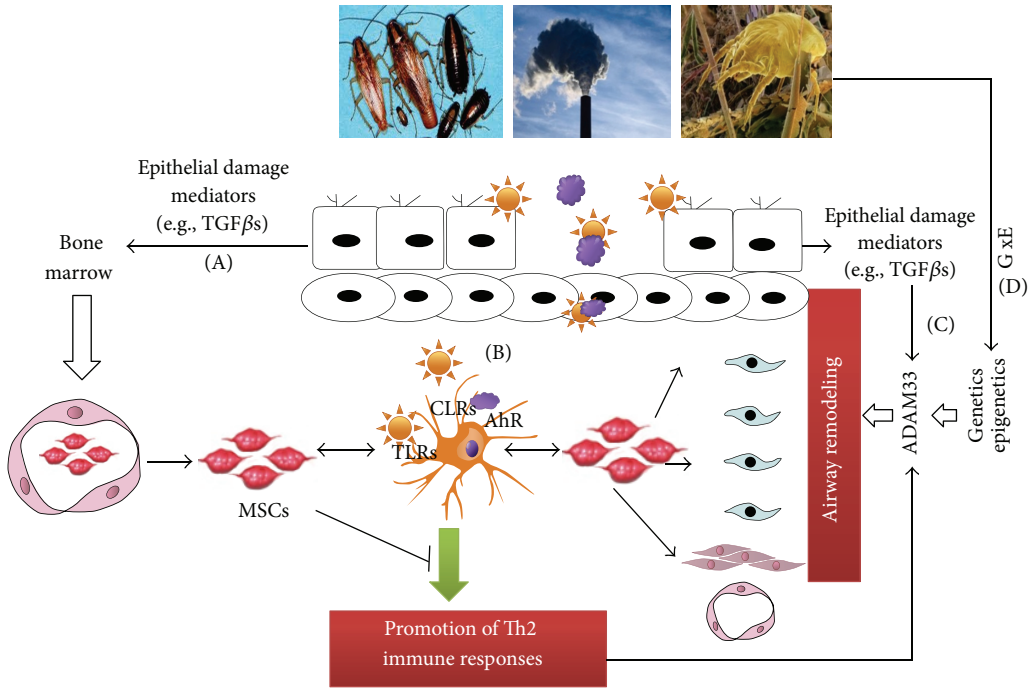


Figure 4: Schematic representation of possible mechanisms for ADAM33 in airway remodeling.

remodeling. TGFβ is a multifunctional cytokine that plays a critical role in cell growth, differentiation, and immune regulation and has been considered a principal mediator of airway remodeling [45–48]. Recent studies have demonstrated that disruption in TGFβ1 signaling imposes a strong predisposition for human allergic diseases [49]. Specifically, increased active TGFβ1 has been observed in airways from asthmatic patients [50] and from experimental mice during allergic airway inflammation [51]. Because TGFβ1 can promote ADAM33 ectodomain shedding and suppress ADAM33 expression, it is possible that active TGFβ1 may cause fibroblast differentiation and proliferation through regulating ADAM33 expression and subsequently lead to airway remodeling. As summarized in Figure 3, we postulate that (1) active TGFβ1 released from damaged/repairing epithelium in response to repetitive allergen and environmental chemical challenges may cause aberrant excessive recruitment of mesenchymal stem cells (MSCs), leading to accumulation of fibroblasts/myofibroblasts and progressive fibrosis and pathological remodeling due to their capability of differentiation into myofibroblasts [52]; ADAM33 may be required for this process. (2) these released active TGFβ1 can directly modulate ADAM33 expression and subsequently functions; (3) allergens and environmental chemicals can disturb airway epithelial integrity and lead to an increased penetration of allergens and chemicals, resulting in activation of innate immune cells (e.g., dendritic cells (DCs)) through C-type lectin receptors (CLRs), Toll-like receptors (TLRs), and aryl hydrocarbon receptor (AhR), which will direct cells of the adaptive immune system to Th2 cell development with the upregulation of ADAM33 mRNA expression [53]; and (4) environmental factors, together with genetic or epigenetic changes of ADAM33 (interaction between environmental and genetic factors), may cause aberrant expression of ADAM33 in epithelial, fibroblasts, and airway smooth muscle cells. It is possible that all of these may synergize, leading to the development of airway remodeling.

6. Conclusions

In this paper, we have reviewed the studies on the replications and meta-analysis of genetic associations for ADAM33 and asthma and its related phenotypes in different populations. While studies, including association studies and meta-analyses, have suggested that several ADAM33 gene polymorphisms (e.g., ST+5, S2, and T1) are important in conferring susceptibility to asthma, the data are controversial, and the true causative variants have not been identified yet. Many factors may be related to the failure to identify the true associations and causal variants, including sample size, environmental modification (interplay between genetic and environmental factors), population mixture, type of clinical asthma, age of onset, and treatment history. Thus, larger scale studies in different ethnic, but age-, sex-, and environmental exposure-matched populations are required to continue these studies. Further, there are few, but important, studies on investigating the effects of on the epigenetic modulation on ADAM33 function. Alterations in epigenetic marks have been shown to alter the patterns of DNA methylation of ADAM33, resulting in potentially adverse biological effects. Thus, epigenetic mechanisms represent a promising direction for the studies on the development of asthma. Finally, while the biological activities of ADAM33 are as yet unknown, it has been suggested that ADAM33 may play a possible role

in airway remodeling because of its expression in epithelium, myo/fibroblasts, and ASMCs and its role in promoting angiogenesis and stimulating cell proliferation and differentiation. It is likely that ADAM33 mediates environmental exposure induced airway inflammation and remodeling that may occur through the activation of TGFβ signaling and several major receptors (CLRs, AhR, and TLRs). Thus, ADAM33 represents a promising target for asthma. However, further studies are clearly needed to discover functional SNPs, the role of genetic/epigenetic factors in conferring genetic susceptibility environmental exposure induced asthma, and biological function in asthma.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This research was supported by the National Institutes of Health (NIH) Grant RO1ES021739-01A1 (to Peisong Gao) and by the Government of India (BT/PR7115/Med/14/955/2006 to Shally Awasthi).

References

[1] M. Masoli, D. Fabian, S. Holt, and R. Beasley, "The global burden of asthma: executive summary of the GINA Dissemination Committee report," *Allergy*, vol. 59, no. 5, pp. 469–478, 2004.

[2] S. Al-Muhsen, J. R. Johnson, and Q. Hamid, "Remodeling in asthma," *Journal of Allergy and Clinical Immunology*, vol. 128, no. 3, pp. 451–462, 2011.

[3] J. A. Elias, Z. Zhu, G. Chupp, and R. J. Homer, "Airway remodeling in asthma," *The Journal of Clinical Investigation*, vol. 104, no. 8, pp. 1001–1005, 1999.

[4] T. M. Ball, J. A. Castro-Rodriguez, K. A. Griffith, C. J. Holberg, F. D. Martinez, and A. L. Wright, "Siblings, day-care attendance, and the risk of asthma and wheezing during childhood," *The New England Journal of Medicine*, vol. 343, no. 8, pp. 538–543, 2000.

[5] G. H. Koppelman, "Gene by environment interaction in asthma," *Current Allergy and Asthma Reports*, vol. 6, no. 2, pp. 103–111, 2006.

[6] P. van Eerdewegh, R. D. Little, J. Dupuis et al., "Association of the ADAM33 gene with asthma and bronchial hyperresponsiveness," *Nature*, vol. 418, no. 6896, pp. 426–430, 2002.

[7] S. T. Holgate, "Mechanisms of asthma and implications for its prevention and treatment: a personal journey," *Allergy, Asthma and Immunology Research*, vol. 5, no. 6, pp. 343–347, 2013.

[8] S. D. Shapiro and C. A. Owen, "ADAM-33 surfaces as an asthma gene," *The New England Journal of Medicine*, vol. 347, no. 12, pp. 936–938, 2002.

[9] R. A. Black and J. M. White, "ADAMs: focus on the protease domain," *Current Opinion in Cell Biology*, vol. 10, no. 5, pp. 654–659, 1998.

[10] C. P. Blobel, "ADAMs: key components in egfr signalling and development," *Nature Reviews Molecular Cell Biology*, vol. 6, no. 1, pp. 32–43, 2005.

[11] P. Primakoff and D. G. Myles, "The ADAM gene family: surface proteins with adhesion and protease activity," *Trends in Genetics*, vol. 16, no. 2, pp. 83–87, 2000.

[12] A. L. Stone, M. Kroenger, and Q. X. A. Sang, "Structure-function analysis of the ADAM family of disintegrin-like and metalloproteinase-containing proteins," *Journal of Protein Chemistry*, vol. 18, no. 4, pp. 447–465, 1999.

[13] S. P. Umland, C. G. Garlisi, H. Shah et al., "Human ADAM33 messenger RNA expression profile and post-transcriptional regulation," *American Journal of Respiratory Cell and Molecular Biology*, vol. 29, no. 5, pp. 571–582, 2003.

[14] T. D. Howard, D. S. Postma, H. Jongepier et al., "Association of a disintegrin and metalloprotease 33 (ADAM33) gene with asthma in ethnically diverse populations," *Journal of Allergy and Clinical Immunology*, vol. 112, no. 4, pp. 717–722, 2003.

[15] M. Werner, N. Herbon, H. Gohlke et al., "Asthma is associated with single-nucleotide polymorphisms in ADAM33," *Clinical and Experimental Allergy*, vol. 34, no. 1, pp. 26–31, 2004.

[16] H. Jongepier, H. M. Boezen, A. Dijkstra et al., "Polymorphisms of the ADAM33 gene are associated with accelerated lung function decline in asthma," *Clinical and Experimental Allergy*, vol. 34, no. 5, pp. 757–760, 2004.

[17] A. Simpson, N. Maniatis, F. Jury et al., "Polymorphisms in a disintegrin and metalloprotease 33 (ADAM33) predict impaired early-life lung function," *American Journal of Respiratory and Critical Care Medicine*, vol. 172, no. 1, pp. 55–60, 2005.

[18] D. L. Lind, S. Choudhry, N. Ung et al., "ADAM33 is not associated with asthma in Puerto Rican or Mexican populations," *American Journal of Respiratory and Critical Care Medicine*, vol. 168, no. 11, pp. 1312–1316, 2003.

[19] B. A. Raby, E. K. Silverman, D. J. Kwiatkowski, C. Lange, R. Lazarus, and S. T. Weiss, "ADAM33 polymorphisms and phenotype associations in childhood asthma," *Journal of Allergy and Clinical Immunology*, vol. 113, no. 6, pp. 1071–1078, 2004.

[20] X. Chi, L. Wang, J. Wang et al., "Association of ADAM33 gene polymorphisms with asthma in a Chinese population," *Clinical Respiratory Journal*, vol. 7, no. 1, pp. 16–20, 2013.

[21] Z. Jie, Z. Hu, C. Bai, and M. Jin, "ADAM33 gene polymorphisms associate with asthma susceptibility and severity in East China han population," *Journal of Asthma*, vol. 48, no. 10, pp. 979–985, 2011.

[22] T. Thongngarm, A. Jameekornrak, C. Limwongse et al., "Association between ADAM33 polymorphisms and asthma in a Thai population," *Asian Pacific Journal of Allergy and Immunology*, vol. 26, no. 4, pp. 205–211, 2008.

[23] S. Awasthi, P. Tripathi, S. Ganesh, and N. Husain, "Association of ADAM33 gene polymorphisms with asthma in Indian children," *Journal of Human Genetics*, vol. 56, no. 3, pp. 188–195, 2011.

[24] S. Awasthi, P. Tripathi, R. Prasad, R. Awasthi, and S. Ganesh, "Association of ADAM33 gene polymorphisms and theirhaplotypes with asthma in an Indian population," *Indian Journal of Medical Sciences*. In press.

[25] P. Tripathi, S. Awasthi, R. Prasad, N. Husain, and S. Ganesh, "Association of ADAM33 gene polymorphisms with adult-onset asthma and its severity in an Indian adult population," *Journal of Genetics*, vol. 90, no. 2, pp. 265–273, 2011.

[26] P. Tripathi, S. Awasthi, R. Prasad, and S. Ganesh, "Haplotypic association of ADAM33 (T+1, S+1 and V−3) gene variants in genetic susceptibility to asthma in Indian population," *Annals of Human Biology*, vol. 39, no. 6, pp. 479–483, 2012.

[27] N. E. Reijmerink, M. Kerkhof, G. H. Koppelman et al., "Smoke exposure interacts with ADAM33 polymorphisms in the development of lung function and hyperresponsiveness," *Allergy*, vol. 64, no. 6, pp. 898–904, 2009.

[28] P. Tripathi, S. Awasthi, and S. Ganesh, "Association of ADAM33 gene polymorphisms with reduction of lung function as measured by peak expiratory flow rate among healthy male smokers and nonsmokers," *The IUP Journal of Genetics & Evolution*, vol. 4, no. 2, pp. 18–26, 2011.

[29] S. Awasthi, P. Tripathi, and R. Prasad, "Environmental risk factors for asthma in Lucknow: a case—control study," *Clinical Epidemiology and Global Health*, vol. 1, no. 3, pp. 115–123, 2013.

[30] J. R. Thompson, J. Attia, and C. Minelli, "The meta-analysis of genome-wide association studies," *Briefings in Bioinformatics*, vol. 12, no. 3, pp. 259–269, 2011.

[31] P. Tripathi and S. Awasthi, "Association of ADAM33 gene polymorphisms with genetic susceptibility to asthma in Asian populations: a meta-analysis," *Indian Journal of Genetics and Molecular Research*, vol. 1, no. 1, pp. 16–21, 2014.

[32] S. Liang, X. Wei, C. Gong, J. Wei, Z. Chen, and J. Deng, "A disintegrin and metalloprotease 33 (ADAM33) gene polymorphisms and the risk of asthma: a meta-analysis," *Human Immunology*, vol. 74, no. 5, pp. 648–657, 2013.

[33] G. G. Song, J. H. Kim, and Y. H. Lee, "Association between ADAM33 S2 and ST+4 polymorphisms and susceptibility to asthma: a meta-analysis," *Gene*, vol. 524, no. 1, pp. 72–78, 2013.

[34] D. Vercelli, "Genetics, epigeneties, and the environment: switching, buffering, releasing," *Journal of Allergy and Clinical Immunology*, vol. 113, no. 3, pp. 381–387, 2004.

[35] Y. Yang, H. M. Haitchi, J. Cakebread et al., "Epigenetic mechanisms silence a disintegrin and metalloprotease 33 expression in bronchial epithelial cells," *Journal of Allergy and Clinical Immunology*, vol. 121, no. 6, pp. 1399.e1–1399.e14, 2008.

[36] Y. Yang, J. Wicks, H. M. Haitchi et al., "Regulation of a disintegrin and metalloprotease-33 expression by transforming growth factor-β," *American Journal of Respiratory Cell and Molecular Biology*, vol. 46, no. 5, pp. 633–640, 2012.

[37] P. J. Yang, R. N. Li, C. C. Huang et al., "The methylation patterns of a disintegrin and metalloproteinase 33 gene (ADAM33) in adult asthma," *International Archives of Allergy and Immunology*, vol. 161, no. 1, pp. 74–80, 2013.

[38] C. V. Breton, H.-M. Byun, M. Wenten, F. Pan, A. Yang, and F. D. Gilliland, "Prenatal tobacco smoke exposure affects global and gene-specific DNA methylation," *American Journal of Respiratory and Critical Care Medicine*, vol. 180, no. 5, pp. 462–467, 2009.

[39] G. Paulissen, N. Rocks, M. M. Gueders et al., "Role of ADAM and ADAMTS metalloproteinases in airway diseases," *Respiratory Research*, vol. 10, article 127, 2009.

[40] S. C. Foley, A. K. Mogas, R. Olivenstein et al., "Increased expression of ADAM33 and ADAM8 with disease progression in asthma," *Journal of Allergy and Clinical Immunology*, vol. 119, no. 4, pp. 863–871, 2007.

[41] J.-Y. Lee, S.-W. Park, H. K. Chang et al., "A disintegrin and metalloproteinase 33 protein in patients with asthma: relevance to airflow limitation," *American Journal of Respiratory and Critical Care Medicine*, vol. 173, no. 7, pp. 729–735, 2006.

[42] P. Tripathi, S. Awasthi, N. Husain, R. Prasad, and V. Mishra, "Increased expression of ADAM33 protein in asthmatic patients as compared to non-asthmatic controls," *The Indian Journal of Medical Research*, vol. 137, no. 3, pp. 507–514, 2013.

[43] F. Lin, A. Song, J. Wu et al., "ADAM33 protein expression and the mechanics of airway smooth muscle cells are highly correlated in ovalbumin-sensitized rats," *Molecular Medicine Reports*, vol. 8, no. 4, pp. 1209–1215, 2013.

[44] R. M. Powell, J. Wicks, J. W. Holloway, S. T. Holgate, and D. E. Davies, "The splicing and fate of ADAM33 transcripts in primary human airways fibroblasts," *American Journal of Respiratory Cell and Molecular Biology*, vol. 31, no. 1, pp. 13–21, 2004.

[45] S. J. McMillan, G. Xanthou, and C. M. Lloyd, "Manipulation of allergen-induced airway remodeling by treatment with anti-TGF-β antibody: effect on the Smad signaling pathway," *Journal of Immunology*, vol. 174, no. 9, pp. 5774–5780, 2005.

[46] J. F. Alcorn, L. M. Rinaldi, E. F. Jaffe et al., "Transforming growth factor-β1 suppresses airway hyperresponsiveness in allergic airway disease," *American Journal of Respiratory and Critical Care Medicine*, vol. 176, no. 10, pp. 974–982, 2007.

[47] R. Halwani, S. Al-Muhsen, H. Al-Jahdali, and Q. Hamid, "Role of transforming growth factor-β in airway remodeling in asthma," *American Journal of Respiratory Cell and Molecular Biology*, vol. 44, no. 2, pp. 127–133, 2011.

[48] X. Peng, S. K. Mathai, L. A. Murray et al., "Local apoptosis promotes collagen production by monocyte-derived cells in transforming growth factor β1-induced lung fibrosis," *Fibrogenesis and Tissue Repair*, vol. 4, no. 1, article 12, 2011.

[49] P. A. Frischmeyer-Guerrerio, A. L. Guerrerio, G. Oswald et al., "TGFβ receptor mutations impose a strong predisposition for human allergic disease," *Science Translational Medicine*, vol. 5, no. 195, pp. 195–194, 2013.

[50] A. Torrego, M. Hew, T. Oates, M. Sukkar, and K. Fan Chung, "Expression and activation of TGF-β isoforms in acute allergen-induced remodelling in asthma," *Thorax*, vol. 62, no. 4, pp. 307–313, 2007.

[51] A. Rosendahl, D. Checchin, T. E. Fehniger, P. ten Dijke, C.-H. Heldin, and P. Sideras, "Activation of the TGF-β/activin-Smad2 pathway during allergic airway inflammation," *American Journal of Respiratory Cell and Molecular Biology*, vol. 25, no. 1, pp. 60–68, 2001.

[52] R. J. McAnulty, "Fibroblasts and myofibroblasts: their source, function and role in disease," *International Journal of Biochemistry and Cell Biology*, vol. 39, no. 4, pp. 666–671, 2007.

[53] Z. Jie, M. Jin, Y. Cai et al., "The effects of Th2 cytokines on the expression of ADAM33 in allergen-induced chronic airway inflammation," *Respiratory Physiology and Neurobiology*, vol. 168, no. 3, pp. 289–294, 2009.

Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2014, Article ID 591479, 13 pages
http://dx.doi.org/10.1155/2014/591479

Research Article

Aryl Hydrocarbon Receptor (AhR) Modulates Cockroach Allergen-Induced Immune Responses through Active TGFβ1 Release

Yufeng Zhou,[1] Sarah Mirza,[1,2] Ting Xu,[1] Priya Tripathi,[1] Beverly Plunkett,[1] Allen Myers,[1] and Peisong Gao[1]

[1] *Johns Hopkins Asthma and Allergy Center, Johns Hopkins University School of Medicine, Baltimore, MD 21224, USA*
[2] *Sher-i-Kashmir Institute of Medical Sciences, Medical College (SKIMS), Kashmir 190001, India*

Correspondence should be addressed to Peisong Gao; pgao1@jhmi.edu

Received 13 December 2013; Revised 14 February 2014; Accepted 15 February 2014; Published 26 March 2014

Academic Editor: Shaoheng He

Background. Aryl hydrocarbon receptor (AhR), a multifunctional regulator that senses and responds to environmental stimuli, plays a role in normal cell development and immune regulation. Recent evidence supports a significant link between environmental exposure and AhR in the development of allergic diseases. We sought to investigate whether AhR plays a role in mediating cockroach allergen-induced allergic immune responses. *Methods*. AhR expression in human lung fibroblasts from asthmatic and healthy individuals and in cockroach extract (CRE) treated human lung fibroblasts (WI-38) was examined. The role of AhR in modulating CRE induced TGFβ1 production was investigated by using AhR agonist, TCDD, antagonist CH122319, and knockdown of AhR. The role of latent TGFβ1 binding protein-1 (LTBP1) in mediating TCDD induced active TGFβ1 release was also examined. *Results*. AhR expression was higher in airway fibroblasts from asthmatic subjects as compared to healthy controls. AhR in fibroblasts was activated by TCDD with an increased expression of cyp1a1 and cyp1b1. Increased AhR expression was observed in CRE-treated fibroblasts. Importantly, CRE induced TGFβ1 production in fibroblasts was significantly enhanced by TCDD but inhibited by CH122319. Reduced TGFβ1 production was further confirmed in fibroblasts with AhR knockdown. Moreover, AhR knockdown inhibited CRE induced fibroblast differentiation. Furthermore, TCDD induced active TGFβ1 release was significantly inhibited by LTBP1 knockdown. *Conclusion*. These results provide evidence for the role of AhR in modulating cockroach allergen-induced immune responses through controlling the active TGFβ1 release, suggesting a possible synergistic effect between exposure to allergens and environmental chemicals on the development of allergic diseases.

1. Introduction

Asthma is the most prevalent serious chronic illness of children in the U.S [1]. While it is generally accepted that environmental chemicals and pollutants can contribute to the occurrence and exacerbation of asthma [2–5], the mechanistic links remain largely unknown. Specifically, environmental chemicals and pollutants have been shown to modulate environmental allergen-induced allergic diseases like asthma [6–8]. AhR is a multifunctional regulator that senses and responds to environmental stimuli and plays a role in normal cell development and immune regulation. It is known that dioxins and dioxin-like compounds, TCDD, PAH, and particulate matter (PM), can activate AhR, which then translocates to the nucleus and dimerizes with AhR nuclear translocator (ARNT). Within the nucleus, the AhR/ARNT heterodimer binds to xenobiotic responsive element (XRE) sequences and leads to changes in gene transcription (e.g., cyp1a1, cyp1b1) and a variety of toxicological effects, such as ROS generation, cell differentiation, and inflammatory cytokine production [9, 10]. Recent discoveries regarding AhR and environmental toxicant interaction and its influence on immune responses [11–14] highlight the potential link between environmental exposure and AhR in modulating allergen-induced allergic

diseases. One intriguing and plausible hypothesis is that the expression of allergic and inflammatory disease could be attributable to those new immune "adjuvant" factors, for example, environmental chemicals, which often coexist with allergens and contribute to progressive fibrosis and pathological remodeling in asthma.

Indeed, recent studies have changed our understanding of asthma from that of a purely inflammatory disease to a disease in which both inflammatory and structural components are equally involved [15]. It has been suggested that there is a strong link between allergen-induced allergic airway inflammation and remodeling [16, 17]. In particular, exposure to cockroach allergen in early life can lead to allergic airway inflammation and an increased risk of developing asthma [18, 19]. One of the central components of airway remodeling is subepithelial fibrosis caused by deposition of collagen in asthma, a process in which fibroblasts and myofibroblast are critical. It has been shown that there is a correlation between the number of myofibroblasts and the degree of subepithelial fibrosis in the airway of asthmatic patients [20]. Of interest to us, allergens can induce an increase in the number of myofibroblasts [21], which may contribute to the progression of subepithelial fibrosis [22]. Recruitment of fibroblasts to the airway in asthma has been suggested to be potentiated by IL13 through a mechanism involving transforming growth factor-beta1 (TGF-β1) and MMPs [23].

TGF-β1 is produced by many cells within the lung, including fibroblasts, and plays a critical role in cell growth, differentiation, and immune regulation, and has been considered a principal mediator of airway remodeling [24–27]. Recent studies have demonstrated that disruption in TGFβ1 signaling imposes a strong predisposition for human allergic diseases [28]. Specifically, increased active TGFβ1 has been observed in airways from asthmatic patients [29] and from experimental mice during allergic airway inflammation [30] (Gao et al. JI 2014 in revision). Furthermore, increased TGFβ1 activity appears to be controlled by latent TGFβ-binding protein-1 (LTBP-1) [31]. Interestingly, primary mouse embryo fibroblasts from AhR−/− mice had increased expression of LTBP1 and higher levels of total and active TGFβ1 that can be partially blocked by antibodies against LTBP1 [32], suggesting that AhR may control LTBP1 expression and subsequently activation of TGFβ1 signaling. Although TGFβ1 signaling and the AhR pathway have been well-studied, detailed information about the interaction between AhR and TGFβ1 signaling remains largely unknown. Several mechanisms have been suggested regarding the regulation of AhR on TGFβ1 signaling, including deregulation of TGFβ1 secretion, suppression of TGFβ1, or downregulation of the LTBP1 expression [11, 32, 33]. Furthermore, studies on ITE, an AhR agonist, have suggested that ITE can disrupt TGFβ1 signaling by inhibiting the nuclear translocation of Smad2/3/4 and block TGFβ1-induced myofibroblast differentiation and extracellular matrix production [34]. TGFβ1, in turn, can suppress the AhR mediated gene expression through deregulation of AhR expression and/or localization [35]. Thus, studies on the cross-regulation between AhR and TGFβ1 signaling might be essential for better understanding of the underlying mechanisms for the synergic effects between environmental chemicals and allergens on the development of allergic diseases. Specifically, we postulated that active TGFβ1, released from fibroblasts or epithelium damaged by repeated environmental exposure, induces cell differentiation and immune regulation and is regulated by AhR.

In the present study, we have specifically focused on the functional significance of AhR in modulating cockroach allergen-induced immune responses through the release of active TGFβ1. We found increased AhR expression in airways of asthmatic patients. Especially, AhR expression was increased in WI-38, a lung fibroblast cell line, after exposure to cockroach allergen. We then demonstrated that TCDD, an AhR agonist, can induce AhR specific downstream genes cyp1a1 and cyp1b1 expression. Importantly, we found the modulating effects of AhR on cockroach allergen-induced TGFβ1 production and fibroblast differentiation. Finally, we demonstrated that TCDD-induced active TGFβ1 was significantly blocked by LTBP knockdown. Taken together, we suggest that AhR plays a role in modulating cockroach allergen-induced immune responses by controlling the active TGFβ1 release, and there is a possible synergistic effect between exposure to allergens and environmental chemicals on the development of allergic diseases.

2. Materials and Methods

2.1. Assessment of AhR in Human Airway. Paraffin-embedded human airway sections (5 μm) from asthmatic ($n = 4$) and healthy individuals ($n = 4$) were used for immunofluorescence analysis. These human airway sections were provided by Dr. Allen (Johns Hopkins Asthma & Allergen Center). While it is not clear about the allergic status for those asthmatic subjects, all healthy individuals were nonallergic individuals. The human airway sections from three nonallergic heavy smokers also included controlling for AhR expression. In brief, nonspecific binding was blocked using 10% blocking serum in PBS for 1 hour. Sections were subsequently incubated with anti-human polyclonal antibody against Aryl hydrocarbon receptor (ab84833, 1 : 20, Abcam) and fibroblast marker antibody ER-TR7 (sc-73355, 1 : 50, Santa Cruz Biotechnology) overnight at 4°C. Normal rabbit IgG (Sigma-Aldrich) and rat IgG were used as a negative control. Sections were then incubated with Alexa Fluor 594-labeled goat anti-rabbit and FITC-labeled rabbit anti-rat secondary antibodies IgG at 1/100 dilutor for 2 hrs at RT. Nuclei were counterstained with 4′,6-diamidino-2-phenylin-dole, dihydrochloride (DAPI) (Sigma-Aldrich). Sections were subsequently dehydrated, mounted, and observed under the fluorescent microscope. The slides were evaluated using micrographs taken by a fluorescent microscope (Olympus BX-5). Imaging software (iVision; Biovision) was used to analyze areas of positive staining.

2.2. Flow Cytometry. WI-38 cells were fixed with BD Cytofix/Cytoperm solution for 30 min and then incubated with specific first antibody or isotype control for 30 min at 4°C in the dark. Then the cells were washed and incubated with fluorescent-conjugated second antibody. The following

antibodies were used: anti-AhR (ab2770, Abcam) and anti-pSmad2/3(cell signaling). The samples were then analyzed on a FACSCalibur flow cytometer (BD Biosystems).

2.3. Immunocytochemical Analysis in WI 38 Cells. Cultured cells were fixed with 10% formalin at room temperature (RT) for 10 minutes and permeabilized for 5 mins with PBS containing Triton X100 and BSA buffer (0.3% TTX, 1% bovine serum albumin: TTX/BSA buffer). The cells were further blocked in 10% blocking serum for 30 min and then incubated with first antibody for 1 hour at RT. After washing with PBS, cells were incubated with fluorescent labelled secondary antibodies for 30 minutes at RT. Nuclei were counterstained with DAPI. Sections were subsequently dehydrated, mounted, and observed under the fluorescent microscope. The following antibodies were used: anti-AhR primary antibody (Abcam, ab2770, 1 : 20); anti-α-SMA (Abcam, ab32575); and anti-vimentin (eBioscience).

2.4. Quantitative Real-Time RT-PCR (qRT-PCR). Total RNA from WI38 was extracted with RNAeasy kit (Qiagen, Valencia, CA). RT-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, USA) according to the manufacturer's protocol. The primers for each gene were designed on the basis of the respective mRNA sequences so that the targets were 100–200 bp in length. Relative mRNA expression was calculated by normalization of all expression levels to actinand then compared to untreated control cells by the $\Delta\Delta$CT method as described previously [36]. The following primers were used:

Actin: F, AGAAAATCTGGCACCACACC; R, CAGAGGCGTACAGGGATAGC; **AhR**: F, GTCGTCTAAGGTGTCTGCTGGA; R, CGCAAACAAAGCCAACTGAGGTG; **cyp1a1**: F, GATTGAGCACTGTCAGGAGAAGC; R, ATGAGGCTCCAGGAGATAGCAG; **cyp1b1**: F, GCCACTATCACTGACATCTTCGG; R, CACGACCTGATCCAATTCTGCC; **ltbp1**: F, TGAATGCCAGCACCGTCATCTC; R, CTGGCAAACACTCTTGTCCTCC; **ltbp-2**: F, CTGCACAGATGACAACGAGTGTC; R, AGAGTGTAGCCAGGGTAGCAGA; **ltbp-3**: F, CGGTCACTACAAGTGCAACTGC; R, CTTGTTCTCGCATTTGCCATCCG; **ltbp-4**: F, TTCCAGTGCAGGACCTGTCCTT; R: GAAGGAGCCTTCGGTGTTAGTG.

2.5. ELISA. Supernatants from cultured fibroblasts (WI38) were collected and measured for active TGFβ1 by ELISA (eBiosciences), according to the manufacturer's instructions. Results were read with a Bio-Rad Bio-Plex instrument (Bio-Rad Laboratories, Hercules, CA).

2.6. Gene Knockdown by siRNA. Transcriptional knockdown was performed by transfection with siRNA oligonucleotide duplexes as a final concentration of 20 nM in DMEM using DharmaFECT transfection reagent (Thermo Scientific, Waltham, MA). A siRNA/transfection reagent complex was formed when siRNA and transfection reagent were mixed for 20 minutes. Then the transfection complex was added to each experimental well and incubated in the serum free media for 6 hours at 37°C. The transfection medium was replaced with complete medium and incubated at 37°C for an additional 24–48 hours. The gene knockdown was confirmed by qRT-PCR and western blotting. AhR specific siRNA was purchased from Thermo Scientific and LTBP1 specific siRNA was purchased from Sigma.

2.7. Western Blotting. Cells were washed twice with ice cold PBS and lysed in RIPA buffer containing protease and phosphatase inhibitor cocktails (Sigma). Protein content was measured with BCA reagent (Pierce). Equivalent protein samples were subjected to SDS-PAGE electrophoresis and then transferred to a polyvinylidene difluoride membrane (Millipore). After blocking with 5% nonfat dry milk in TBST, the membrane was incubated with primary anti-AhR (Abcam), anti-LTBP1 (GeneTex), anti-α-SMA (Abcam, ab32575), anti-vimentin (eBioscience), or anti-β-actin (clone C4, Santa Cruz) antibody as a loading control for normalization. Proteins reactive with primary Abs were visualized with HRP-conjugated secondary Ab and ECL reagents (Amersham). The levels of proteins were quantified by ImageJ (National Institutes of Health, USA) for the densitometric analysis of the band intensities and normalized to those of β-actin.

2.8. Statistical Analysis. Data are expressed as the means $\pm$ SEM for each group. Statistical significance for normally distributed samples was assessed using an independent two-tailed Student's t-test or with analysis of variance by using GraphPad Prism version 5.1 software (GraphPad Software, La Jolla, CA). Differences with $P < 0.05$ were considered statistically significant.

3. Results

3.1. Increased AhR Expression in Fibroblasts from Asthmatic Patients. To examine whether there was a differential expression for AhR in asthmatic and healthy individuals, we performed immunofluorescence analysis for both AhR and fibroblast marker ER-TR7 in human airway sections. Compared to healthy individuals (Figure 1, middle panel), the airway sections from asthmatic patients showed significant expression of AhR, increased fibroblasts marker ER-TR7, and thickening of basal membranes (Figure 1, top panel). Particularly, AhR was predominantly expressed in fibroblasts and basal membranes. Interestingly, significantly increased AhR expression was also observed in airway fibroblasts from heavy smokers (Figure 1, bottom panel). These findings suggest an increased AhR expression in fibroblasts from asthmatic patients and possibly from those who are repeatedly exposed to smoking.

3.2. Increased AhR Expression in CRE-Treated Human Lung Fibroblasts. To delineate the role of AhR in the regulation of fibroblast's function and its mechanisms, we used human lung fibroblast cell line as an *in vitro* model. To validate AhR expression in fibroblasts, we detected AhR expression in WI-38, a human lung fibroblast cell line, by

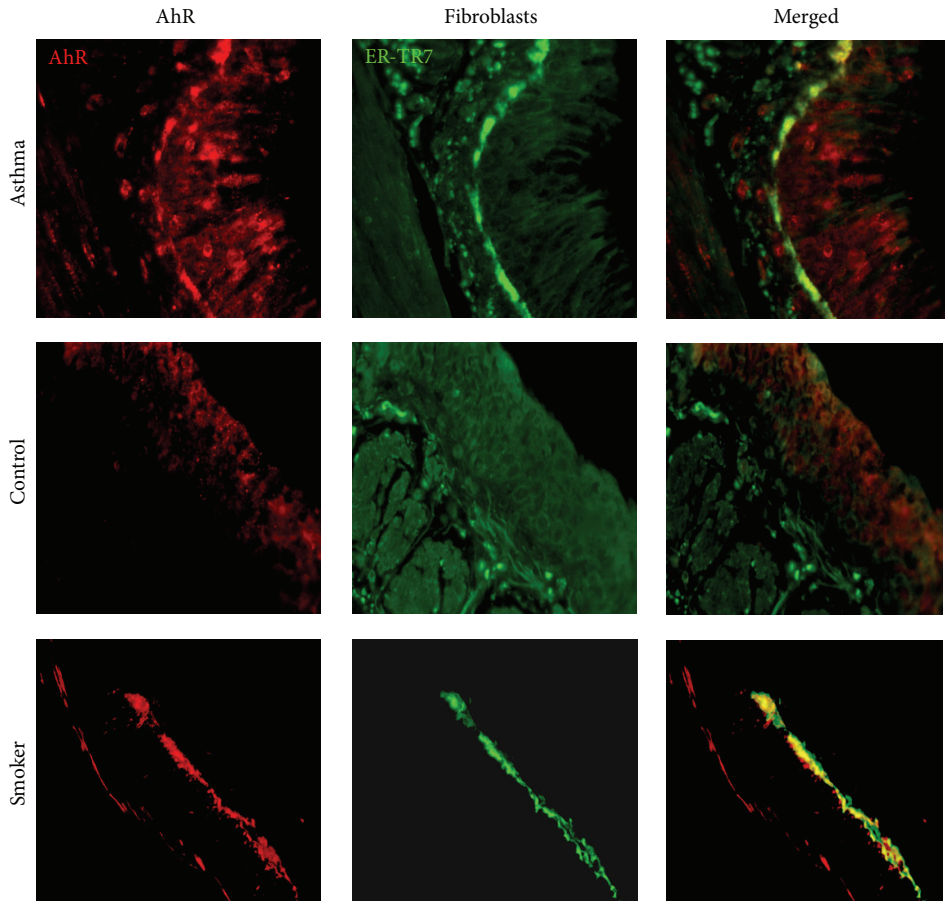


Figure 1: AhR expression in human airway. Immunofluorescence analysis of AhR expression in the airway, particularly fibroblasts from asthmatics (top), healthy individuals (middle), and heavy smokers (bottom), for antibodies against AhR (red) and fibroblasts marker (ER-TR-7, green). Figure 1 represents 4 individuals from each group.

flow cytometry and western blot (data not shown). We found that AhR was constitutively expressed in fibroblasts (Figure 2(a)). We next examined whether AhR is functional; we treated fibroblasts using different doses of TCDD known AhR ligands (0.1 nM and 1 nM) for 2 to 48 hours; expression of AhR downstream genes cyp1a1 (Figure 2(b)) and cyp1b1 (Figure 2(c)) was examined by RT-PCR. Compared to those untreated fibroblasts, an increased expression was noted in TCDD treated fibroblasts for cyp1a1 in a dose- and time-dependent manner. There was nearly a 2-fold increase in cyp1a1 expression after treatment with 1.0 nM TCDD for 48 hours. Similarly, an 18.5-fold increase was observed for cyp1b1 when 1.0 nM TCDD was used to treat fibroblasts for 48 hours, suggesting that TCDD can activate the AhR pathway in fibroblasts. Furthermore, to investigate whether CRE can induce AhR expression, we treated fibroblasts with 50 μg/mL CRE for 2–48 hours and AhR expression was examined by RT-PCR. An increased AhR expression was seen with a peak level at 4 hours for a 5.7-fold increase when compared with untreated cells (Figure 2(d)). Increased AhR expression in a dose-dependent manner was further observed by immunofluorescence analysis (Figure 2(e)), suggesting that cockroach allergen can induce AhR expression which may be critical in modulating allergen-induced immune responses.

3.3. AhR Modulates CRE Induced TGFβ1 Production in Fibroblasts. To investigate whether AhR can modulate cockroach allergen-induced TGFβ1 production that may control cell differentiation and immune regulation, we treated fibroblasts with CRE (50 μg/mL) in the presence or absence of AhR agonist TCDD or antagonist CH122319 and detected the levels of active TGFβ1 in supernatants of cultured and treated fibroblasts. We found that TCDD induced a significant release of active TGFβ1 by fibroblasts that were treated with TCDD at various doses (1.0, 10 nM) and times (24, 48 hours, Figure 3(a)). Both cockroach allergen and TCDD as an individual induced increased levels of active TGFβ1 by fibroblasts. Interestingly, the increased TGFβ1 was further enhanced when both of them were combined (Figure 3(b)). In contrast, AhR antagonist CH122319 inhibited CRE+TCDD induced TGFβ1 production (Figure 3(b)). Cockroach allergen-induced active TGFβ1 release and the inhibition of CH122319 were further confirmed by using natural purified cockroach allergen, Bla g2 (Figure 3(c)).

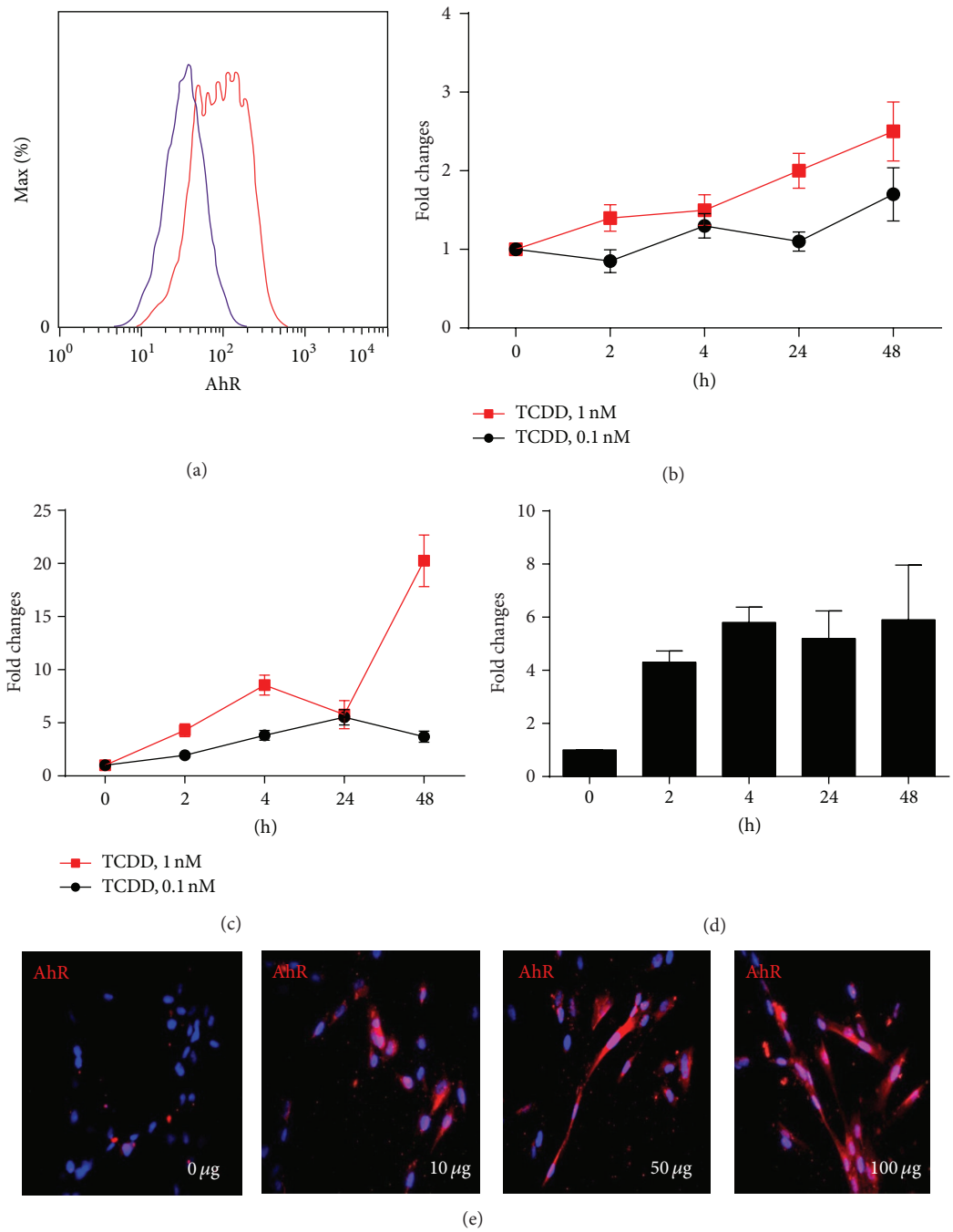


Figure 2: AhR expression in CRE-treated human lung fibroblasts. (a) AhR expression in WI-38 was detected using antibody against AhR (red line) and IgG2 (blue line) by flow cytometry. (b-c) Fibroblasts were treated with different doses of TCDD (0.1 nM and 1 nM) for 2 to 48 hours; expressions of cyp1a1 (b) and cyp1b1 (c) were examined by RT-PCR. (d) Fibroblasts were treated with 50 μg/mL CRE for 2–48 hours and AhR expression was examined by RT-PCR. (e) Immunofluorescence analysis of AhR expression in CRE-treated fibroblasts at various doses (0–100 μg/mL). Bars represent mean ± SEM of 3 independent experiments.

These findings suggest that AhR can modulate cockroach allergen-induced TGFβ1 production.

3.4. Reduced Levels of Active TGFβ1 in Fibroblasts with AhR Knockdown. To further examine the modulation of AhR on cockroach allergen-induced TGFβ1 production, we knocked down AhR in fibroblasts using siRNAs. The AhR knockdown was validated by RT-PCR (Figure 4(a)), which showed at least 60% knockdown for si-RNA-2, and by western blotting (Figure 4(b)). AhR expression in Figure 4(b) was further quantified by ImageJ for the densitometric analysis of the band intensities and normalized to those

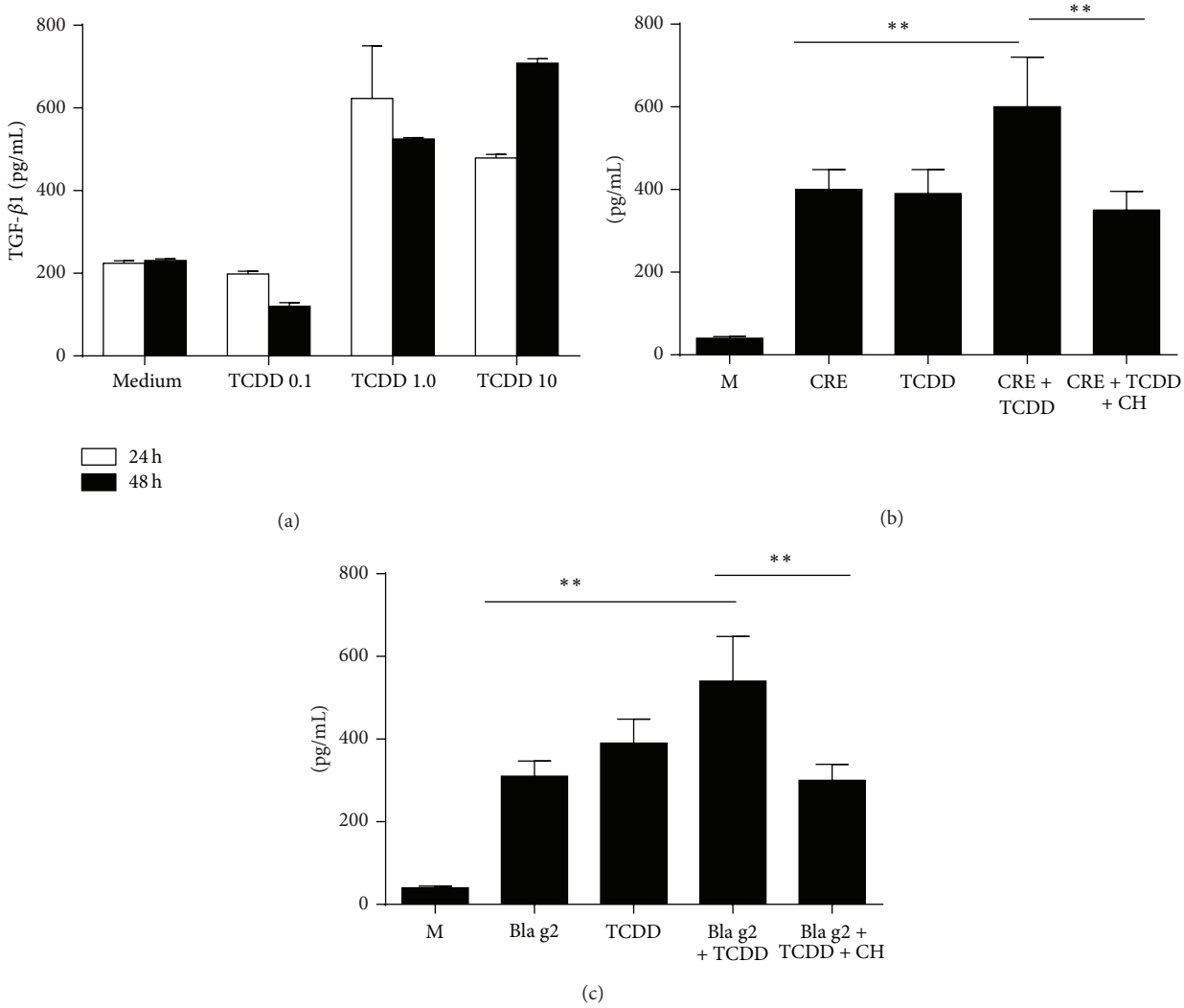


Figure 3: AhR modulates cockroach allergen-induced TGFβ1 production. (a) TCDD can induce TGFβ1 secretion by fibroblasts and (b) cockroach allergen-induced TGFβ1 secretion by fibroblasts can be further enhanced by TCDD but inhibited by AhR antagonist CH122319 (c). (c) Cockroach allergen-induced TGFβ1 production and the inhibitory role of CH122319 were further confirmed when Bla g2 was used. Bars represent mean ± SEM of 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

of β-actin and then compared to scramble si-RNA treated group (Figure 4(c)). There was at least 70% knockdown for si-RNA-2. We next used fibroblasts with AhR knocked down by si-AhR-2 to detect TGFβ1 expression at the RNA levels by RT-PCR (Figure 4(d)) and active TGFβ1 secretion by ELISA (Figure 4(e)). We found that TGFβ1 expression at the RNA level or in secretion was significantly reduced for fibroblasts with AhR knockdown. To examine whether AhR knockdown can affect the activation of TGFβ signaling, we treated those fibroblasts with or without AhR knockdown with 5 ng/mL TGFβ1 and measured phosphorylated Smad2/3 (p-Smad2/3) at various times by flow cytometry (Figure 4(f)). We noted an increased activation of Smad2/3 at 15 min for all these treated cells but we noted a decline at 30 mins and 120 mins. Interestingly, fibroblasts with AhR knockdown showed remarkable reduction in the levels of p-Smad2/3 at 120 mins as compared to the control cells. These data suggest that there may be a crosstalk between AhR pathway and TGFβ1 pathway.

3.5. AhR Modulates CRE Induced Fibroblast Differentiation. To examine whether AhR controls fibroblast differentiation induced by CRE, we cultured fibroblasts with and without AhR knockdown and treated with CRE (50 μg/mL) for 24 and 72 hours. Differentiation of fibroblasts was evaluated by the expression of α-SMA with DAPI for nuclei immune-staining. While no clear change was noted in the α-SMA expression for fibroblasts with and without AhR knockdown at basal levels and 24 hours, an increased expression was seen when treated with CRE for 72 hours for fibroblasts without AhR knockdown. The α-SMA positive staining in fibroblasts was quantified and analyzed by Imaging software (Figure 5(b)).

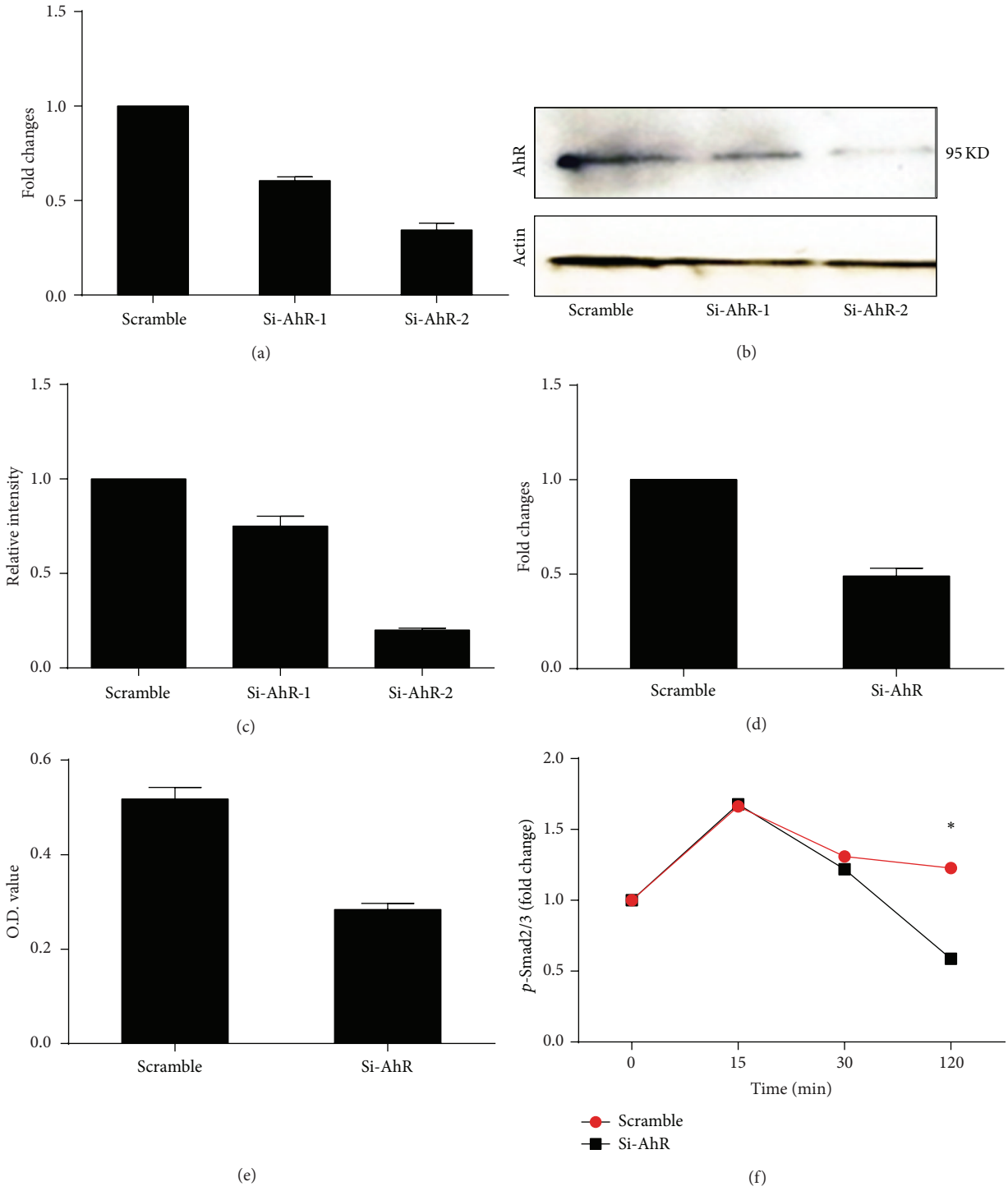


Figure 4: TGFβ1 production was reduced in fibroblasts with AhR knockdown by siRNA. (a–c) AhR knockdown by siRNAs was confirmed by RT-PCR (a) and western blotting (b). (c) AhR expression in (b) was quantified by ImageJ and normalized to those of β-actin and then compared to scramble si-RNA treated group. (d) TGFβ1 expression in fibroblasts treated with scrambled siRNA and si-AhR. (e) Levels of secreted TGFβ1 stimulated with CRE by fibroblasts treated with scramble si-RNA and si-AhR. (f) WI38 cells were stimulated with 5 ng/mL TGFβ1 for indicated time after AhR knockdown for 48 h; p-Smad2/3 was detected with flow cytometry. Bars represent mean ± SEM of 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

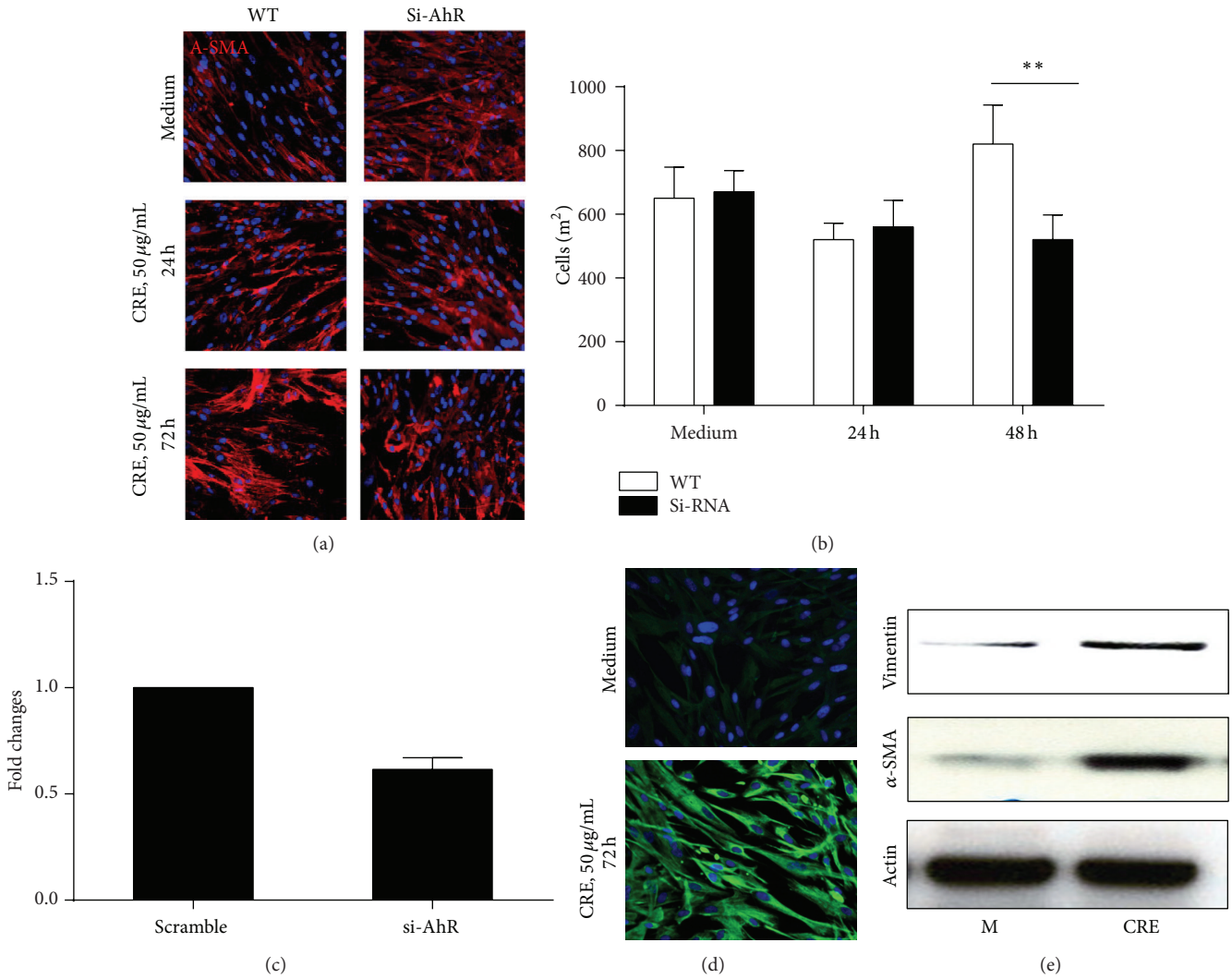


FIGURE 5: AhR controls fibroblast differentiation induced by CRE. (a) Differentiation of fibroblasts with and without AhR knockdown was evaluated by the expression of α-SMA with DAPI for nuclei immune-staining after cells were treated with CRE (50 μg/mL) for 24 and 72 hours. (b) Positive staining for α-SMA was analyzed by Imaging software (iVision; Biovision). (c) α-SMA expression was detected by RT-PCR in fibroblasts with or without AhR knockdown. Bars represent mean ± SEM of 3 independent experiments, $^{**}P < 0.01$. (d) Vimentin was detected by immune-staining after cells were treated with CRE (50 μg/mL) for 72 hours. (e) Both α-SMA and vimentin expression were detected by western blots after WI38 cells were treated with 50 μg/mL CRE for 72 h.

A significantly greater expression of α-SMA was observed for fibroblasts with CRE treatment. Finally, reduced α-SMA expression was detected by RT-PCR in fibroblasts with AhR knockdown as compared to those without gene knockdown (Figure 5(c)), suggesting that AhR may control cockroach allergen-induced differentiation. CRE induced fibroblast differentiation was further confirmed by immune-staining (Figure 5(d)) and western blots (Figure 5(e)) with another myofibroblast marker, vimentin.

3.6. Increased LTBP1 Expression in TCDD Treated Fibroblasts. TGFβ1 activity has been shown to be controlled by LTBP-1 [31]. Furthermore, it has been suggested that AhR may control LTBP1 expression, and subsequently activation of TGFβ1 signaling [32]. To investigate whether the AhR ligand can activate LTBP1 that may control TGFβ1 release, we treated fibroblasts using different doses of TCDD (0.1 nM and 10 nM) for different times (2 to 48 hours), and expression of LTBP1 to 4 at the RNA levels was measured by RT-PCR. Of these, increased LTBP1 was observed after treatment with TCDD (Figure 6(a)). The peak level was at 4 h for 0.1 nM and 24 h for 1 nM TCDD, respectively. Reduced expression of LTBP-2 was detected (Figure 6(b)), while no significant changes of LTBP-3 and 4 were detected (data not shown). To see the importance of increased LTBP-1 in AhR modulating active TGFβ1 release, we pretreated fibroblasts with LTBP-1 specific siRNA. We found that TCDD induced active TGFβ1

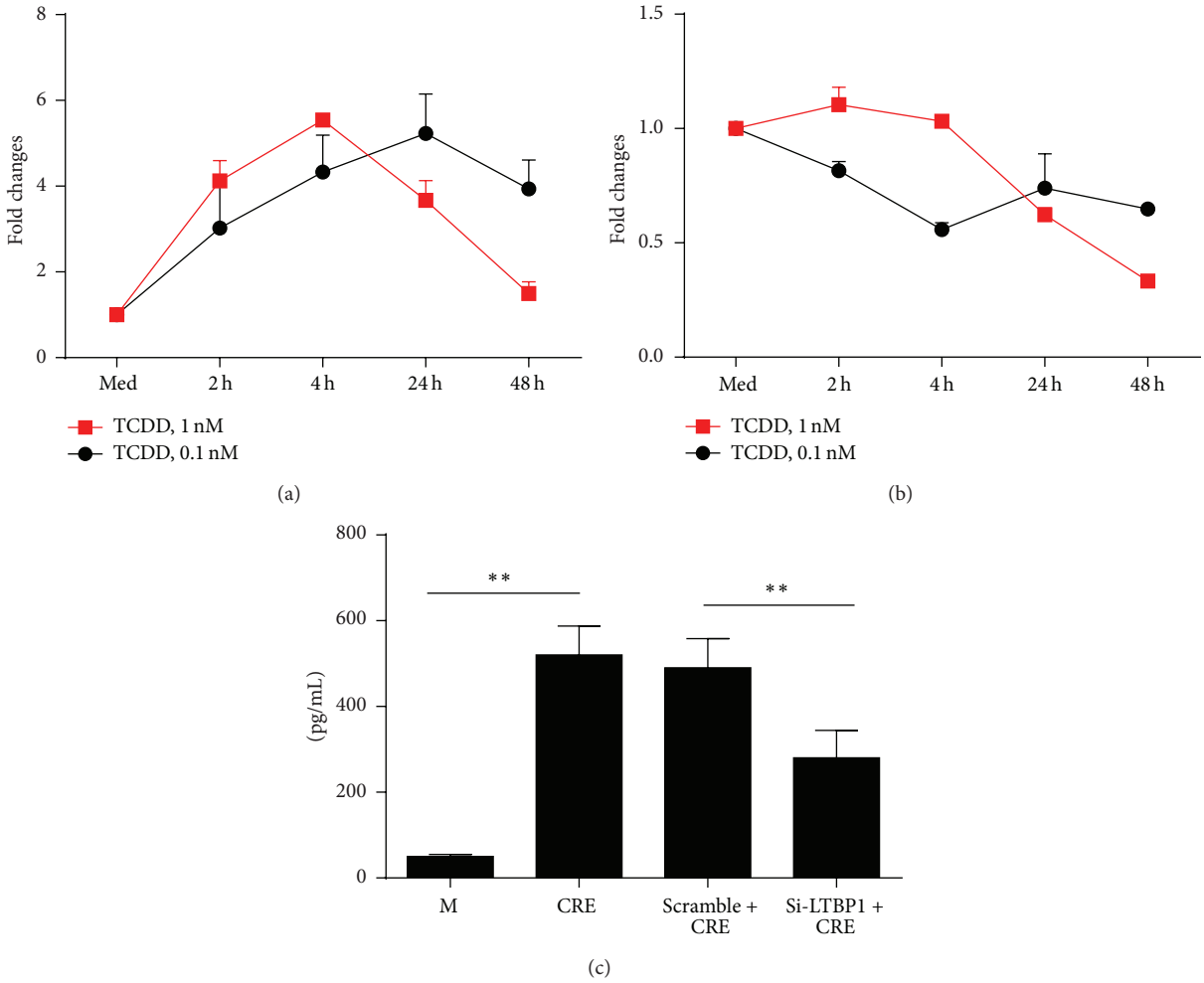


FIGURE 6: AhR controls LTBP-1 expression in fibroblasts. (a-b) Fibroblasts were treated with TCDD at 0.1 nM and 1 nM for 2 to 48 hours; expression of LTBP1 (a) and LTBP-2 (b) was detected by RT-PCR. Each point represents mean ± SEM of at least 3 independent experiments. (c) WI38 cells were treated with LTBP1 siRNA, scramble RNA, or medium control for 24 h and then treated with 50 μg/mL CRE for 24 h; active form TGFβ1 in the supernatant was detect with ELISA.

was significantly inhibited when LTBP-1 was knockdown (Figure 6(c)), suggesting that LTBP-1 may be critical in AhR controlling the release of active TGFβ1.

4. Discussion

In the present study, we investigated the functional significance of AhR in modulating cockroach allergen-induced immune responses by controlling the release of active TGFβ1. We found an increased AhR expression in the airways of asthmatic patients, mainly in airway epithelium and fibroblasts. While it is recognized that dioxins and dioxin-like compounds, TCDD, PAH, and PM, can activate AhR and lead to ROS generation, cell differentiation, and inflammatory cytokine production, we, for the first time, found that AhR ligands could enhance cockroach allergen-induced active TGFβ1 production. The findings may suggest a possible biological link between environmental exposure and AhR in modulating allergen-induced allergic diseases. Because of the increased expression of AhR in epithelium, and predominantly in fibroblasts of the thickening basal membrane from asthmatic patients, it is possible that AhR as a sensor for environmental chemicals and allergens contributes to progressive fibrosis and pathological remodeling in asthma.

TGFβ1 has been shown to be critical in cell growth, differentiation, and immune regulation, and a principal mediator of airway remodeling [24–27]. Our recent studies have observed an increased active TGFβ1 in airways from cockroach allergen-induced mouse models (Gao et al., submitted 2013). In this study, we found that cockroach allergen can induce an increased production of active TGFβ1 in fibroblasts. It is known that TGFβ1 and AhR signaling pathways can crossregulate each other in a cell-specific manner [37]. We found that TCDD as an AhR agonist can enhance cockroach allergen-induced TGFβ1 production, but CH122319 as an antagonist can inhibit allergen-induced TGFβ1 secretion. TCDD that we used in this study has

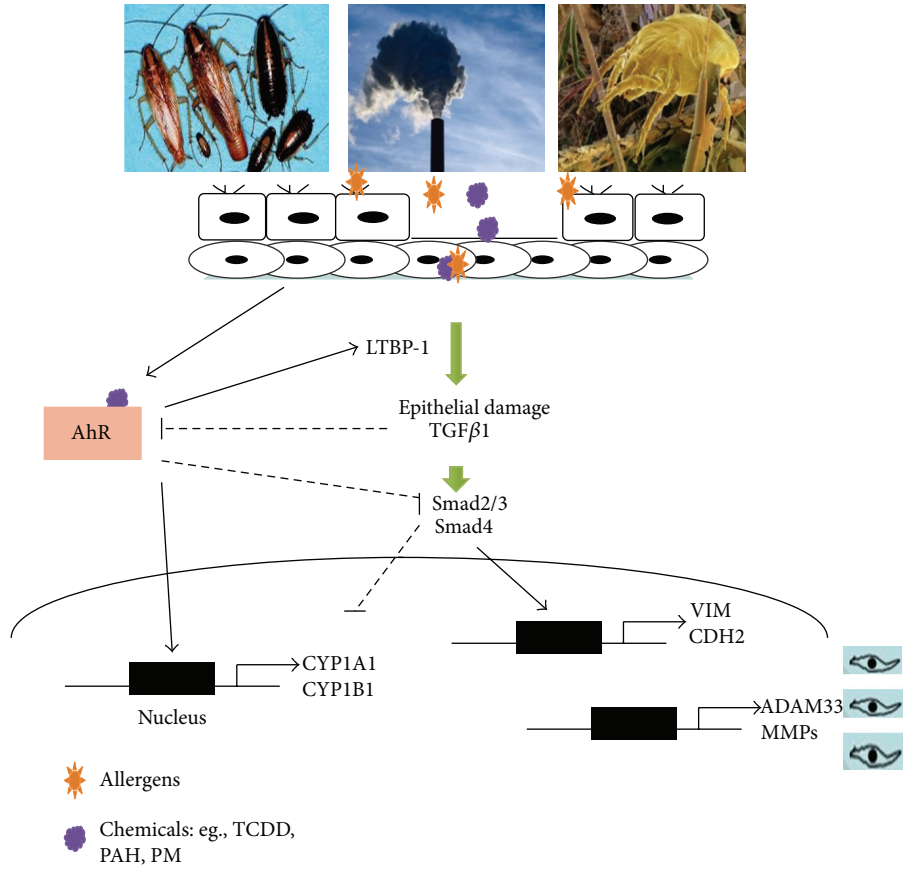


Figure 7: Proposed model of the role of AhR in modulating environmental chemicals and allergens induced activation of TGFβ1 signaling (modified from an article by Denison and Nagy 2003) [38].

been considered as the most potent AhR ligand known and has been shown to be only slightly metabolized and to be relatively slowly excreted [38, 39]. The role of AhR in regulating cockroach allergen-induced TGFβ1 release was further confirmed by using Bla g2, a purified cockroach allergen with undetectable endotoxin levels, and by using a human fibroblast cell line (WI38) with or without AhR knockdown. Particularly, the fibroblasts with AhR knockdown showed reduction of the levels of active TGFβ1 as compared to the control cells. Furthermore, those cells with AhR knockdown showed remarkable reduction in the levels of p-Smad2/3. These findings suggest that AhR may be a positive regulator of cockroach allergen-induced TGFβ1 signaling in human fibroblasts. Our findings seem to be contradictory to several previous studies suggesting a negative role of AhR in the initiation of food allergic responses [40, 41], regulation of TGFβ1 secretion, and LTBP1 expression [11, 32, 42]. For instance, ITE, an AhR agonist, has been demonstrated to disrupt TGFβ1 signaling by inhibiting the nuclear translocation of Smad2/3/4 and to block TGFβ1-induced myofibroblast differentiation and extracellular matrix production [34]. In contrast, some other studies suggest that constitutive AhR activity positively controls TGFβ1, TGFβ2, and LTBP-1 in malignant glioma cells [43]. Thus, we postulate that there may be significant complexity of the regulatory mechanisms of AhR on TGFβ1 signaling with coregulation of multiple other pathways.

A significant correlation has been observed between the number of myofibroblasts and the degree of subepithelial fibrosis in the airway of asthmatic patients [20], and fibroblasts have been recognized as major players by differentiation into myofibroblasts that may control the development subepithelial fibrosis in asthma. Further, TGF-β1 has been suggested to recruit fibroblasts to the airway in asthma [23]. We thus investigated whether AhR controls fibroblast differentiation after exposure to cockroach allergen. We found that, while there was no clear change in α-SMA expression (a marker for myofibroblasts) for fibroblasts with and without AhR knockdown at basal levels and at 24 hours, inhibited differentiation of fibroblasts was observed in AhR knocked-down fibroblasts with CRE treatment for 72 hours. This was further confirmed by showing a reduced α-SMA expression at the transcriptional level. Interestingly, a significant reduction in fibroblast proliferation was also noted at 72 hours (data not shown). Thus, we, for the first time, illustrate that AhR may control cockroach allergen-induced fibroblast differentiation that is critical in controlling subepithelial fibrosis and airway remodelling.

To explore the possible underlying mechanisms of AhR modulating allergen-induced TGFβ1 signaling, we investigated LTBP1, which has been shown to be critical in controlling TGFβ1 activity [31]. AhR has been shown to regulate Ltbp-1 transcription by a mechanism involving recruitment of coactivators such as CREB1 and corepressors such as HDAC2 to the Ltbp-1 promoter [44]. We thus hypothesized that AhR may regulate LTBP-1 transcription, control gene expression, and subsequently activate TGFβ1 signaling [32]. Indeed, an increased LTBP1 was observed after treatment with TCDD with the peak levels of expression at early time points (4 h). In contrast, LTBP-2 expression was reduced, and LTBP-3 and 4 remained unchanged after treatment with TCDD (data not shown). Most importantly, AhR modulating active TGFβ1 release was significantly blocked when LTBP-1 was knocked down. The findings support our initial hypothesis that LTBP-1 is critical in controlling AhR mediated TGFβ1 secretion. Although the mechanism is not clear regarding the regulation of active TGFβ1 by LTBP-1, studies have suggested that LTBP-1 contributes to TGF-beta1 activation, possibly through a process involving extracellular protease activities [31]. However, our findings in human fibroblasts showed that activation of AhR pathway can increase LTBP-1 expression, but in contrast, studies on the primary mouse embryo fibroblasts from AhR−/− mice also showed an increased expression of LTBP1 and higher levels of active TGFβ1 [32]. So far, the reasons for the discrepancies between studies from human and mouse fibroblasts with or without AhR knockdown are largely unknown, a subject which would be of interest to pursue in the future.

Taken together, this study provides evidence for the contribution of AhR to the mechanism regulating cockroach allergen-induced activation of TGFβ1. In particular, studies on the interplay between AhR and TGFβ1 pathways may better help us understand the potential mechanisms regarding the environmental chemical exposure modulating allergen-induced immune responses. As shown in Figure 7, we propose a model of the role of AhR in modulating environmental chemicals and allergen-induced activation of TGFβ1 signaling (partially modified based on the article by Starsichova et al. 2012) [35]. Briefly, epithelial cells damaged by repeated exposure to environmental chemicals and allergens can release active TGFβ1, which is largely controlled by LTBP-1. On the other hand, environmental chemicals bind AhR, leading to its activation, which plays a role in the control of LTBP-1 transcription, activation of TGFβ1 signaling, and subsequently the control of cell proliferation, differentiation, and airway remodeling. These studies provide an important basis for a further detailed investigation of the interaction between AhR and TGFβ1 signaling in environmental chemicals and allergens induced inflammation and repair/remodeling in asthma.

Abbreviations

CRE: Cockroach extract
AhR: Aryl hydrocarbon receptor (AhR)
TCDD: 2,3,7,8-Tetrachlorodibenzo-p-dioxin
TGFβ: Transforming growth factor β
cyp1a1: Cytochrome P450, family 1, subfamily B
cyp1b1: Cytochrome P450, family 1, subfamily B
LTBP-1: Latent TGFβ-binding protein-1 (LTBP-1)
α-SMA: α-Smooth muscle actin.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This research was supported by the National Institutes of Health (NIH), Grants 1R21AI088406 and RO1ES021739. The authors thank Liang Yan for her excellent technical assistance.

References

[1] M. Masoli, D. Fabian, S. Holt, and R. Beasley, "The global burden of asthma: executive summary of the GINA dissemination committee report," *Allergy*, vol. 59, no. 5, pp. 469–478, 2004.

[2] D. Jenerowicz, W. Silny, A. Danczak-Pazdrowska, A. Polanska, A. Osmola-Mankowska, and K. Olek-Hrab, "Environmental factors and allergic diseases," *Annals of Agricultural and Environmental Medicine*, vol. 19, no. 3, pp. 475–481, 2012.

[3] B.-F. Hwang, Y.-L. Lee, Y.-C. Lin, J. J. K. Jaakkola, and Y. L. Guo, "Traffic related air pollution as a determinant of asthma among Taiwanese school children," *Thorax*, vol. 60, no. 6, pp. 467–473, 2005.

[4] N. N. Hansel, M. C. McCormack, A. J. Belli et al., "In-home air pollution is linked to respiratory morbidity in former smokers with chronic obstructive pulmonary disease," *American Journal of Respiratory and Critical Care Medicine*, vol. 187, no. 10, pp. 1085–1090, 2013.

[5] K. K. Nishimura, J. M. Galanter, L. A. Roth et al., "Early-life air pollution and asthma risk in minority children. The GALA II and SAGE II studies," *American Journal of Respiratory and Critical Care Medicine*, vol. 188, no. 3, pp. 309–318, 2013.

[6] J.-T. Lee, J.-Y. Son, and Y.-S. Cho, "The adverse effects of fine particle air pollution on respiratory function in the elderly," *Science of the Total Environment*, vol. 385, no. 1–3, pp. 28–36, 2007.

[7] K. Ito, R. Mathes, Z. Ross, A. Nádas, G. Thurston, and T. Matte, "Fine particulate matter constituents associated with cardiovascular hospitalizations and mortality in New York City," *Environmental Health Perspectives*, vol. 119, no. 4, pp. 467–473, 2011.

[8] J. Zhou, K. Ito, R. Lall, M. Lippmann, and G. Thurston, "Time-series analysis of mortality effects of fine particulate matter components in Detroit and seattle," *Environmental Health Perspectives*, vol. 119, no. 4, pp. 461–466, 2011.

[9] F. Ohtake, K.-I. Takeyama, T. Matsumoto et al., "Modulation of oestrogen receptor signalling by association with the activated dioxin receptor," *Nature*, vol. 423, no. 6939, pp. 545–550, 2003.

[10] A. Puga, C. R. Tomlinson, and Y. Xia, "Ah receptor signals cross-talk with multiple developmental pathways," *Biochemical Pharmacology*, vol. 69, no. 2, pp. 199–207, 2005.

[11] F. J. Quintana, A. S. Basso, A. H. Iglesias et al., "Control of Treg and TH17 cell differentiation by the aryl hydrocarbon receptor," *Nature*, vol. 453, no. 7191, pp. 65–71, 2008.

[12] M. Veldhoen, K. Hirota, A. M. Westendorf et al., "The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins," *Nature*, vol. 453, no. 7191, pp. 106–109, 2008.

[13] K. Masuda, A. Kimura, H. Hanieh et al., "Aryl hydrocarbon receptor negatively regulates LPS-induced IL-6 production through suppression of histamine production in macrophages," *International Immunology*, vol. 23, no. 10, pp. 637–645, 2011.

[14] Y. Zhou, H. Y. Tung, Y. M. Tsai et al., "Aryl hydrocarbon receptor controls murine mast cell homeostasis," *Blood*, vol. 121, no. 16, pp. 3195–3204, 2013.

[15] S. Al-Muhsen, J. R. Johnson, and Q. Hamid, "Remodeling in asthma," *Journal of Allergy and Clinical Immunology*, vol. 128, no. 3, pp. 451–462, 2011.

[16] S. Phipps, F. Benyahia, T.-T. Ou, J. Barkans, D. S. Robinson, and A. B. Kay, "Acute allergen-induced airway remodeling in atopic asthma," *American Journal of Respiratory Cell and Molecular Biology*, vol. 31, no. 6, pp. 626–632, 2004.

[17] C. M. Lloyd and D. S. Robinson, "Allergen-induced airway remodelling," *European Respiratory Journal*, vol. 29, no. 5, pp. 1020–1032, 2007.

[18] D. L. Rosenstreich, P. Eggleston, M. Kattan et al., "The role of cockroach allergy and exposure to cockroach allergen in causing morbidity among inner-city children with asthma," *The New England Journal of Medicine*, vol. 336, no. 19, pp. 1356–1363, 1997.

[19] E. C. Matsui, R. A. Wood, C. Rand et al., "Cockroach allergen exposure and sensitization in suburban middle-class children with asthma," *Journal of Allergy and Clinical Immunology*, vol. 112, no. 1, pp. 87–92, 2003.

[20] C. E. Brewster, P. H. Howarth, R. Djukanovic, J. Wilson, S. T. Holgate, and W. R. Roche, "Myofibroblasts and subepithelial fibrosis in bronchial asthma," *American Journal of Respiratory Cell and Molecular Biology*, vol. 3, no. 5, pp. 507–511, 1990.

[21] M. J. Gizycki, E. Ädelroth, A. V. Rogers, P. M. O'Byrne, and P. K. Jeffery, "Myofibroblast involvement in the allergen-induced late response in mild atopic asthma," *American Journal of Respiratory Cell and Molecular Biology*, vol. 16, no. 6, pp. 664–673, 1997.

[22] S. T. Holgate, "Pathogenesis of asthma," *Clinical and Experimental Allergy*, vol. 38, no. 6, pp. 872–897, 2008.

[23] J. L. Ingram, M. J. Huggins, T. D. Church et al., "Airway fibroblasts in asthma manifest an invasive phenotype," *American Journal of Respiratory and Critical Care Medicine*, vol. 183, no. 12, pp. 1625–1632, 2011.

[24] S. J. McMillan, G. Xanthou, and C. M. Lloyd, "Manipulation of allergen-induced airway remodeling by treatment with anti-TGF-β antibody: effect on the Smad signaling pathway," *Journal of Immunology*, vol. 174, no. 9, pp. 5774–5780, 2005.

[25] J. F. Alcorn, L. M. Rinaldi, E. F. Jaffe et al., "Transforming growth factor-β1 suppresses airway hyperresponsiveness in allergic airway disease," *American Journal of Respiratory and Critical Care Medicine*, vol. 176, no. 10, pp. 974–982, 2007.

[26] R. Halwani, S. Al-Muhsen, H. Al-Jahdali, and Q. Hamid, "Role of transforming growth factor-β in airway remodeling in asthma," *American Journal of Respiratory Cell and Molecular Biology*, vol. 44, no. 2, pp. 127–133, 2011.

[27] X. Peng, S. K. Mathai, L. A. Murray et al., "Local apoptosis promotes collagen production by monocyte-derived cells in transforming growth factor β1-induced lung fibrosis," *Fibrogenesis and Tissue Repair*, vol. 4, no. 1, article 12, 2011.

[28] P. A. Frischmeyer-Guerrerio, A. L. Guerrerio, G. Oswald et al., "TGFbeta receptor mutations impose a strong predisposition for human allergic disease," *Science Translational Medicine*, vol. 5, no. 195, Article ID 195ra94, 2013.

[29] A. Torrego, M. Hew, T. Oates, M. Sukkar, and F. C. Kian, "Expression and activation of TGF-β isoforms in acute allergen-induced remodelling in asthma," *Thorax*, vol. 62, no. 4, pp. 307–313, 2007.

[30] A. Rosendahl, D. Checchin, T. E. Fehniger, P. Ten Dijke, C.-H. Heldin, and P. Sideras, "Activation of the TGF-β/activin-Smad2 pathway during allergic airway inflammation," *American Journal of Respiratory Cell and Molecular Biology*, vol. 25, no. 1, pp. 60–68, 2001.

[31] A. Gomez-Duran, S. Mulero-Navarro, X. Chang, and P. M. Fernandez-Salguero, "LTBP-1 blockade in dioxin receptor-null mouse embryo fibroblasts decreases TGF-β activity: role of extracellular proteases plasmin and elastase," *Journal of Cellular Biochemistry*, vol. 97, no. 2, pp. 380–392, 2006.

[32] B. Santiago-Josefat, S. Mulero-Navarro, S. L. Dallas, and P. M. Fernandez-Salguero, "Overexpression of latent transforming growth factor-β binding protein 1 (LTBP-1) in dioxin receptor-null mouse embryo fibroblasts," *Journal of Cell Science*, vol. 117, no. 6, pp. 849–859, 2004.

[33] X. Chang, Y. Fan, S. Karyala et al., "Ligand-independent regulation of transforming growth factor β1 expression and cell cycle progression by the aryl hydrocarbon receptor," *Molecular and Cellular Biology*, vol. 27, no. 17, pp. 6127–6139, 2007.

[34] G. M. Lehmann, X. Xi, A. A. Kulkarni et al., "The aryl hydrocarbon receptor ligand ITE inhibits TGFβ1-induced human myofibroblast differentiation," *American Journal of Pathology*, vol. 178, no. 4, pp. 1556–1567, 2011.

[35] A. Starsichova, E. Hruba, E. Slabakova et al., "TGF-beta1 signaling plays a dominant role in the crosstalk between TGF-beta1 and the aryl hydrocarbon receptor ligand in prostate epithelial cells," *Cellular Signalling*, vol. 24, no. 8, pp. 1665–1676, 2012.

[36] P. Gao, D. N. Grigoryev, N. M. Rafaels et al., "CD14, a key candidate gene associated with a specific immune response to cockroach," *Clinical and Experimental Allergy*, vol. 40, no. 9, pp. 1353–1364, 2010.

[37] I. Tritschler, D. Gramatzki, D. Capper et al., "Modulation of TGF-β activity by latent TGF-β-binding protein 1 in human malignant glioma cells," *International Journal of Cancer*, vol. 125, no. 3, pp. 530–540, 2009.

[38] M. S. Denison and S. R. Nagy, "Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals," *Annual Review of Pharmacology and Toxicology*, vol. 43, pp. 309–334, 2003.

[39] N. I. Kerkvliet, L. B. Steppan, W. Vorachek et al., "Activation of aryl hydrocarbon receptor by TCDD prevents diabetes in NOD mice and increases Foxp3$^+$ T cells in pancreatic lymph nodes," *Immunotherapy*, vol. 1, no. 4, pp. 539–547, 2009.

[40] V. J. Schulz, J. J. Smit, K. J. Willemsen et al., "Activation of the aryl hydrocarbon receptor suppresses sensitization in a mouse peanut allergy model," *Toxicological Sciences*, vol. 123, no. 2, pp. 491–500, 2011.

[41] V. J. Schulz, M. van Roest, M. Bol-Schoenmakers et al., "Aryl hydrocarbon receptor activation affects the dendritic cell phenotype and function during allergic sensitization," *Immunobiology*, vol. 218, no. 8, pp. 1055–1062, 2013.

[42] U. A. Bussmann and J. L. Barañao, "Interaction between the aryl hydrocarbon receptor and transforming growth factor-β

signaling pathways: evidence of an asymmetrical relationship in rat granulosa cells," *Biochemical Pharmacology*, vol. 76, no. 9, pp. 1165–1174, 2008.

[43] D. Gramatzki, G. Pantazis, J. Schittenhelm et al., "Aryl hydrocarbon receptor inhibition downregulates the TGF-B/Smad pathway in human glioblastoma cells," *Oncogene*, vol. 28, no. 28, pp. 2593–2605, 2009.

[44] A. Gomez-Duran, E. Ballestar, J. M. Carvajal-Gonzalez et al., "Recruitment of CREB1 and histone deacetylase 2 (HDAC2) to the mouse Ltbp-1 promoter regulates its constitutive expression in a dioxin receptor-dependent manner," *Journal of Molecular Biology*, vol. 380, no. 1, pp. 1–16, 2008.

Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2014, Article ID 291618, 11 pages
http://dx.doi.org/10.1155/2014/291618

Research Article

Reduction of the Number of Major Representative Allergens: From Clinical Testing to 3-Dimensional Structures

Ying He,[1] Xueting Liu,[1] Yuyi Huang,[1] Zehong Zou,[1] Huifang Chen,[1] He Lai,[1] Lida Zhang,[2] Qiurong Wu,[1] Junyan Zhang,[1] Shan Wang,[1] Jianguo Zhang,[1] Ailin Tao,[1] and Baoqing Sun[3]

[1] *Guangdong Provincial Key Laboratory of Allergy & Immunology, The State Key Laboratory of Respiratory Disease, The Second Affiliated Hospital of Guangzhou Medical University, 250 Changgang Road East, Guangzhou 510260, China*
[2] *Plant Biotechnology Research Center, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 200030, China*
[3] *Guangdong Provincial Key Laboratory of Allergy & Immunology, The State Key Laboratory of Respiratory Disease, The First Affiliated Hospital of Guangzhou Medical University, 151 Yanjiang Road, Guangzhou 510120, China*

Correspondence should be addressed to Ailin Tao; taoailin@gzhmu.edu.cn and Baoqing Sun; sunbaoqing@vip.163.com

Received 10 December 2013; Accepted 7 February 2014; Published 23 March 2014

Academic Editor: Huiyun Zhang

Vast amounts of allergen sequence data have been accumulated, thus complicating the identification of specific allergenic proteins when performing diagnostic allergy tests and immunotherapy. This study aims to rank the importance/potency of the allergens so as to logically reduce the number of allergens and/or allergenic sources. Meta-analysis of 62 allergenic sources used for intradermal testing on 3,335 allergic patients demonstrated that in southern China, mite, sesame, spiny amaranth, *Pseudomonas aeruginosa*, and house dust account for 88.0% to 100% of the observed positive reactions to the 62 types of allergenic sources tested. The *Kolmogorov-Smironov* Test results of the website-obtained allergen data and allergen family featured peptides suggested that allergen research in laboratories worldwide has been conducted in parallel on many of the same species. The major allergens were reduced to 21 representative allergens, which were further divided into seven structural classes, each of which contains similar structural components. This study therefore has condensed numerous allergenic sources and major allergens into fewer major representative ones, thus allowing for the use of a smaller number of allergens when conducting comprehensive allergen testing and immunotherapy treatments.

1. Introduction

Over the years, vast amounts of data regarding worldwide allergy diagnosis and treatment have been accumulated. More recently, allergen researchers have probed this data resulting in the complete cDNA sequencing of a large number of allergens, as well as the determination of their three-dimensional (3D) structure in some cases. However, systematic evaluation of the importance and/or potency of the allergens or their sources has not been investigated thus complicating the identification of specific allergenic proteins when performing diagnostic allergy tests. On the other hand, many patients react to a large number of proteins, and allergic cross-reactivity has been described on many levels [1]. The relatedness of pollen and plant food allergens was recently described based on sequence and/or structure similarity [2, 3], and many kinds of allergens were able to be classified into just a few protein families with a restricted number of biochemical functions [4].

Our previous results showed that 478 allergens retrieved online could be clustered into eight groups, regardless of their biological source [5]. It would be a desirable goal if extensive research on a large number of allergens could be transformed into intensive research on just a few major allergens, hereafter referred to as "representative allergens." Major allergens are proteins that substantially bind to IgE from more than 50 percent of the patients with that specific allergy [6]. Major allergens have therefore been employed as internal standards in order to standardize allergen vaccines [7]. A detailed characterization of the importance of major allergens and

their biological sources would allow for the improvement of allergen standardization and thus help to obtain more effective and safer modalities for the diagnosis and therapy of many allergic diseases.

In the present study, we reviewed our clinical intradermal test (IDT) data from 2001 to 2003 on 62 kinds of allergenic sources and compared the amino acid sequences and 3D structures of the major allergens obtained from different biological sources, with the goal of logically reducing these allergenic sources to a few species, as well as progressively clustering the 280 major allergens obtained from the ExPASy Proteomics Server (June 20, 2012) into several of the most representative major allergens. The results obtained could therefore facilitate more consistent and straightforward allergen research.

2. Methods

2.1. Allergic Patients and IDT. Over the past 32 years, we have intradermally tested (IDT) more than 90,000 patients referred to our allergy clinic. The IDT results of 62 allergen extracts on 3,335 patients in a three-year period (2001–2003) are shown in Figure 1 (children less than three years old were excluded). In our department, IDT has always been exhibited to be effective and safe and was reported to be diagnostically better than the skin prick test [8]. Nevertheless, informed consent for testing was obtained from all patients or their guardians, and the current study was approved by the Ethics Committee of the Second Affiliated Hospital of Guangzhou Medical University.

All allergens extracts were prepared in a sterile environment followed by toxicity and potency evaluation according to an in-house standard protocol as described [9]. After filtration to sterilize, protein levels were quantitated by the Kjeldahl Method [10]. The allergenicity of the extracts was further tested by mice assay to keep the consistency of different batches, followed by the adjustment of stock solution to the standard concentration. All extracts were aliquoted into 10 mL portions at a concentration of 1 : 10 or 1 : 100 and then stored at 4°C until just prior to use.

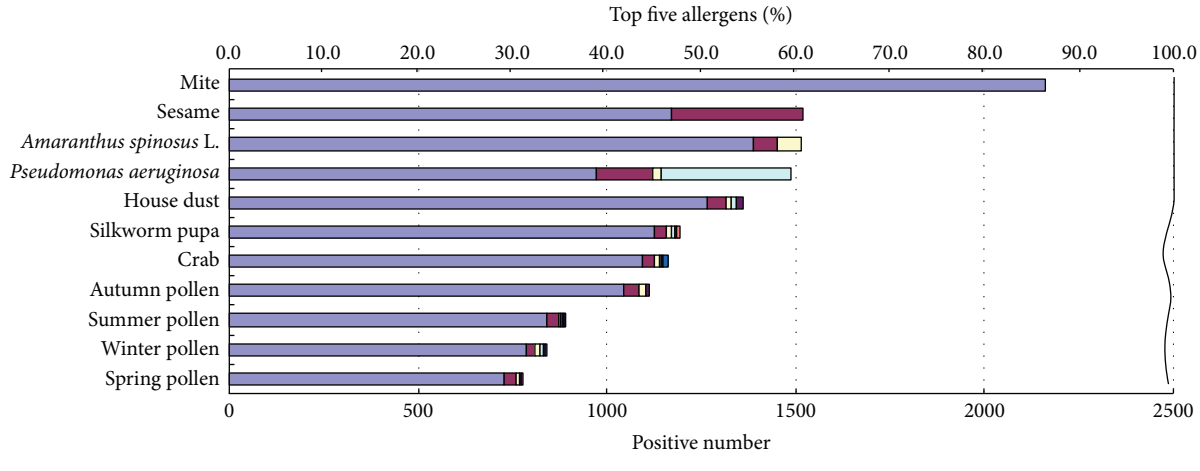
On the day of the test, the allergen extracts were brought to room temperature and diluted immediately in the solvent (Hengda Pharmacy Co. Ltd, Shanxi, China). Each patient received IDT using the same batch of allergen extracts. IDT was performed as follows: 10–20 μL of each allergen dilution was intradermally injected into each patient with 24–30 allergens tested at a time. Injected allergens were arranged vertically on the upper arm(s) with an interval of 2.5–3.0 cm. A positive control (histamine dihydrochloride, 10 mg/mL) and a negative control (solvent) were also included, and there was no duplicate testing. After 15 min, the size of the wheal was determined by measuring the diameter in two perpendicular directions and then halving the sum. Since bacterial proteins always exhibit a late phase reaction, those results were measured after 24 hrs. The reaction was regarded as positive if the calculated wheal diameter was more than 5 mm.

2.2. Relationship of the Allergenic Sources. To assess whether the distribution of positive reactions to each allergenic source reflects a correlation between different allergenic sources, the number of patients with positive IDT reactions to each allergenic source was counted. Patients showing multiple positive reactions to different allergenic sources were counted once for each individual allergenic source they reacted to. All the allergenic sources were ranked according to patient counts, since there may be cosensitization to different allergenic sources.

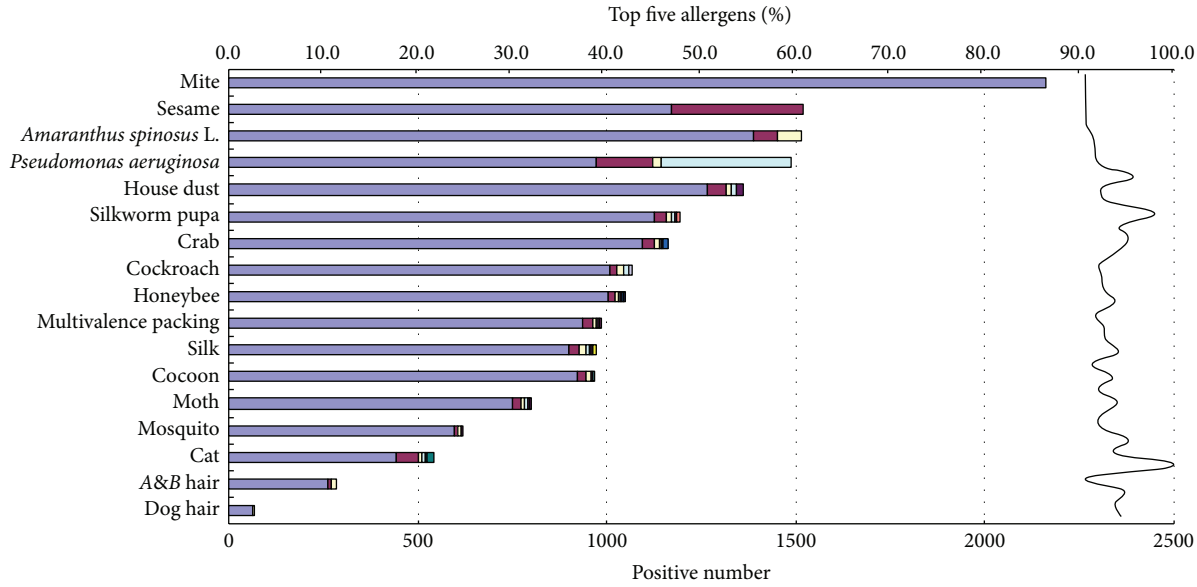
2.3. Overall Distribution of the Allergens Worldwide. The nonredundant allergen data were extracted from the IUIS allergen list (June 20, 2012) available at the website http://www.allergen.org/. The overall research status of allergens worldwide was analyzed as follows: the number of allergenic species and the related allergen numbers within each taxonomic category were recorded. The consistency of the two distributions thereof was investigated by *Kolmogorov-Smirnov test*, which measures the maximum difference between two cumulative distribution functions and calculates the probability that the two observed distributions would exhibit a difference at least that large if the samples were drawn from identical populations [11]. The parameters tested are as follows: $D_{n,n1\sim n2}$ = Maximum $(S_{n1}|x| - S_{n2}|x|)$, $D_{n,0.01} = 1.63/\sqrt{n}$, $D_{n,0.05} = 1.36/\sqrt{n}$, $D_{n,0.10} = 1.23/\sqrt{n}$, $n = n_1 * n_2/(n_1 + n_2)$, where n_1 and n_2 denote the number of allergenic species and the number of allergenic proteins within each taxonomic category, respectively.

2.4. Clustering of Major Allergens by Amino Acid Sequences. Another set of data related to the amino acid sequences of major allergens was retrieved on June 20, 2012, by searching UniProtKB/Swiss-Prot (http://www.expasy.org/) using the keyword "major allergen" and was selected from the IUIS allergen list (http://www.allergen.org/) by IgE-binding potency. The phylogenetic relationship among the major allergens was inferred by the free package Clustal W 1.83 [12] and MEGA5.0 [13] using the alignments of the amino acid sequences. In the output tree, each single, line-linked, large, and dense group of allergens was taken as one cluster or subcluster. The uppermost allergen sequence in each large and dense cluster in the output tree was retained and taken as the core sequence, with other sequences eliminated. Progressive clustering was repeated by manual iterative selection and alignment of the core sequences. The alignment cycle was stopped when any two allergens were no longer able to be clustered into one subcluster. These allergens are hereafter referred to as "major representative allergens."

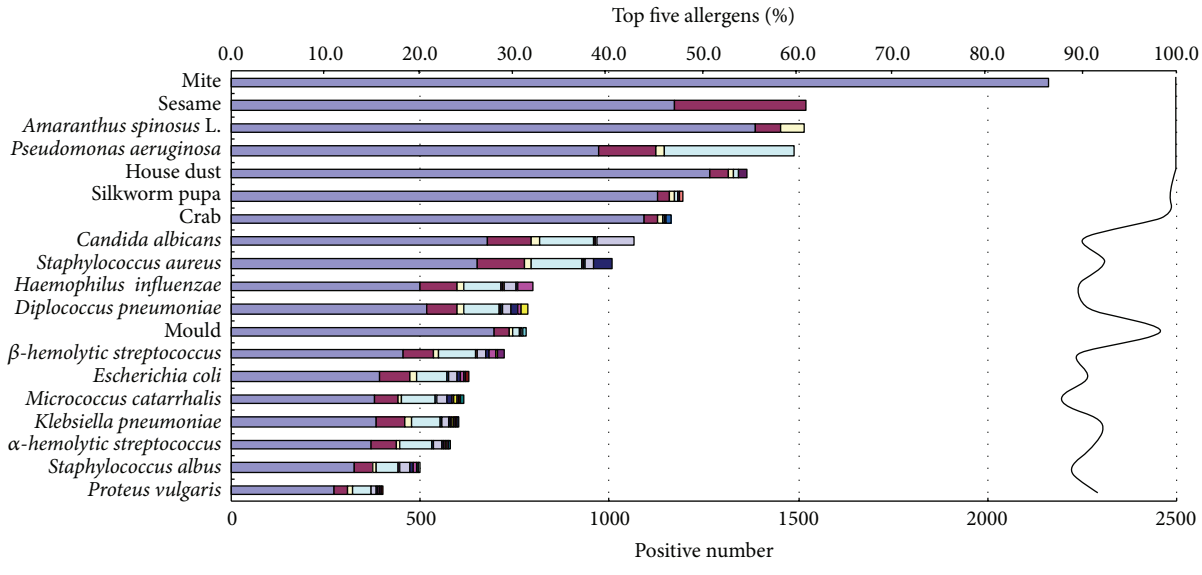
The same clustering procedure above mentioned was applied on the corresponding allergens in which Allergen Family Featured Peptides (AFFPs) are located. AFFPs are allergen-specific peptides panned from nonredundant allergens and harbor perfect information with noise fragments eliminated because of their similarity with nonallergens. 534 AFFPs can correctly discriminate 2290 allergens at



(a)



(b)



(c)

Figure 1: Continued.

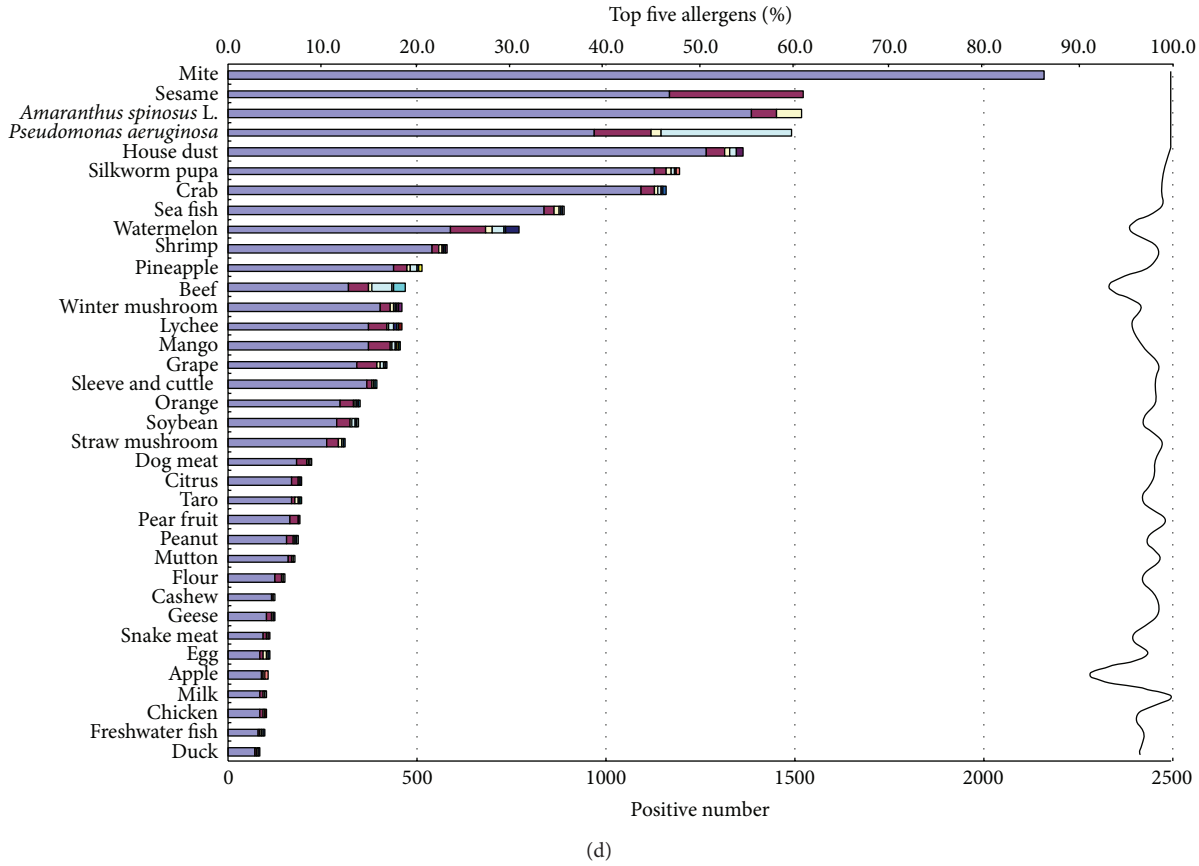


Figure 1: IDT results of 62 allergenic sources on 3,335 patients. The allergenic sources were grouped into 4 panels: (a) pollen; (b) contactant; (c) microbes; (d) food. The top 5 most potent allergenic sources were compared to all other allergenic sources and displayed on each panel. "*A&B hair*" in (b) denotes mix of animal fur and bird feather. The same bar color represents cosensitization among different allergenic sources. And the length of different color bars indicates the proportion of positively sensitized subjects. The different color at the very top of each bar represents the specific positives of each allergenic source. The rightmost curve denotes the total proportion in each allergenic source of the positive cases cosensitized to the top five allergenic sources. Spring pollen is produced by *Acacia confusa* Merr., pine tree, cedar, paper mulberry (*Broussonetia papyrifera*), waxberry (*Morella rubra*), Chinese Mulberry (*Morus australis* Poir.), and Chinese fan palm (*Livistona chinensis* R.). Summer pollen originates from maize, Australian pine (*Casuarina equisetifolia*), Chinaberry (*Melia azedarach*), and *Eucalyptus camaldulensis* Dehnh. Autumn pollen comes from *Mallotus apelta*, *Humulus scandens*, mugwort (*Artemisia vulgaris*), *Vitex negundo*, and *Dioscorea benthamii*. Winter pollen is from cajeput (*Melaleuca leucadendra* L.) and *Bauhinia blakeana* Dunn.

the highest sensitivity and specificity and make the underlying software SORTALLER outperform other methods at present [14], which demonstrates that 534 AFFPs have a powerful representativeness.

2.5. Comparison of the 3D Structures of the Major Representative Allergens. To compare the three-dimensional (3D) structures of the major representative allergens, the 3D structure of each major representative allergen was modeled in SWISS-MODEL workspace, a web-based integrated service dedicated to protein structure homology modeling and assessment that can be accessible via the ExPASy Bioinformatics Resource Portal [15]. The modeling results were viewed in SWISS-PdbViewer v3.7, an integrated sequence-to-structure workbench [16], followed by adjusting the presenting orientation to facilitate the comparison of the 3D structures of the different major representative allergens.

3. Results

3.1. Distribution of Positive Reactions to Allergenic Sources in Southern China. Intradermal tests using 62 allergenic sources were performed on 3,335 allergic patients. 3,084 of these patients reacted to at least one of the allergenic sources, with the positive frequency being 89.8%. All the allergenic sources used were aligned according to the number of patients with positive reactions. For convenience, the 62 allergenic sources were divided into four general groups, that is, contact allergens, food allergens, pollen allergens, and microbial allergens. The top seven most potent allergenic sources across all groups were then analyzed as to the frequency of their positive reactions when compared to the positive reactions seen with the specific allergens in any particular group. As shown in Figure 1, different allergenic sources show different degrees of positive reactions. Mites

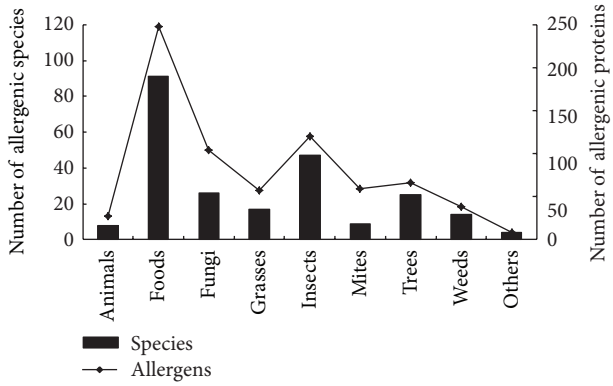


Figure 2: The consistency of two distributions: allergen related species distribution (■) and the distribution of the number of allergenic proteins within each species (▲). The allergens data were downloaded from the IUIS website (http://www.allergen.org/) on June 20, 2012. Estimated parameter for *Kolmogorov-Smirnov test* is 0.06642, less than the threshold values ($D_{n,0.01}$ = 0.1212, $D_{n,0.05}$ = 0.1011), thus statistically validating the consistency of the two distributions.

(*Dermatophagoides pteronyssinus* and *D. farinae*) were the number one allergenic source, resulting in more than 70% of the positive reactions among all the four groups. Reaction to sesame (*Sesamum indicum* L.) ranked second with 49.3% of the positive reactions. Spiny amaranth (*Amaranthus spinosus* L.), *Pseudomonas aeruginosa*, and house dust were next with 49.2%, 48.3%, and 44.2% of the positive reactions, respectively. In total, these five allergenic sources express 88.0% to 100% of the positive reactions when compared with microbial allergens, 90.6% to 100% when compared with food allergens, 96.3% to 100% when compared with contact allergens, and 99% to 100% when compared with pollen allergens. That is to say, these five allergenic sources are the most potent and show the most reactivity among the patients we tested; hence we have designated them as the major representative allergenic sources.

3.2. Species Distribution of Allergens Studied Worldwide. At the date of data retrieval for this study (June 20, 2012), 727 nonredundant allergens were listed in the allergen website (http://www.allergen.org/), relating nine categories and 241 species altogether (Figure 2). In terms of categories, foods contain the highest number of allergenic species with insects and fungi being the next most frequent. For allergenic proteins within each category, foods are also number one with fungus and insect allergens ranking second and third. Animals, weeds, and grasses possess the least number of species. In terms of species, the most abundant allergens are possessed by ragweed, timothy, olive, mite, cat, *Aspergillus fumigatus*, peanut, latex, and so forth. The number of allergenic species and the number of allergens in each category constitute two distributions (Figure 2). *Kolmogorov-Smirnov test* shows no significant statistical difference ($P < 0.05$) between the number of allergenic species in each category and the number of allergens in each category (Table 1). It indicates that the two distributions were drawn from an identical population, and that the number of allergens is closely related to the number of allergenic species. That is to say, in terms of the allergen research realm worldwide, allergens evolved in parallel from one species to another; no emphasis was prescribed to a certain species/allergen.

3.3. Progressive Clustering of the Major Allergens and AFFPs. Previously, we retrieved online 478 allergen sequences and clustered them into eight groups by sequence similarity [5]. In this study, we focused on major allergens and retrieved 280 entries, and 59 major allergens were retained after initial reduction. Clustering results showed that the 59 major allergens were initially classified into seven clusters (Figure 3(a)). Two or more neighboring clusters were combined to form a new data source for further clustering. This procedure was iterated until the last clustering exhibited 21 allergens that were distantly related to each other (Figure 3(b)). Further alignment showed that several pairs of allergens could be respectively grouped together, but with less than 15% pairwise positives in amino acid sequences and most of them exhibited different tertiary structures (See next part). Therefore, no further clustering was assigned to these allergens, and tertiary structure analysis was performed on the identified 21 major allergens.

Groups with remote homology (<20%–35% in local region) were represented by single entries. This method allowed us to reduce 534 AFFPs into 21 allergens (Figure 4) through five cycles of "cluster-selection-alignment" step, the same as that for major allergens. All the core peptides contain 3–5 matching residues with adjacent mismatches.

3.4. Overall Structure Description of the 21 Major Representative Allergens. 3D structure modeling was completed for most of the above 21 allergens by an automated mode or template identification mode in a SWISS-MODEL workspace [15]. Only O82015 [17], homologous to olive allergen Ole e 1 [18], and Q01940, a major allergen Mal f 1 from *Malassezia furfur* [19], had no identifiable protein structures by a SWISS-MODEL search. Therefore, their 3D structures were modeled by homology to related proteins from the nearby superfamilies Q04656 [20] and O05871 [21]. The 3D structures of the 21 major representative allergens are depicted in Figure 5.

When inspected from spatial structural orientations and surface exposures of the allergens, all of the 21 allergens were shuffled against the initial clustering and interestingly fell into seven structural classes (Figure 5). However, this classification is complicated by the existence of similar structural scenarios in different structural classes.

(I) Up-and-down β-barrel: includes P80384, the major allergen Lep d 2 from Fodder mite (*Lepidoglyphus destructor*) [22], as well as other group 2 allergens from dust mite species, for example, Der f 2 (P49278) from *Dermatophagoides farinae* [23].

(II) β-meander and/or ψ-loop constituted calyx, shortened as $\beta(\alpha)$ calyx, such as hexagon, cradle, and globose twins: includes allergens Q01940 [19], Q9FY19 [24], P18632 [25], Q40967 [26], Q84 UI0 [27], and O82015 [17].

Table 1: Parameter estimate for *Kolmogorov-Smirnov test* (Complementary for Figure 2)*.

Group	Frequence		Cumulate Frequence		Cumulate Frequency		$S_{n1}\|x\| - S_{n2}\|x\|$
	Species no.	Allergens no.	Species no.	Allergens no.	Species no.	Allergens no.	
Others	4	8	4	8	0.01660	0.01100	−0.00560
Animals	8	27	12	35	0.04979	0.04814	−0.00165
Mites	9	59	21	94	0.08714	0.12930	0.04216
Weeds	14	38	35	132	0.14523	0.18157	0.03634
Grasses	17	57	52	189	0.21577	0.25997	0.04420
Trees	25	66	77	255	0.31950	0.35076	0.03126
Fungi	26	104	103	359	0.42739	0.49381	**0.06642**
Insects	47	120	150	479	0.62241	0.65887	0.03646
Foods	91	248	241	727	1	1	0

*The allergen data analyzed were downloaded from the IUIS website (http://www.allergen.org/) on June 20, 2012. $D_{n,241\sim727}$ = Maximum ($S_{n1}|x| - S_{n2}|x|$) = 0.06642, while the threshold values were $D_{n,0.01} = 0.1212$, $D_{n,0.05} = 0.1011$.

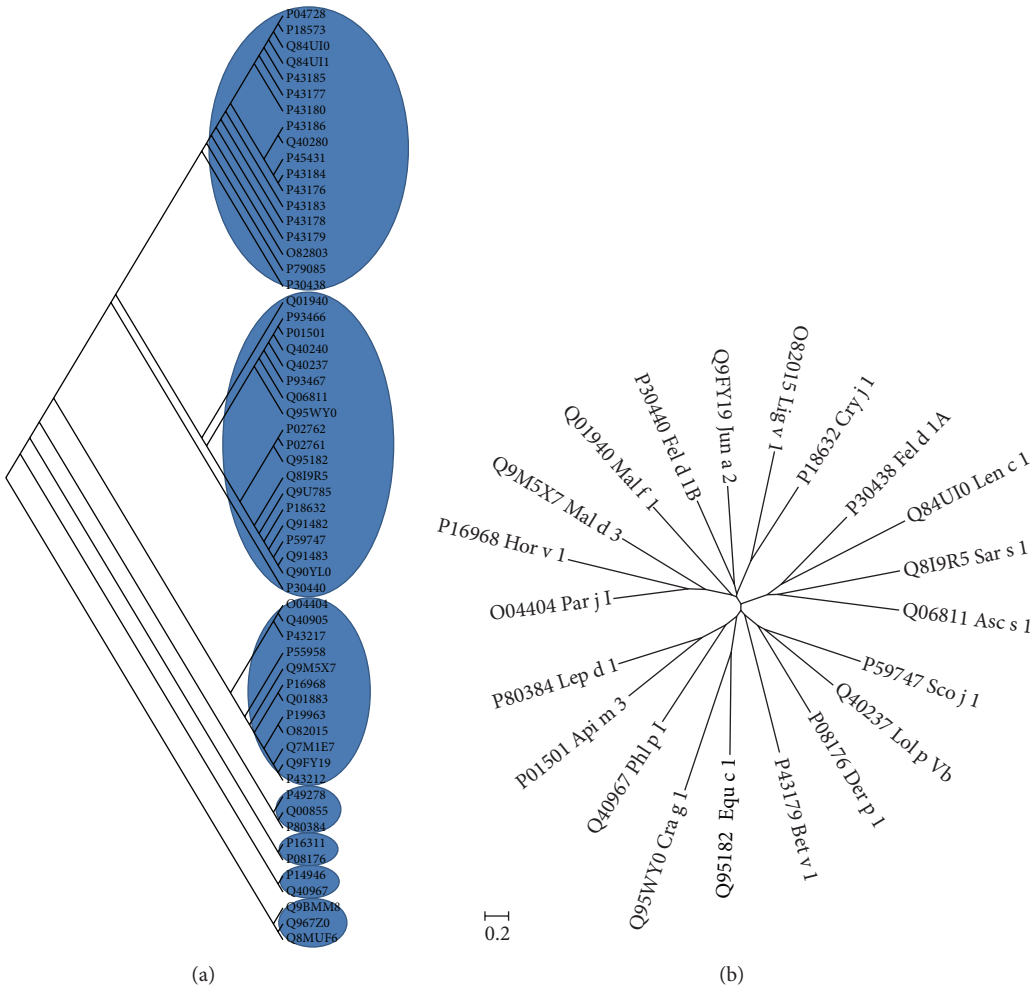


Figure 3: Progressive clustering of the major allergens acquired in UniProtKB/Swiss-Prot database. (a) Phylogeny tree of 59 major allergens constructed and tested by Maximum Liklihood from 280 major allergens. (b) Bootstrap consensus tree of 21 major representative allergens reduced from 59 major allergens.

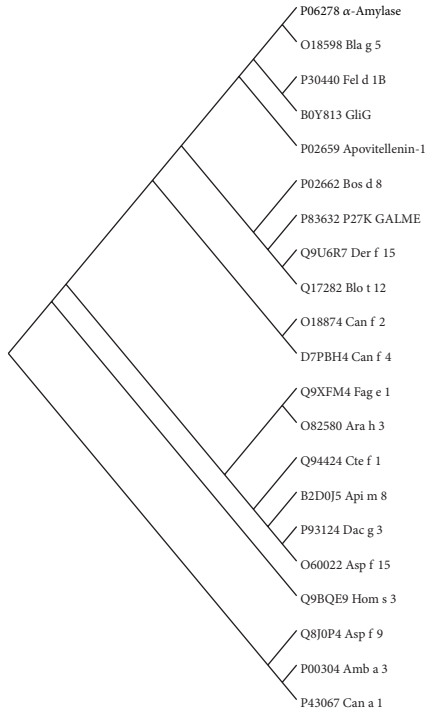


Figure 4: Maximum Parsimony Tree of 21 representative allergens reduced from the allergens corresponding to 534 AFFPs.

(III) α-β structured crane: includes Q8I9R5, an allergen from *Sarcoptes scabiei* type hominis [28].

(IV) α-β arranged banana string: includes Q95182 [29] and P43179 [30].

(V) $\alpha(\beta)$ formed complex: includes P08176 [31] and Q06811 [32].

(VI) α-helix built clips: a large group including P30438, P30440, P59747 [33], Q40237 [34], O04404 [35], Q9M5X7, and P16968 [36]. Interestingly, P30438 and P30440, chain 1 and chain 2 of cat allergen Fel d 1 [37], configure a pair of chiral molecules on the 3D level. O04404, Q9M5X7, and P16968, originating from different taxonomic species but classified into the same initial Cluster 7, here exhibited structures similar to each other.

(VII) α-helix spiral cord: includes P01501 [38] and Q95WY0 [39], both of which came from the same initial cluster III. P01501 is an allergen Api m 3 from honeybee (*Apis mellifera*), also a main toxin of bee venom with strong hemolytic activity. Q95WY0 is the major oyster allergen and tropomyosin from the pacific oyster *Crassostrea gigas*. These two allergens exhibited similar structures but displayed different lengths of their spiral cord.

4. Discussion

There is a tight link between allergen diagnosis and immunotherapy. After obtaining the results of allergen intradermal testing, specific IgE diagnosis, and/or even challenge assays, those allergen(s) with the highest positive scores would typically be chosen for use in immunotherapy on the allergic patients. The advent of molecular biology and bioinformatics heralded an unprecedented breakthrough in the development of recombinant allergens engineered to have the same immunological characteristics as natural allergens. Many studies have therefore used recombinant allergens in place of their natural counterparts. Unfortunately, allergen screening from one species to another in parallel only displays an ostensible prosperity of allergen study and could not pinpoint the importance of each allergen. The accumulation of a large quantity of overlapping data is threatening to undermine the achievements of allergen research. Whereto allergen research goes becomes a compelling question.

Facing this question, we firstly cast a meta-analysis on 62 allergenic sources used for intradermal testing on 3,335 patients in a three-year period. The result demonstrated that 88% to 100% of the patients were cosensitized to the top five allergenic sources and assumed that these five allergenic sources would have positive immunotherapeutic effects on the majority of the patients and that the remaining allergenic sources would have minor effects on the patients when used for immunotherapy. All these data corroborate that mite, sesame (*Sesamum indicum* L.), spiny amaranth (*Amaranthus spinosus* L.), *Pseudomonas aeruginosa*, and house dust are the five most prevalent allergenic sources and can well represent the 62 allergenic sources identified in southern China.

Basically, each extract from a single individual allergenic source is a mixture containing about ten allergenic proteins. Certainly, house dust is a mixture as well and contains different allergenic proteins. Hence, using house dust for diagnosis is similar to general screening of allergies with allergen mixes, such as fx1, fx5, mx1, and Phadiatop of UniCAP [40]. The allergens in the top five allergenic sources hence constitute a potent and limited allergen aggregate. Besides the cross-reactivity among some allergens thereof, as deduced from the cosensitization to different allergenic sources, what their relationship is in the repertoire becomes an urgent question. Our unpublished clinical data demonstrated that the allergen preparations from either *D. pteronyssinus* or *D. farinae* can be administered to patients allergic to either mite source and can achieve similar immunotherapeutic effects, which suggests the mutual substitution of the two allergens. This result, corroborating Weber's summary [41], also suggests that it is possible to allow the substitution of the closely related allergenic sources by major representative ones. It is tempting to further think that allergens existing in the five major allergenic sources can be reduced to fewer nonredundant and nonhomologous ones. Hence, it is crucial to converge efforts on the typical representative allergens for further research.

A systematic classification of all allergens by protein taxonomic family and even by structure has long been needed. A former study found that only 52 motifs matched 644 of 779 allergen sequences from all types of sources [42]. Mueller and colleagues argued that primary sequence comparisons could sometimes miss conserved elements of a protein, which can only be seen at the structural level, and that a comparative structural modeling approach could reveal

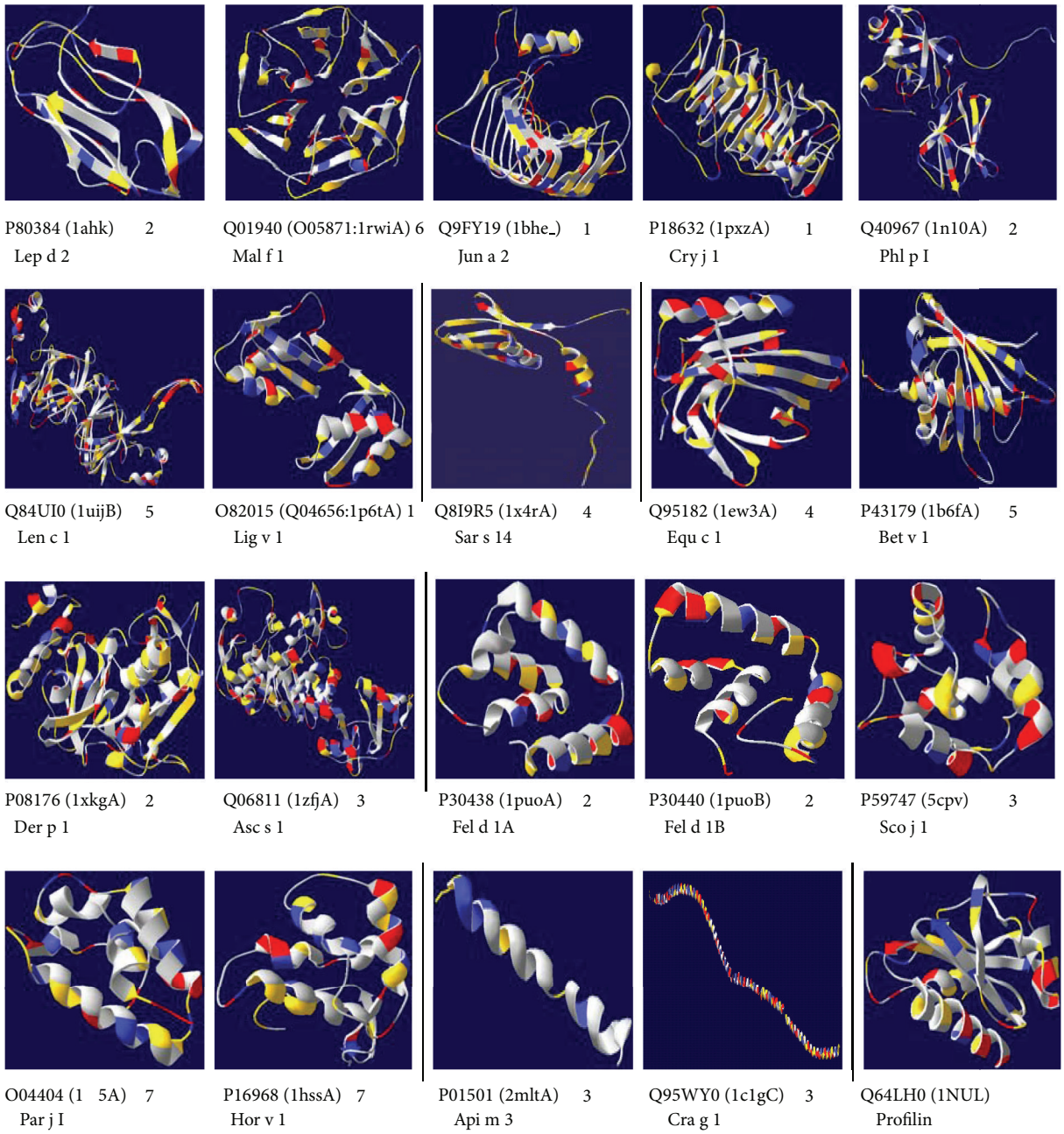


Figure 5: Ribbon diagrams of profilin (Q64LH0) and 19 out of the 21 major representative allergens subtracted. The 7 structural classes are separated by vertical lines. PDB codes are shown in brackets; the following numbers indicate the initial sequence clustering groups. Q64LH0 was not retrieved as a major allergen thus without initial cluster number. Q9M5X7 and Q40237 were omitted for space consideration.

these structural similarities undetectable at the sequence level [43]. Based on these results, the present study focused on major allergens over general ones and performed progressive clustering and manual subtraction of sequence redundancy of major allergens. Twenty-one major representative allergens were subsequently retained and were further classified into seven structural patterns, with many allergens from different sequence groups compiled in one structural class, thus validating the limitation of sequence comparison [44]. Structure class VI, for example, includes contact allergens, pollen allergens, and food allergens. Although they have low sequence homology, these allergens share similar structure scenarios with each other. Moreover, we also found that panallergen profilin (e.g., Q64LH0 [5]) exhibits a configuration of α-β-α layers (Figure 5) and is analogous to 2 EF-hand configuration (polcalcin, e.g., P59747 [33]) and even more similar if the mesial layer is replaced by an α helix-formed clip. Further analysis showed that different kinds of allergens, no matter whether they are in the same structure class or not, would share similar structure scenarios in part

of their component elements. These results not only theoretically confirmed the clinical relevance between profilin and polcalcin [45], but also suggested the relevant relationship between 2 EF-hand calcium-binding proteins (P59747 [33]) and Poa p IX/Phl p VI allergen family (Q40237 [34]), cereal trypsin/alpha-amylase inhibitor family (P16968 [36]), nonspecific lipid-transfer protein (O04404 [35]), and even uteroglobin (P30438 [37]) and Ole e 1 (O82015 [17]). Major allergen Ole e 1, for example, also harbors a profilin structure-like component element (Figure 5), suggesting its panallergen characteristics [18]. All the results mentioned above are theoretically supported by remote homology modeling and protein profile comparison [46, 47] and thus have drawn a picture of a cross-reactivity network among taxonomically different allergens and even allergens with nil or low sequence similarity and explained the underlying basis of the universal existence of cosensitization to different allergenic sources by individual patients disclosed by the present study.

The 3D structures of a continuously increasing number of allergens are currently being solved. Therefore, it is possible to produce recombinant allergens that exactly mimic their natural wild types and even to produce genetically engineered hypoallergens with nil or low IgE reactivity but retained T-cell reactivity. As the present study demonstrates, clinical cosensitization to multiple allergenic sources can be attributed to a few major allergenic sources; major allergens from different species can be logically reduced to 21 major representative allergens and even grouped into 7 or less structure classes with similar structure scenarios shared by different allergens. It is tempting to think that there might not be a need to unequivocally search all the undeveloped species for totally novel allergen genes or to equivalently test numerous allergenic sources on patients. Thus, it is cost-efficient, practical, and crucial to have the recombinant allergen research focused on the major representative allergens or core AFFPs for immunotherapy strategy development and diagnosis formulation.

We recently noticed that single or fewer major allergens can not only be used to diagnose the genuine sensitization of patients to a given allergen or to the cross-sensitization to several allergenic sources, but also be used for allergen-specific immunotherapy to yield the same effects as the whole allergen mixtures in allergic patients [14, 48, 49]. Intensive clinical evidence has also proven that specific immunotherapy with one kind of allergenic reagent can prevent both the progression of allergies and the acquisition of new allergic sensitizations [50]. All these conclusions corroborate our results.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Ying He and Xueting Liu equally contributed to this study.

Acknowledgments

This work was supported by Great Project (2011ZX08011-005) from the Major Program of National Science and Technology of China and the National Natural Science Foundation of China (nos. 81373128, 81301948).

References

[1] F. Ferreira, T. Hawranek, P. Gruber, N. Wopfner, and A. Mari, "Allergic cross-reactivity: from gene to the clinic," *Allergy*, vol. 59, no. 3, pp. 243–267, 2004.

[2] C. Radauer and H. Breiteneder, "Pollen allergens are restricted to few protein families and show distinct patterns of species distribution," *Journal of Allergy and Clinical Immunology*, vol. 117, no. 1, pp. 141–147, 2006.

[3] J. A. Jenkins, S. Griffiths-Jones, P. R. Shewry, H. Breiteneder, and E. N. C. Mills, "Structural relatedness of plant food allergens with specific reference to cross-reactive allergens: an in silico analysis," *Journal of Allergy and Clinical Immunology*, vol. 115, no. 1, pp. 163–170, 2005.

[4] C. Radauer, M. Bublin, S. Wagner, A. Mari, and H. Breiteneder, "Allergens are distributed into few protein families and possess a restricted number of biochemical functions," *Journal of Allergy and Clinical Immunology*, vol. 121, no. 4, pp. 847–852, 2008.

[5] A.-L. Tao and S.-H. He, "Cloning, expression, and characterization of pollen allergens from Humulus scandens (Lour) Merr and *Ambrosia artemisiifolia L*," *Acta Pharmacologica Sinica*, vol. 26, no. 10, pp. 1225–1232, 2005.

[6] J. A. Nordlee, S. L. Taylor, J. A. Townsend, L. A. Thomas, and R. K. Bush, "Identification of a Brazil-nut allergen in transgenic soybeans," *The New England Journal of Medicine*, vol. 334, no. 11, pp. 688–692, 1996.

[7] U. Seppälä, C. Dauly, S. Robinson, M. Hornshaw, J. N. Larsen, and H. Ipsen, "Absolute quantification of allergens from complex mixtures: a new sensitive tool for standardization of allergen extracts for specific immunotherapy," *Journal of Proteome Research*, vol. 10, no. 4, pp. 2113–2122, 2011.

[8] C. Fernández, R. Cárdenas, D. Martín et al., "Analysis of skin testing and serum-specific immunoglobulin E to predict airway reactivity to cat allergens," *Clinical and Experimental Allergy*, vol. 37, no. 3, pp. 391–399, 2007.

[9] B. Qiao, "Allergen preparation and dispensing," in *Allergology*, Y. Shitai, Ed., pp. 713–750, Science Press, Beijing, China, 1998 (Chinese).

[10] W. R. Campbell and M. I. Hanna, "The determination of nitrogen by modified Kjeldahl Methods," *The Journal of Biological Chemistry*, vol. 119, no. 1, pp. 1–7, 1937.

[11] D. A. Morrow, E. M. Antman, S. A. Murphy et al., "The risk score profile: a novel approach to characterising the risk of populations enrolled in clinical studies," *European Heart Journal*, vol. 25, no. 13, pp. 1139–1145, 2004.

[12] M. A. Larkin, G. Blackshields, N. P. Brown et al., "Clustal W and clustal X version 2.0," *Bioinformatics*, vol. 23, no. 21, pp. 2947–2948, 2007.

[13] S. Kumar, M. Nei, J. Dudley, and K. Tamura, "MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences," *Briefings in Bioinformatics*, vol. 9, no. 4, pp. 299–306, 2008.

[14] L. Zhang, Y. Huang, Z. Zou et al., "SORTALLER: predicting allergens using substantially optimized algorithm on allergen

family featured peptides," *Bioinformatics*, vol. 28, no. 16, pp. 2178–2179, 2012.

[15] K. Arnold, L. Bordoli, J. Kopp, and T. Schwede, "The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling," *Bioinformatics*, vol. 22, no. 2, pp. 195–201, 2006.

[16] T. Schwede, J. Kopp, N. Guex, and M. C. Peitsch, "SWISS-MODEL: an automated protein homology-modeling server," *Nucleic Acids Research*, vol. 31, no. 13, pp. 3381–3385, 2003.

[17] E. Batanero, M. A. Gonzalez de la Peña, M. Villalba, R. I. Monsalve, M. Martin-Esteban, and R. Rodriguez, "Isolation, cDNA cloning and expression of Lig v 1, the major allergen from privet pollen," *Clinical and Experimental Allergy*, vol. 26, no. 12, pp. 1401–1410, 1996.

[18] J. de Dios Alché, M. M'rani-Alaoui, A. J. Castro, and M. I. Rodríguez-García, "Ole e 1, the major allergen from olive (*Olea europaea* L.) pollen, increases its expression and is released to the culture medium during in vitro germination," *Plant and Cell Physiology*, vol. 45, no. 9, pp. 1149–1157, 2004.

[19] M. Schmidt, A. Zargari, P. Holt et al., "The complete cDNA sequence and expression of the first major allergenic protein of *Malassezia furfur*, Mal f 1," *European Journal of Biochemistry*, vol. 246, no. 1, pp. 181–185, 1997.

[20] M. L. Kennerson, G. A. Nicholson, S. G. Kaler et al., "Missense mutations in the copper transporter gene ATP7A cause X-linked distal hereditary motor neuropathy," *The American Journal of Human Genetics*, vol. 86, no. 3, pp. 343–352, 2010.

[21] M. C. Good, A. E. Greenstein, T. A. Young, H.-L. Ng, and T. Alber, "Sensor domain of the *Mycobacterium tuberculosis* receptor Ser/Thr protein kinase, PknD, forms a highly symmetric β propeller," *Journal of Molecular Biology*, vol. 339, no. 2, pp. 459–469, 2004.

[22] L. Kaiser, G. Gafvelin, E. Johansson, M. van Hage-Hamsten, and O. Rasool, "Lep d 2 polymorphisms in wild and cultured *Lepidoglyphus destructor* mites," *European Journal of Biochemistry*, vol. 270, no. 4, pp. 646–653, 2003.

[23] S. Ichikawa, T. Takai, T. Inoue et al., "NMR study on the major mite allergen Der f 2: its refined tertiary structure, epitopes for monoclonal antibodies and characteristics shared by ML protein group members," *Journal of Biochemistry*, vol. 137, no. 3, pp. 255–263, 2005.

[24] M. Yokoyama, M. Miyahara, K. Shimizu, K. Kino, and H. Tsunoo, "Purification, identification, and cDNA cloning of Jun a 2, the second major allergen of mountain cedar pollen," *Biochemical and Biophysical Research Communications*, vol. 275, no. 1, pp. 195–202, 2000.

[25] M. Usui, A. Saito, N. Taniguchi, N. Nishijima, H. Azakami, and A. Kato, "Reduction of antigenicity of Cry j I, major allergen of Japanese cedar pollen, by the attachment of polysaccharides," *Bioscience, Biotechnology and Biochemistry*, vol. 67, no. 11, pp. 2425–2430, 2003.

[26] A. Petersen, G. Schramm, A. Bufe, M. Schlaak, and W.-M. Becker, "Structural investigations of the major allergen Phl p I on the complementary DNA and protein level," *Journal of Allergy and Clinical Immunology*, vol. 95, no. 5, part 1, pp. 987–994, 1995.

[27] G. López-Torrejón, G. Salcedo, M. Martín-Esteban, A. Díaz-Perales, C. Y. Pascual, and R. Sánchez-Monge, "Len c 1, a major allergen and vicilin from lentil seeds: protein isolation and cDNA cloning," *Journal of Allergy and Clinical Immunology*, vol. 112, no. 6, pp. 1208–1215, 2003.

[28] K. Fischer, D. C. Holt, P. Harumal, B. J. Currie, S. F. Walton, and D. J. Kemp, "Generation and characterization of cDNA clones from *Sarcoptes scabiei* var. *hominis* for an expressed sequence tag library: identification of homologues of house dust mite allergens," *The American Journal of Tropical Medicine and Hygiene*, vol. 68, no. 1, pp. 61–64, 2003.

[29] A. Immonen, T. Kinnunen, P. Sirven et al., "The major horse allergen Equ c 1 contains one immunodominant region of T cell epitopes," *Clinical and Experimental Allergy*, vol. 37, no. 6, pp. 939–947, 2007.

[30] A. Gieras, P. Cejka, K. Blatt et al., "Mapping of conformational IgE epitopes with peptide-specific monoclonal antibodies reveals simultaneous binding of different IgE antibodies to a surface patch on the major birch pollen allergen, Bet v 1," *Journal of Immunology*, vol. 186, no. 9, pp. 5333–5344, 2011.

[31] E. Adam, K. K. Hansen, O. F. Astudillo et al., "The house dust mite allergen Der p 1, unlike Der p 3, stimulates the expression of interleukin-8 in human airway epithelial cells via a proteinase-activated receptor-2-independent mechanism," *The Journal of Biological Chemistry*, vol. 281, no. 11, pp. 6910–6923, 2006.

[32] Y. Xia, H. J. Spence, J. Moore et al., "The ABA-1 allergen of Ascaris lumbricoides: sequence polymorphism, stage and tissue-specific expression, lipid binding function, and protein biophysical properties," *Parasitology*, vol. 120, no. 2, pp. 211–224, 2000.

[33] M. Perez-Gordo, J. Cuesta-Herranz, A. S. Maroto et al., "Identification of sole parvalbumin as a major allergen: study of cross-reactivity between parvalbumins in a Spanish fish-allergic population," *Clinical and Experimental Allergy*, vol. 41, no. 5, pp. 750–758, 2011.

[34] E. K. Ong, I. J. Griffith, R. B. Knox, and M. B. Singh, "Cloning of a cDNA encoding a group-V (group-IX) allergen isoform from rye-grass pollen that demonstrates specific antigenic immunoreactivity," *Gene*, vol. 134, no. 2, pp. 235–240, 1993.

[35] R. González-Rioja, J. A. Asturias, A. Martínez, F. M. Goñi, and A. R. Viguera, "Par j 1 and Par j 2, the two major allergens in *Parietaria judaica*, bind preferentially to monoacylated negative lipids," *FEBS Journal*, vol. 276, no. 6, pp. 1762–1775, 2009.

[36] M. Mena, R. Sanchez-Monge, L. Gomez, G. Salcedo, and P. Carbonero, "A major barley allergen associated with baker's asthma disease is a glycosylated monomeric inhibitor of insect α-amylase: cDNA cloning and chromosomal location of the gene," *Plant Molecular Biology*, vol. 20, no. 3, pp. 451–458, 1992.

[37] M. Emara, P.-J. Royer, Z. Abbas et al., "Recognition of the major cat allergen fel d 1 through the cysteine-rich domain of the mannose receptor determines its allergenicity," *The Journal of Biological Chemistry*, vol. 286, no. 15, pp. 13033–13040, 2011.

[38] T. Grunwald, B. Bockisch, E. Spillner, J. Ring, R. Bredehorst, and M. W. Ollert, "Molecular cloning and expression in insect cells of honeybee venom allergen acid phosphatase (Api m 3)," *Journal of Allergy and Clinical Immunology*, vol. 117, no. 4, pp. 848–854, 2006.

[39] P. S. C. Leung and K. H. Chu, "cDNA cloning and molecular identification of the major oyster allergen from the Pacific oyster *Crassostrea gigas*," *Clinical and Experimental Allergy*, vol. 31, no. 8, pp. 1287–1294, 2001.

[40] C. Nilsson, G. Lilja, M. Nordlund, M. Berthold, and M. P. Borres, "Phadiatop Infant detects IgE-mediated diseases among pre-school children: a prospective study," *Pediatric Allergy and Immunology*, vol. 23, no. 2, pp. 160–166, 2012.

[41] R. W. Weber, "Cross-reactivity of pollen allergens: recommendations for immunotherapy vaccines," *Current Opinion in Allergy and Clinical Immunology*, vol. 5, no. 6, pp. 563–569, 2005.

[42] M. B. Stadler and B. M. Stadler, "Allergenicity prediction by protein sequence," *The FASEB Journal*, vol. 17, no. 9, pp. 1141–1143, 2003.

[43] J. L. Mueller, D. R. Ripoll, C. F. Aquadro, and M. F. Wolfner, "Comparative structural modeling and inference of conserved protein classes in *Drosophila* seminal fluid," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 37, pp. 13542–13547, 2004.

[44] J. Peng and J. Xu, "Low-homology protein threading," *Bioinformatics*, vol. 26, no. 12, pp. i294–i300, 2010.

[45] R. Barderas, M. Villalba, C. Y. Pascual, E. Batanero, and R. Rodríguez, "Profilin (Che a 2) and polcalcin (Che a 3) are relevant allergens of *Chenopodium album* pollen: isolation, amino acid sequences, and immunologic properties," *Journal of Allergy and Clinical Immunology*, vol. 113, no. 6, pp. 1192–1198, 2004.

[46] M. Nielsen, C. Lundegaard, O. Lund, and T. N. Petersen, "CPHmodels-3.0-remote homology modeling using structure-guided sequence profiles," *Nucleic Acids Research*, vol. 38, supplement 2, pp. W576–W581, 2010.

[47] Y. Wang, R. I. Sadreyev, and N. V. Grishin, "PROCAIN: protein profile comparison with assisting information," *Nucleic Acids Research*, vol. 37, no. 11, pp. 3522–3530, 2009.

[48] C. Möbs, C. Slotosch, H. Löffler, T. Jakob, M. Hertl, and W. Pfützner, "Birch pollen immunotherapy leads to differential induction of regulatory T cells and delayed helper T cell immune deviation," *Journal of Immunology*, vol. 184, no. 4, pp. 2194–2203, 2010.

[49] M. Bublin, M. Pfister, C. Radauer et al., "Component-resolved diagnosis of kiwifruit allergy with purified natural and recombinant kiwifruit allergens," *Journal of Allergy and Clinical Immunology*, vol. 125, no. 3, pp. 687–694, 2010.

[50] M. Pifferi, G. Baldini, G. Marrazzini et al., "Benefits of immunotherapy with a standardized *Dermatophagoides pteronyssinus* extract in asthmatic children: a three-year prospective study," *Allergy*, vol. 57, no. 9, pp. 785–790, 2002.

Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2014, Article ID 476357, 14 pages
http://dx.doi.org/10.1155/2014/476357

Review Article

Importins and Exportins Regulating Allergic Immune Responses

Ankita Aggarwal and Devendra K. Agrawal

Department of Medical Microbiology & Immunology and Center for Clinical & Translational Science, Creighton University School of Medicine, Omaha, NE 68178, USA

Correspondence should be addressed to Devendra K. Agrawal; dkagr@creighton.edu

Received 9 December 2013; Revised 18 January 2014; Accepted 21 January 2014; Published 9 March 2014

Academic Editor: Peisong Gao

Nucleocytoplasmic shuttling of macromolecules is a well-controlled process involving importins and exportins. These karyopherins recognize and bind to receptor-mediated intracellular signals through specific signal sequences that are present on cargo proteins and transport into and out of the nucleus through nuclear pore complexes. Nuclear localization signals (NLS) present on cargo molecules to be imported while nuclear export signals (NES) on the molecules to be exported are recognized by importins and exportins, respectively. The classical NLS are found on many transcription factors and molecules that are involved in the pathogenesis of allergic diseases. In addition, several immune modulators, including corticosteroids and vitamin D, elicit their cellular responses by regulating the expression and activity of importin molecules. In this review article, we provide a comprehensive list of importin and exportin molecules and their specific cargo that shuttled between cytoplasm and the nucleus. We also critically review the role and regulation of specific importin and exportin involved in the transport of activated transcription factors in allergic diseases, the underlying molecular mechanisms, and the potential target sites for developing better therapeutic approaches.

1. Introduction

The trafficking of molecules between the cytoplasm and the nucleus in eukaryotes is regulated by nuclear pore complexes (NPCs or nucleoporins), which are cylindrical structures containing about 100 different polypeptides and embedded in the double membrane of the nuclear envelope. The regulation of bidirectional movement of molecules within a cell is critical in the exchange of molecules in and out of the nucleus and precise control of signal transduction processes as well as gene expression, cell cycle progression, and other cellular responses. Generally, the molecules up to 38–50 kDa in size may passively diffuse through the nuclear pore complexes. However, molecules larger than 50 kDa require machinery whereby mobile targeting receptors called karyopherins recognize and bind to receptor-mediated intracellular signals through specific signal sequences that are present on substrate proteins. Depending upon the movement of macromolecules from cytoplasm to nucleus or nucleus to cytoplasm, these karyopherins target the substrates that contain nuclear localization signal (NLS) or nuclear export signals (NES). The classical amino acid sequence in the NLS is PKKKRRV that mediates the nuclear translocation of large molecules from the cytoplasm [1]. The nuclear export signals (NES) typically contain the sequence LQLPPLERLTL, which directs the proteins to leave the nucleus [2].

The first conventional NLS is recognized by an adaptor protein, importin-α (also known as karyopherin-α), that functions as the NLS receptor. The importin-α interacts with importin β (also known as karyopherin-β), which mediates the process of docking to the nucleoporins. The protein containing NLS is transported through NPC to the nucleus by importin α/β heterodimeric complex. This translocation of the substrate-receptor complex through the NPC requires energy provided by a low molecular weight Ras-family GTPase, Ran, which is present in the nucleus. After translocation to the nucleus, the importin heterodimeric complex dissociates by the action of Ran-GTP, whereby the importin-α and the substrate enter and accumulate in the nucleoplasm while importin-β binds to Ran-GTP and accumulates at the NPC [2]. Thus, the import of protein substrates bound to NLS receptors in the cytoplasm does not require Ran-GTP. However, the release of the protein substrate in the nucleus is dependent upon Ran-GTP binding to the complex.

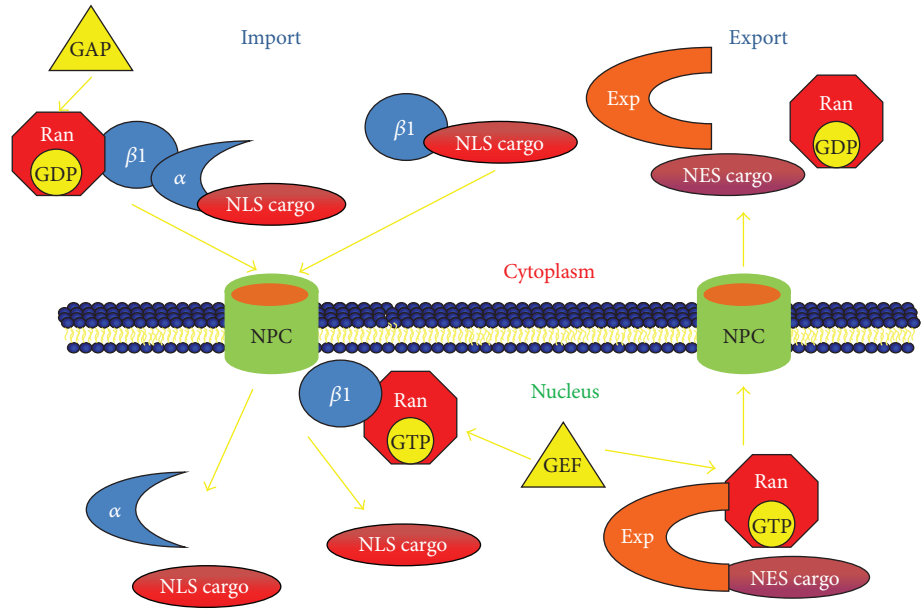


Figure 1: Nucleocytoplasmic transport of macromolecules via importin-exportin pathway. In the cytoplasm, importin-α forms a heterotrimeric complex with importin-β1, RanGDP, and NLS containing cargo protein. This heterotrimeric complex passes through the NPC into the nucleus and RanGTP binds to importin-β1 and disassembles the complex in the nucleus. The binding of exportin to NES within the cargo protein is triggered by RanGTP in the nucleus and is exported back to the cytoplasm through nuclear pore complex (NPC). In the cytoplasm, the dissociation of the complex is mediated through the hydrolysis of RanGTP to RanGDP by GAP (GTPase activating protein) that forces the binding of GDP to Ran. GEF (guanine nucleotide exchange factor) is a nucleoplasmic factor that stimulates exchange of GDP with GTP forming RanGTP in the nucleus.

Finally, importin α/β returns back to the cytoplasm to import additional protein molecules into the nucleus (Figure 1).

Nuclear export of proteins occurs through NES in an analogous manner by exportins, which are homologous to importin-β. However, this requires the binding of Ran-GTP to protein-NES-exportin complex. After the cytoplasm has been reached, the hydrolysis of RanGTP to RanGDP by Ran GTPase-activating protein occurs, resulting in the dissociation of the complex of exportin and protein. The direction in which protein is carried is generally determined by the gradient of Ran GTPase because the majority of RanGDP and Ran-GTPase-activating proteins are found in the cytoplasm, whereas Ran-GTP and Ran-guanine nucleotide exchange factors (Ran-GEF) are primarily present in the nucleus [3].

Human importins and exportins are classified into four subfamilies. First family is importin αP that consists of importin α1 [4], second family is importin αQ, including importins α3 and α4 [5], third family is importin αS including importins α5, α6, and α7 [5–7], and the fourth family is importin β that consists of importins β1, β2, and β3, importins 7, 8, and 12, exportins 1, 5, and 6, and exportin t [8]. However, there are few human importin/exportin molecules that have not been yet categorized into a subfamily. These are importins 4, 9, 11, and 13, transportin 2, and exportins 4 and 7. Different subfamily members share around 50% sequence identity. The similarity in sequence within a subfamily is around 85%. Despite of these subtle differences in the sequence, they possess similar pattern of recognition of NLS on cargo proteins. However the experimental evidence is provided by many studies validating the fact that different importin/exportin molecules possess distinct properties in terms of interacting with the NLS of specific cargo proteins [6, 9, 10]. A comprehensive summary of human importins and exportins along with their alternative names, short names, and the gene symbols given by NCBI (National Center for Biotechnology Information) is provided in Table 1. There also are nonconventional nuclear transport mechanisms that are distinct from the classical concept of importing proteins which are responsible for the import of molecules into the nucleus by directly binding to specific importin β1 homologs from importin β family.

Karyopherins recognize various cargo molecules and bind to them by interacting with NLS or NES present on cargo molecules. However, the specificity of importins or exportins for each cargo would depend upon the structure and direct interaction of importin-cargo molecules. In Table 2, a summary of cargos that bind directly to importins and exportins is provided.

2. Molecules Involved in Allergic Immune Response

Inflammation is a hallmark of allergic diseases, including asthma, allergic rhinitis, and atopic dermatitis. In most of the allergic diseases, inflammation is primarily characterized by the predominance of Th2 lymphocytes and the specific cellular response of Th2 cytokines, IL-4, IL-5, IL-9, and IL-13. IL-4 mediates the class switching of IgM secreted by

Table 1: Names and symbols of human importins and exportins.

Transporters	Alternative name/s	Short name/s	NCBI symbols
Importin α1/ Importin α2	Karyopherin α2 RAG cohort protein 1 SRP1-α	RCH1 SRP1	KPNA2
Importin α3	Importin α Q1 Karyopherin α4	Qip1	KPNA4
Importin α4	Importin α Q2 Karyopherin α3	Qip2 SRP-1γ	KPNA3
Importin α5	Karyopherin α1 Nucleoprotein interactor 1 RAG cohort protein 2	NPI-1 SRP1-β RCH2	KPNA1
Importin α6	Karyopherin α5	—	KPNA5
Importin α7	Karyopherin α6	IPOA7	KPNA6
Importin β1	Importin 90 Karyopherin β1 Nuclear factor p97 Pore targeting complex 97 kDa subunit	PTAC97 NTF97	KPNB1
Importin β2	Transportin 1 Karyopherin β2 M9 region interaction protein	KPNB2 MIP1 TRN	TNPO1
Importin β3	Importin 5 Karyopherin β3 Ran-binding protein 5	Imp5 KPNB3 RanBP5	IPO5
Importin 4	Importin 4b Ran-binding protein 4	Imp4b RanBP4 IMP4B	IPO4
Importin 7	Ran-binding protein 7	Imp7 RanBP7	IPO7
Importin 8	Ran-binding protein 8	Imp8 RanBP8	IPO8
Importin 9	Ran-binding protein 9	Imp9 RanBP9 KIAA1192 HSPC273	IPO9
Importin 11	Ran-binding protein 11	Imp11 RanBP11	IPO11
Importin 12	Transportin 3 Transportin-SR	Imp12 TRN-SR IPO12	TNPO3
Importin 13	Karyopherin 13	Kap13 RanBP13 KIAA0724	IPO13
Transportin 2	Karyopherin β2b	—	TNPO2
Exportin 1	Chromosome region maintenance 1 protein homolog	Exp1 CRM1	XPO1
Exportin 4	—	Exp4 KIAA1721	XPO4
Exportin 5	Ran-binding protein 21	Exp5 KIAA1291 RanBP21	XPO5
Exportin 6	Ran-binding protein 20	Exp6 KIAA0370 RanBP20	XPO6
Exportin 7	Ran-binding protein 16	Exp7 KIAA0745 RanBP16	XPO7
Exportin t	tRNA exportin	—	XPOT

plasma cells to allergen specific IgE, IL-5 is a major cytokine involved in eosinophilic inflammation, and IL-13 is partly involved in class-switching event and plays a key role in goblet cell hyperplasia and mucus production [11]. IL-9 was initially described as a growth factor for T-lymphocyte and mast cells and is released from many cell types, including Th2 cells, eosinophils, mast cells, and neutrophils. IL-9 can induce multiple effects in the initiation, exacerbation, and maintenance of allergic airway inflammation and airway remodeling (Figure 2).

Patients with allergic respiratory diseases have a biased immune response towards Th2 phenotype, contrasting that of healthy individuals in which the host-defense mechanisms maintain a balance between Th1 and Th2 phenotypes. The differentiation of Th2 cells is regulated by zinc finger transcriptional factor GATA-3, suggesting a key role of GATA-3 in mediating allergic immune response [12]. There is a pivotal role of bronchial smooth muscle cells in the pathogenesis of airway inflammation [13]. The increase in bronchial smooth muscle mass narrows the airway lumen, which further obstructs the airflow. There are a number of inflammatory cytokines secreted by bronchial smooth muscles such as TNF-α, IL-6, IL-17, IL-8, and IL-1β. Indeed, nearly every structural cell in the lung, including epithelial cells and infiltrated cells, becomes activated during allergic immune response. Thus, airway inflammation, due to the effect of various cytokines and mediators released by the cells in the lung, is a hallmark of allergic diseases.

The transcription, translation, and release of cytokines during allergic response depend upon specific signals, activation of intracellular kinases, and transcription factors present in the cytoplasm (Figure 2). STAT6 is vital to inducing the expression of Th2 cytokines, initiating allergic immune responses in many allergic diseases [14, 15]. The activated NF-κB is one of the transcriptional factors that trigger airway hyperresponsiveness and allergic airway inflammation by rapidly inducing the expression of various genes involved in the pathology of allergic diseases [16]. However along with AP-1, it can further enhance the production of proinflammatory cytokines such as IL-1 and TNF-α [17]. Th17 cells are another subset of T helper cells that are differentiated from naïve T cells in the presence of TGF-β, and a very potent inflammatory cytokine, IL-6, is credited with inducing pathology in allergic inflammatory diseases [18]. RORγt is a transcriptional factor responsible for the differentiation of Th17 cells via signal transducer and activator of

Table 2: Specificity of different classes of importins/exportins in transporting cargo to and from the nucleus.

Transporters	Organism	Cells/Tissue	Cargo (proteins) specific to each importin or exportin	Reference
Importin α1	Human	ROS 17/2.8, UMR-106, MC3T3-E1, and SaOS-2	Type 1 parathyroid hormone receptor	[62]
Importin α2	Human	HuT-78 cells	GATA-3	[63]
Importin α3	Human	A5A9 lung cells, human Bronchial smooth muscle cells	NF-κB p50/p65	[25, 50]
Importin α3	Mouse	Mouse lung tissue	NF-κB p65	[11]
Importin α4	Human	A5A9 lung cells	NF-κB p50/p65	[25]
Importin α5	Human	COS-1	STAT3	[45]
		HepG2 HuH7 hepatoma cells	STAT1-STAT2	[43]
Importin β1	Human	HEK293	PRPF31	[64–69]
		HUVEC	NF-κB	
		C58	CREB	[33]
		CO57	PTHrP	
		HeLa	SRY/SOX-9	[68]
			Cyclin B1	
			NFAT	
Importin β1	Mouse	Sf9	NFAT	[69]
Importin β2	Human	HeLa	HPV16E6 oncoprotein	[70]
Importin β3	Human	HeLa	c-Jun	[71, 72]
			Ribosomal proteins	
Importin 4	Human	HeLa	Vitamin D receptor	[73, 74]
			HIF1-α	
Importin 5	Human	HeLa	c-Jun	[70]
Importin 7	Human	HeLa	c-Jun	[71]
			Histone H1	[75, 76]
			CREB	[33]
			SMAD3	[48]
			Glucocorticoid receptor	[55]
Importin 8	Human	HaCaT	SMADs	[76, 77]
		COS-1	Signal recognition particle protein 19	
Importin 9	Human	HeLa	c-Jun	[71]
			PP2A (PR65)	[78]
Importin 11	Human	BHK	L12	[79, 80]
			UbcM2	
Importin 13	Human	HeLa	c-Jun	[71] [57]
		Airway epithelial cells	Glucocorticoid receptor	
Importin α/β	Human	HTC	HIV-1 integrase	[81, 82]
		MCF-7	P53	
Exportin 1	Human	NIH-3H3	Cyclin D1	[14, 26, 83, 84]
		HeLa	IκB	
			NFAT	
Exportin 4	Mouse	HeLa	elF-5A	[15]
Exportin 5	Human	BHK	Staufen2	[85]
Exportin 6	Human		Actin	[86]
Exportin 7	Human	HeLa	P50RhoGAP	[87]
		BHK		
Exportin t	Human	HeLa	tRNA	[72, 88]

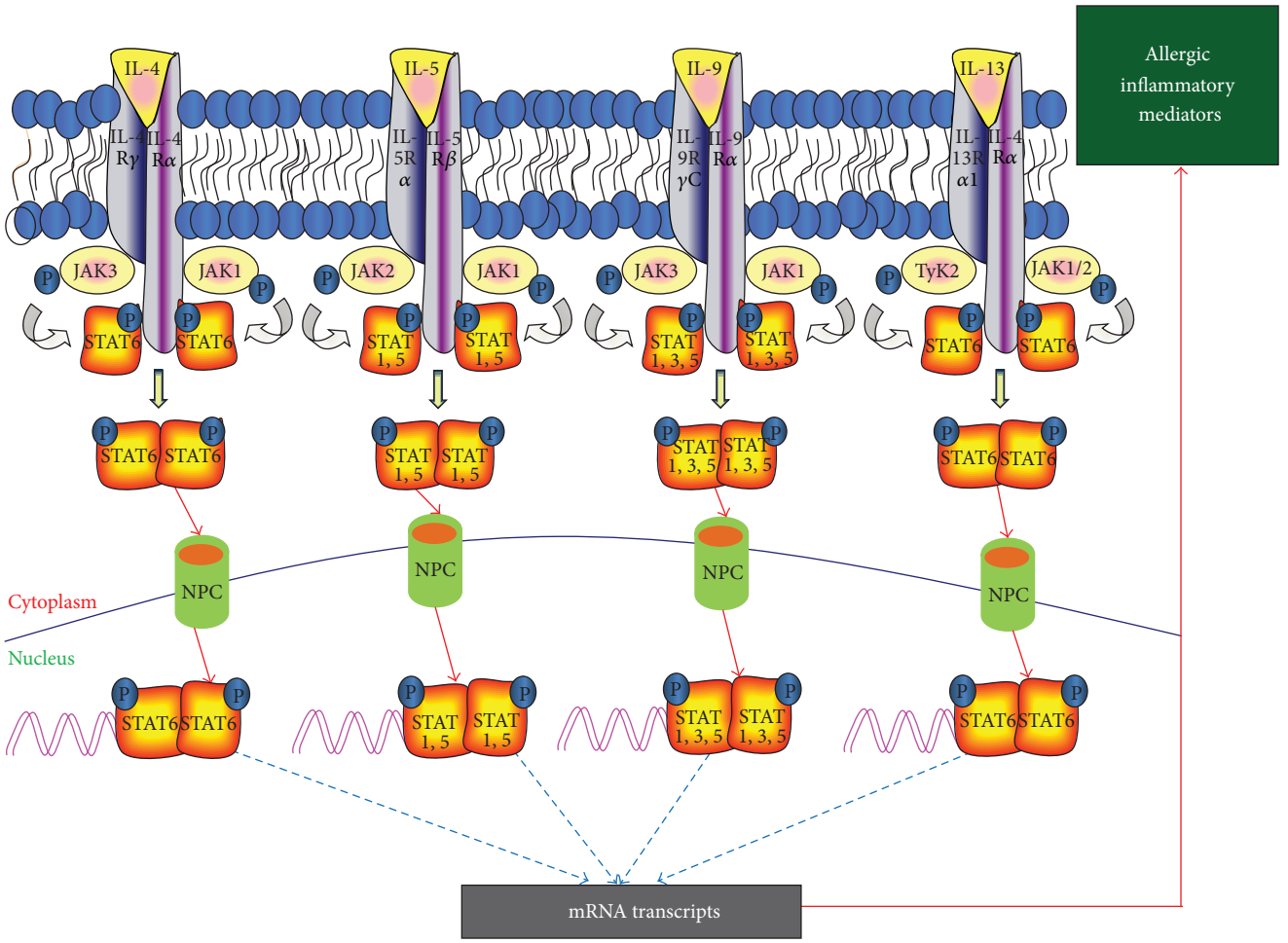


Figure 2: Schematic diagram showing the involvement of transcriptional factors in response to key mediators of allergic inflammation: binding of Th2 cytokines to their specific receptors and the activation of the downstream JAK/STAT pathways specific to each cytokine are shown. JAK: Janus activated kinase; NPC: nuclear pore complex; STAT: signal transducer and activator of transcription; TyK2: tyrosine kinase 2.

the transcription 3 (STAT3)-RORγt pathway. The activation and functionality of transcriptional factors determine the fate of T cell differentiation and the cytokine secretion according to the lineage of the T cell. Th1 cells express Th1 master regulator, T-box transcriptional factor (T-bet), which is induced via STAT4 or STAT1 signaling pathways [19]. Runx3 is another transcriptional factor whose expression is upregulated by Th1 cells. If the conditions favoring the differentiation of Th1 cells are present, then the differentiation of Th2 cells will be inhibited. IL-4 encoding genes are silenced by T-bet and Runx3 [20], suggesting that targeting IL-4 signaling is one of the therapeutic approaches to attenuating allergic airway inflammation. On the other hand, Th2 cell differentiation is accompanied by IL-4 through STAT6, which in turn induces GATA-3 the master regulator for Th2 cell differentiation [21].

The activation of the respective transcriptional factors is a key component to the induction or alleviation of allergic inflammation. The transcriptional factors responsible for inducing or reducing allergic inflammation must enter the nucleus of the target cell and bind to their specific response elements to exhibit their functional role. Small molecules up to ~40 kDa can easily diffuse through the nuclear pore complexes, whereas the larger molecules require help from import and export molecules to get through the nuclear membrane. The import-export machinery responsible for such translocation consists of importins and exportins. In the following section, the role of specific importin or exportin molecules in the transport of specific transcription factors involved in allergic inflammation will be discussed.

3. Effect of Importins/Exportins on Key Transcription Factors Involved in Allergic Inflammation Rel or NF-κB

Since importins and exportins play a crucial role in transporting macromolecules into and out of the nucleus, their regulation is critical in cellular responses (Figure 3). Rel/NF-κB p50/p65 is actively involved in cell differentiation, host

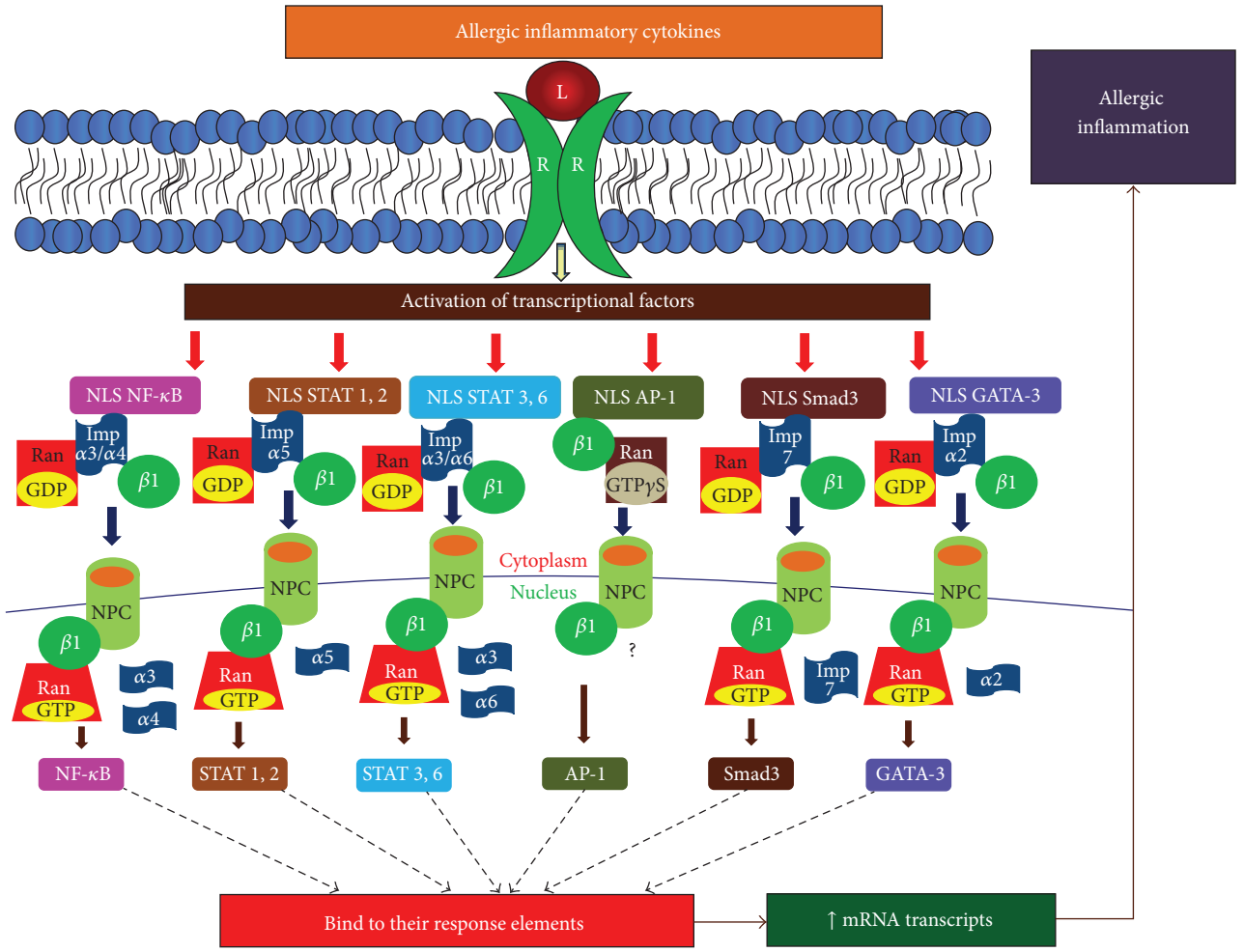


FIGURE 3: Binding of specific importin molecules to transcription factors: the figure shows the recognition of nuclear localization signal (NLS) by specific importin (Imp) molecules on activated transcriptional factors in the cytoplasm, binding of importin β, and transport of the cargo to the nucleus through nuclear pore complex (NPC).

immune response and in the transcription of many inflammatory cytokines [22]. Rel/NF-κB activation is critical for the induction and stimulation of allergic airway inflammation. Indeed, increased activation of activated Rel/NF-κB has been found in immune cells and structural cells of the lungs in asthmatic subjects [23, 24]. TNF-α, IL-17, and IL-1β are crucial proinflammatory cytokines that are secreted by many cells in the airway during allergic airway inflammation and activate NF-κB [24]. In the latent state, Rel/NF-κB remains in the inactive state complexed with IκB-IKK complex in the cytosol. IKK complex consists of three distinct subunits: IKKα and IKKβ as catalytic subunits and IKKγ (also called as NEMO) as a regulatory subunit to sense scaffold and to integrate the upstream signals to activate catalytic subunits. Once signaled, IKK complex is activated, leading to phosphorylation of IKKβ. This results in the ubiquitination of IκBα followed by degradation by the 26S proteasome. This releases the Rel/NF-κB dimers containing primarily p50–p65 subunits to enter the nucleus and activate target gene expression. Due to the large size of Rel/NF-κB dimers, the translocation event is triggered by importins (Figure 3). NF-κB subunits p50 and RelA contain classical nuclear localization signals that are unmasked after ubiquitination and degradation of IκBα prior to their translocation to the nucleus by importin α/β heterodimer [25].

Fagerlund and colleagues have presented strong evidence in their study to suggest that importins α3 and α4 are responsible for the translocation of TNF-α-stimulated active subunits of NF-κB to the nucleus from the cytoplasm [25] and the subsequent export back into the cytoplasm with the support of exportin, CRM1 (chromosome region maintenance 1) [26, 27]. This exportin has sequence similarity with karyopherin β1 and interacts with nucleoporin Nup214 and exports NF-κB to the cytoplasm, thereby inhibiting its accumulation in the nucleus. Thus, importin α3 and importin α4 together with CRM1 are critical karyopherins involved in the import and export of NF-κB in nucleocytoplasmic translocation during allergic airway inflammation (Figure 3).

Recently, several molecules have been identified which might have a significant effect on CRM1-dependent export

of NF-κB and retention of NF-κB in the nucleus. These molecules include prohibitin [11, 27, 28], HSCARG [29], poly(ADP-ribose)polymerase-1 [30], and heat shock protein 72 (Hsp72) [31]. However, the potential presence of additional endogenous mediators as a natural defense mechanism in response to NF-κB activity cannot be ruled out. The underlying mechanisms by which these molecules influence import nucleocytoplasmic shuttling of NF-κB are unclear and warrant further investigation. Nonetheless, there is a potential to develop novel therapeutics in controlling allergic inflammation and the treatment of allergic diseases due to increased activity of NF-κB.

4. AP-1

Activator protein-1 (AP-1) is another transcriptional factor that plays a crucial role in the induction of cellular differentiation, gene expression, and apoptosis. Active AP-1 enhances the secretion of proinflammatory cytokines, including TNF-α and IL-1. In response to allergic inflammatory mediators, both AP-1 and NF-κB are activated and these transcription factors can synergistically induce the transcription and generation of inflammatory mediators to induce allergic and autoimmune diseases. AP-1 consists primarily of two components, c-jun and c-fos, and the active AP-1 is made up of homodimer or heterodimer of these subunits [32]. The homodimer or heterodimer of activated AP-1 enters into the nucleus primarily through importin β1 that recognizes subunits of AP-1 in importin-α-independent manner (Figure 3). In fact, Importin β1 binds to AP-1 subunits, jun and fos, with higher affinity than that of importin-α [33]. Thus, by controlling the binding of importin β1, the nuclear translocation of AP-1 may be regulated and could thus be useful in the attenuation of inflammation in allergic diseases.

There are no reports in human cells in regard to the export of AP-1. However, in yeast it has been found that β-karyopherin-like nuclear exporter, Crm1p, recognizes and binds to the nuclear export sequence on AP-1 in the presence of RanGTP, and this process is inhibited by oxidation [34].

5. GATA-3

GATA-3 is a transcriptional factor that mediates the differentiation and proliferation of Th2 cells [35]. Th2 cytokines, IL-4, IL-5, IL-9, and IL-13, are predominantly regulated by GATA-3 and critically involved in the pathogenesis of allergic airway inflammation. IL-4 and IL-13 are mainly responsible for inducing antigen specific IgE while IL-5 is involved in eosinophilic inflammation. GATA-3 plays a pivotal role in mediating allergic diseases, such as allergic rhinitis, asthma, and atopic dermatitis, and therefore is a key target in developing better therapeutic approaches. Indeed, the knockdown of GATA-3 under both *in vivo* and *in vitro* conditions reduces Th2 cytokines, resulting in inhibited allergic inflammation [36, 37]. GATA-3 contains a classical nuclear import signal that is recognized by importin α and thus translocates to the nucleus [37]. The deletion of the region, which is critical for the interaction of the NLS in AP-1 and importin α, hinders nuclear translocation of activated AP-1 [38]. The p38 MAPK-mediated serine phosphorylation on GATA-3 is critical for nuclear import of GATA-3 from cytoplasm after its interaction with importin α [37]. Upon arrival in the nucleus, GATA-3 binds to its response element in the promoter region of Th2 cytokines, increases their gene expression, thus stimulates IgE class switching by increased production of IL-4 and IL-13, and induces eosinophilic inflammation (Figure 3).

An endogenous inhibitor, MAPK phosphatase-1 (MKP-1), inhibits the phosphorylation and activation of p38 MAPK [37]. The inhibition of p38-MAPK leads to the downregulation of Th2 cytokines [39]. The interaction between importin α and NLS on GATA-3 is affected due to the inhibition of p38 MAPK, resulting in the attenuation of nuclear translocation of GATA-3 from cytoplasm (Figure 3). This, in turn, leads to the downregulation of Th2 cytokines and reduced allergic airway inflammation. The results from animal studies also support the fact that the inhibition of the phosphorylation of p38 MAPK causes a reduction in allergic airway inflammation. This is further supported by findings in which the suppressed activity of p38 MAPK reduced eosinophilic inflammation in mice and guinea pigs that were exposed to OVA [40]. Mice sensitized and challenged with OVA were aerosolized with the SB239063, a potent inhibitor of p38 MAPK inhibitor, showed reduced eosinophilic inflammation, and attenuated airway hyperresponsiveness and mucus production, and there was downregulation in IL-4, IL-5, and IL-13 in the bronchoalveolar lavage fluid. These findings suggest that GATA-3 is regulated via its nuclear import gene importin α. Thus, potential inhibitors of importin α binding to the NLS on GATA-3 could be therapeutically useful in regulating allergic inflammation.

There is no data available at this time on the export of GATA-3 from nucleus to cytoplasm. However, the potential role of CRM-1 cannot be ruled out.

6. STAT6, STAT1, and STAT2

Th2 cytokines, IL-4 or IL-13, activate signal transducer and activator of transcription (STAT) 6 by binding to their receptor IL-4Rα that mediates JAK1 and JAK3, and further activates STAT6 classical pathway. The critical role of STAT6 on IgE class switching, Th2 differentiation, and IL-4 mediated responses is supported by the fact that such effects are drastically impaired in STAT6 knockout mice [41]. STAT6, upon tyrosine phosphorylation, becomes active and translocates to the nucleus by importin α-importin β1 receptors (Figure 3). STAT6 also possesses the binding sites for importins α3 and α6 [42]. Little is known about the STAT6 complex crystal structure; however, the data available in the literature suggests that it can be regulated by targeting the importins responsible for its translocation into the nucleus. This suggests an important role of inhibiting or downregulating importins to alleviate allergic diseases.

Both STAT1 and STAT2 are activated in response to type I interferons, IFN-α, IFN-β, and IFN-ω. Both STAT1

and STAT2 have an arginine/lysine-rich nuclear localization signal. The tyrosine-phosphorylation on Tyr-701 and Tyr-69 on STAT1 and STAT2, respectively, results in the dimerization of STAT1-STAT2, and this heterodimer translocates to the nucleus. The NLS on STAT1-STAT2 heterodimer is recognized by and binds to two importin α5 molecules that are responsible for their translocation into the nucleus (Figure 3) [43].

7. STAT3, RORγt, and Smad3

IL-6, a proinflammatory cytokine responsible for differentiating Th0 cells into pathogenic Th17 cells, activates STAT3 transcription factor. STAT3 is also responsible for the Th17 cell differentiation. It binds to importin α3 and importin α6 and is actively transported into the nucleus with the help of these karyopherins [44]. It has also been shown that nuclear import of STAT3 occurs independent of tyrosine phosphorylation. According to another study, it has been shown to have binding sites for importin α5 and importin α7 as well; however, importin α7 has weak interactions with STAT3 [45] (Figure 3).

IL-6 and TGF-β contribute to induce the expression of RORγt (transcriptional factor, required for the initiation and survival of Th17 cell). Other than our knowledge of its involvement in mediating allergic immune diseases, there is no information on its nuclear versus cytoplasmic movement. However, there is no karyopherin-recognizable nuclear localization sequence present on RORγt [46, 47]. Hence, the translocation of RORγt from the cytoplasm into the nucleus is carried by S6K2, a nuclear counterpart of S6K1, which is induced by PI3 K-Akt-mTORC1 axis [47]. The S6K2 possess functional classical NLS at its C-terminus. It binds to RORγt and then carries it into the nucleus from cytoplasm. Since PI3 K-Akt-mTORC1 axis suppresses Gfi1 expression to positively regulate Th17 differentiation and enhances the S6K2 expression responsible for nuclear translocation of RORγt, the PI3K-Akt-mTORC1 axis-S6K2 is a novel target to limit the differentiation of pathogenic Th17 cell differentiation and could thus be a useful therapeutic approach to control allergic inflammation.

Smad3 is activated in response to TGF-β, which is not only a key factor involved in the expression of RORγt and differentiation of Th17 cells, but also critical in the resolution phase of inflammation by induction of fibrosis at the site allergic reactions. Both importin β and importin 7 are utilized in the transport of activated Smad3 from cytoplasm to nucleus [48, 49] (Figure 3).

8. Allergic Immune Regulation by Importins and Exportins

Recently, we found that vitamin D decreases airway hyperresponsiveness and allergic airway inflammation via its receptor VDR (vitamin D receptor) that acts to downregulate importin α3 in ovalbumin-sensitized and -challenged mice [11]. Although there are several mechanisms proposed in the literature on the beneficial effect of vitamin D in alleviating allergic immune responses, one of the potential targets is importins [50]. Calcitriol, an activated form of vitamin D, stimulates VDR and exerts its beneficial effect by suppressing cell growth, cell proliferation and cell differentiation, and immune regulation. VDR interacts with its response elements in the nucleus and alters the transcription of the subsequent target genes involved in the biological response. We also found that vitamin D reduces allergic responses by increasing the protein expression of prohibitin, a molecule that regulates the activity of importins and its binding to inflammatory transcription factors [51].

Prohibitin (PHB) is a protein that is expressed ubiquitously in cell and mitochondrial membranes and in the nucleus. It is a multifunctional protein that is implicated in various regulatory functions such as proliferation, differentiation, apoptosis, transcription, and protein folding [52]. The expression of PHB decreases in the tissues under inflammatory conditions, including inflammatory bowel disease and airway inflammation, suggesting its potential role as an anti-inflammatory gene [53]. Overexpression and/or restoration of PHB in intestinal epithelial cells downregulated importin α3, an importin involved in NF-κB nuclear translocation [27]. Recently, under both *in vitro* and *in vivo* conditions, we found the downregulation of importin α3 at both the mRNA and protein levels by prohibitin in response to the activation of calcitriol-activated VDR [11, 34]. In vitamin D-deficient allergic asthmatic mice there was a significant reduction in prohibitin due to an upregulation of importin α3 and thus increased translocation of activated NF-κB from the cytoplasm into the nucleus. In vitamin D-sufficient and vitamin D-supplemented mice, the VDR compartments remain intact to keep sufficient amount of prohibitin levels for controlling cellular activity. This regulates importin α3-mediated nuclear translocation of NF-κB and thus reduces allergic airway inflammation and airway hyperresponsiveness in response to allergen challenge in allergen-sensitized mice [11]. Thus, in inflammatory conditions, a decrease in expression of either or both VDR and PHB leads to an increase in importin α3 expression and its activity, resulting in the exaggeration of allergic inflammatory response.

Corticosteroids are potent anti-inflammatory agents and regulate the activity of several transcriptional factors involved in allergic inflammation [54]. Although there could be several potential mechanisms for the underlying anti-inflammatory effects of corticosteroids, one of the major effects is the inhibition of nuclear translocation of activated transcription factors, including GATA-3 and NF-κB (Figure 4). Corticosteroids are highly efficient in suppressing Th2 cytokines in the airways of individuals with allergic asthma. The anti-inflammatory effect by corticosteroids is exerted via binding to cytosolic glucocorticoid receptors (GR). After interacting with their receptors, corticosteroid-GR complex translocates to the nucleus from the cytoplasm where they bind to the promoter region of steroid-sensitive genes containing glucocorticoid response elements (GREs). The activated glucocorticoid receptors suppress inflammatory action exerted by NF-κB via interaction with coactivator molecules. This is achieved by inhibiting either the nuclear translocation of NF-κB by upregulating endogenous regulatory molecules,

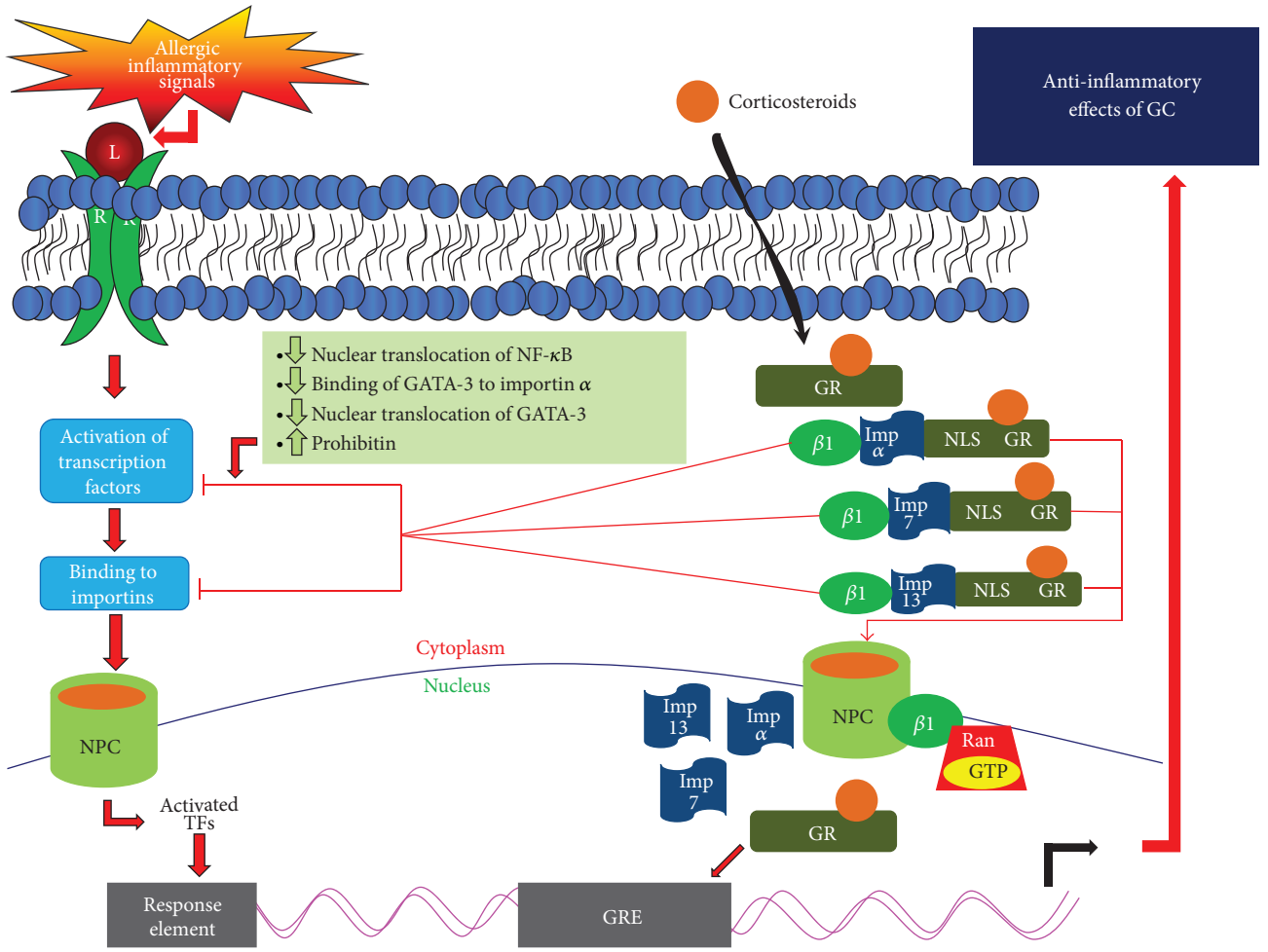


FIGURE 4: Activation of glucocorticoid receptor (GR) and control of allergic inflammation:corticosteroids are lipophilic in nature and cross the bilipid membrane to bind to their receptors in the cytoplasm. The nuclear localization signal (NLS) on steroid-receptor complex can be recognized by importin α, importin 7, or importin 13 to transport the steroid-receptor complex to the nucleus to bind to glucocorticoid-response element (GRE) to induce the transcription of several genes that elicit anti-inflammatory effect. Also, the steroid-receptor complex in the cytoplasm can also induce effects on the nuclear transport of activated transcription factors by either inhibiting the binding of activated transcription factors to importins or increasing the inhibitory molecules, such as prohibitin, in the cytoplasm.

including prohibitin, or by inducing more inhibitory subunits, IκB, of NF-κB, to keep them inactive in the cytoplasm (Figure 4).

The major event to exert anti-inflammatory effect is mediated via the nuclear translocation of corticosteroid-GR complex. The nuclear translocation signals responsible for nuclear transport and retention of activated GR are NL1 and NL2, where NL1 is similar in sequence to Simian virus 40 and the NL2 of GR is poorly defined [55, 56]. These nuclear localization signals are present on importins (Figure 4). Additionally, their nuclear export is mediated by chromosomal region maintenance (CRM-1) pathway.

Several importins have been found to be involved in the nuclear translocation of corticosteroid-GR complex. NL1 of GR binds to importin α and is responsible for importing corticosteroids into the nucleus. In addition, the other importins involved in nuclear translocation of corticosteroid-GR complex include importin 7, importin 8, and importin 13 [29, 48, 57, 58]. However, whether the NLS1 sequence, to which these importins bind, is the same or different is not very well understood [55] even though the presence of both NLS1 and NLS2 on GR and its binding to importin-7 has been found in nonmammalian cells, suggesting the crucial role of both NLSs for translocation of GR into nucleus [55]. Importin β stimulates the binding of GR to importin α, whether it is involved in the translocation of GR into the nucleus is poorly defined.

Recently, Hakim and colleagues [58] confirmed earlier findings of Xiao et al. [48] that importin 7 is critical in the translocation of glucocorticoid receptor from the cytoplasm into the nucleus (Figure 4). Also, the expression of the cofactor of importin 7 complex, RanGTP, is reduced during oxidative stress-induced corticosteroid insensitivity, as shown in the presence of hydrogen peroxide [58]. Interestingly, the degree of loss of importin 7 correlates well with the reduction in GR nuclear translocation and insensitivity

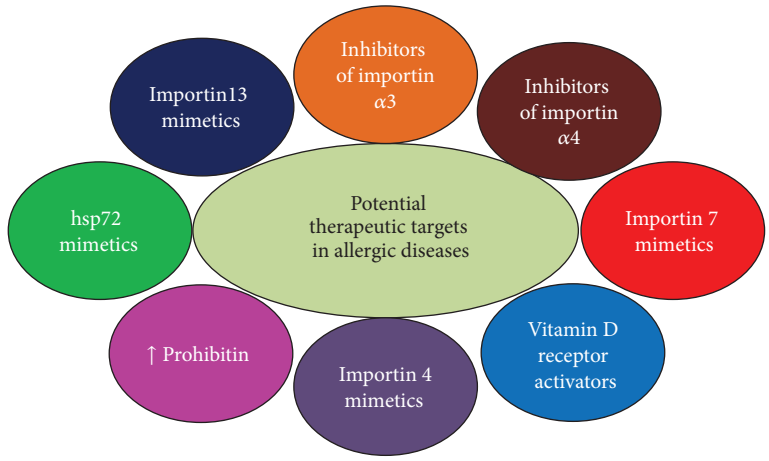


Figure 5: Schematic diagram showing various target sites for intervention in allergic diseases: there are many sites in the importin-exportin system in inflammatory cells involved in allergic diseases. Detailed knowledge on the involvement and role of specific importin molecule and/or the synthesis of mimetics/activators of endogenous inhibitors would help in the development of better therapeutic approaches in allergic diseases.

of corticosteroids [58]. In several cases of severe asthma and chronic obstructive pulmonary disease (COPD), corticosteroids are not very effective [59]. In chronic inflammation, biomarkers of oxidative stress such as oxidant hydrogen peroxide and 8-isoprostane are upregulated, leading to exacerbated inflammation. This uncontrolled oxidative stress results in glucocorticoid insensitivity in the biosystem [60]. The diminished expression of importin 7 can knock down the protective effect of glucocorticoid receptors in human macrophages cell line, which might support the critical role of importin 7 in transporting GR from cytoplasm to the nucleus [58]. However, whether or not the insensitivity of corticosteroids in severe asthmatic and COPD patients is due to the defect in importin 7 is not known and warrants further studies in allergic subjects.

One of the mechanisms of corticosteroid-induced inhibition of Th2 response could be due to the competition between GR and GATA-3 to bind to importin α since both GR and GATA-3 utilize this karyopherin. Maneechotesuwan et al. [60] reported that fluticasone, a synthetic corticosteroid, either under *in vitro* conditions or upon inhalation causes suppression in nuclear transport of GATA-3 in human T lymphocytes. In this study, the interaction between GR and importin-α was found to be very effective even at low concentration of fluticasone. There was a reduction in GATA-3-importin α complex and an increase in GR-importin-α interactions when fluticasone was inhaled by asthmatic patients [60].

GR can also be carried into the nucleus by importin 7 and importin 13 [55, 58, 59] (Figure 4). Since NF-κB and AP-1 activity can also be inhibited by GR, it is likely that GR has a higher affinity for the karyopherins than the inflammatory transcription factors and/or activated GR induces the production of endogenous inhibitors of importins involved in the translocation of inflammatory transcription factors. Therefore, any defect either in the activation of GR or its translocation into the nucleus could be responsible for the lack of responsiveness or decreased response to corticosteroids. On the other hand, there could be variants of importins that enhance the entry of GR to the nucleus and thus enhance the response to corticosteroids. Indeed, a genetic variant in importin 13 has been found to be associated with improved PC_{20} (i.e., decreased airway hyperresponsiveness) to methacholine in mild-to-moderate asthmatic children [61]. Accordingly, potential polymorphisms in various importins in allergic subjects could dictate proinflammatory or anti-inflammatory response.

9. Outstanding Questions and Future Directions

Since the nuclear import and export of the proteins occur in highly systematic and organized manner, it is critical to understand the precise regulatory mechanisms of the importin and exportin molecules with respect to their synthesis and function. This would assist in targeting specific sites to allow for the development of better therapeutic approaches in allergic diseases (Figure 5). Since several importin molecules are nonspecific in nature, antagonists or antibodies specifically against a particular importin might not prove to be clinically beneficial. However, additional knowledge regarding the precise control over the recruitment and activation of a few key karyopherins could be beneficial in the development of better therapeutic approaches. In addition, further studies regarding the absence and presence of endogenous mediators that regulate karyopherins in clinical allergic conditions would be informative. Potential polymorphisms and genetic variants in key importin molecules and the epigenetic control of karyopherins are also wide open fields for further investigation (Figure 5). At present, there are no studies on the nucleocytoplasmic shuttling of Foxp3 and T-bet, key transcription factors associated with allergic diseases. Overall, the potential role of importins and exportins in the regulation of allergic immune response is

indeed both fascinating and challenging and thus warrants further investigation.

Disclaimer

The content of this review is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

This work was supported by research Grants R01AI075315, R01HL073349, and R01HL116042 from the National Institutes of Health, USA, to D. K. Agrawal. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the paper apart from those disclosed.

References

[1] M. A. Zanta, P. Belguise-Valladier, and J. P. Behr, "Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 1, pp. 91–96, 1999.

[2] I. K. H. Poon and D. A. Jans, "Regulation of nuclear transport: central role in development and transformation?" *Traffic*, vol. 6, no. 3, pp. 173–186, 2005.

[3] O. J. Gruss, R. E. Carazo-Salas, C. A. Schatz et al., "Ran induces spindle assembly by reversing the inhibitory effect of importin α on TPX2 activity," *Cell*, vol. 104, no. 1, pp. 83–93, 2001.

[4] K. Weis, I. W. Mattaj, and A. I. Lamond, "Identification of hSRP1α as a functional receptor for nuclear localization sequences," *Science*, vol. 268, no. 5213, pp. 1049–1053, 1995.

[5] M. Köhler, S. Ansieau, S. Prehn, A. Leutz, H. Haller, and E. Hartmann, "Cloning of two novel human importin-α subunits and analysis of the expression pattern of the importin-α protein family," *FEBS Letters*, vol. 417, no. 1, pp. 104–108, 1997.

[6] M. Köhler, C. Speck, M. Christiansen et al., "Evidence for distinct substrate specificities of importin α family members in nuclear protein import," *Molecular and Cellular Biology*, vol. 19, no. 11, pp. 7782–7791, 1999.

[7] P. Cortes, Z. S. Ye, and D. Baltimore, "RAG-1 interacts with the repeated amino acid motif of the human homologue of the yeast protein SRP1," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 16, pp. 7633–7637, 1994.

[8] D. Görlich and U. Kutay, "Transport between the cell nucleus and the cytoplasm," *Annual Review of Cell and Developmental Biology*, vol. 15, pp. 607–660, 1999.

[9] M. V. Nachury, U. W. Ryder, A. I. Lamond, and K. Weis, "Cloning and characterization of hSRP1γ, a tissue-specific nuclear transport factor," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 2, pp. 582–587, 1998.

[10] Y. Miyamoto, N. Imamoto, T. Sekimoto et al., "Differential modes of nuclear localization signal (NLS) recognition by three distinct classes of NLS receptors," *Journal of Biological Chemistry*, vol. 272, no. 42, pp. 26375–26381, 1997.

[11] T. Agrawal, G. K. Gupta, and D. K. Agrawal, "Vitamin D supplementation reduces airway hyperresponsiveness and allergic airway inflammation in a murine model," *Clinical & Experimental Allergy*, vol. 43, no. 6, pp. 672–683, 2013.

[12] S. Finotto, G. T. de Sanctis, H. A. Lehr et al., "Treatment of allergic airway inflammation and hyperresponsiveness by antisense-induced local blockade of GATA-3 expression," *Journal of Experimental Medicine*, vol. 193, no. 11, pp. 1247–1260, 2001.

[13] Y. Bossé, K. Maghni, and T. J. Hudson, "1α,25-dihydroxyvitamin D3 stimulation of bronchial smooth muscle cells induces autocrine, contractility, and remodeling processes," *Physiological Genomics*, vol. 29, no. 2, pp. 161–168, 2007.

[14] R. H. Kehlenbach, A. Dickmanns, and L. Gerace, "Nucleocytoplasmic shuttling factors including Ran and CRM1 mediate nuclear export of NFAT in vitro," *Journal of Cell Biology*, vol. 141, no. 4, pp. 863–874, 1998.

[15] G. Lipowsky, F. R. Bischoff, P. Schwarzmaier et al., "Exportin 4: a mediator of a novel nuclear export pathway in higher eukaryotes," *EMBO Journal*, vol. 19, no. 16, pp. 4362–4371, 2000.

[16] I. E. Wertz and V. M. Dixit, "Regulation of death receptor signaling by the ubiquitin system," *Cell Death and Differentiation*, vol. 17, no. 1, pp. 14–24, 2010.

[17] Z. Han, D. L. Boyle, A. M. Manning, and G. S. Firestein, "AP-1 and NF-κB regulation in rheumatoid arthritis and murine collagen-induced arthritis," *Autoimmunity*, vol. 28, no. 4, pp. 197–208, 1998.

[18] J. Louten, K. Boniface, and R. de Waal Malefyt, "Development and function of TH17 cells in health and disease," *Journal of Allergy and Clinical Immunology*, vol. 123, no. 5, pp. 1004–1011, 2009.

[19] A. A. Lighvani, D. M. Frucht, D. Jankovic et al., "T-bet is rapidly induced by interferon-γ in lymphoid and myeloid cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 26, pp. 15137–15142, 2001.

[20] I. M. Djuretic, D. Levanon, V. Negreanu, Y. Groner, A. Rao, and K. M. Ansel, "Transcription factors T-bet and Runx3 cooperate to activate Ifng and silence Il4 in T helper type 1 cells," *Nature Immunology*, vol. 8, no. 2, pp. 145–153, 2007.

[21] J. Zhu, L. Guo, C. J. Watson, J. Hu-Li, and W. E. Paul, "Stat6 is necessary and sufficient for IL-4's role in TH2 differentiation and cell expansion," *Journal of Immunology*, vol. 166, no. 12, pp. 7276–7281, 2001.

[22] D. K. Agrawal, K. Ariyarathna, and P. W. Kelbe, "(S)-Albuterol activates pro-constrictory and pro-inflammatory pathways in human bronchial smooth muscle cells," *Journal of Allergy and Clinical Immunology*, vol. 113, no. 3, pp. 503–510, 2004.

[23] M. R. Edwards, N. W. Bartlett, D. Clarke, M. Birrell, M. Belvisi, and S. L. Johnston, "Targeting the NF-kappaB pathway in asthma and chronic obstructive pulmonary disease," *Pharmacology & Therapeutics*, vol. 121, no. 1, pp. 1–13, 2009.

[24] P. J. Barnes and M. Karin, "Nuclear factor-κB—a pivotal transcription factor in chronic inflammatory diseases," *New England Journal of Medicine*, vol. 336, no. 15, pp. 1066–1071, 1997.

[25] R. Fagerlund, L. Kinnunen, M. Köhler, I. Julkunen, and K. Melén, "NF-κB is transported into the nucleus by importin α3 and importin α4," *Journal of Biological Chemistry*, vol. 280, no. 16, pp. 15942–15951, 2005.

[26] S. H. Lee and M. Hannink, "The N-terminal nuclear export sequence of IκBα is required for RanGTP-dependent binding to CRM1," *Journal of Biological Chemistry*, vol. 276, no. 26, pp. 23599–23606, 2001.

[27] A. L. Theiss, A. K. Jenkins, N. I. Okoro, J. M. A. Klapproth, D. Merlin, and S. V. Sitaraman, "Prohibitin inhibits tumor necrosis factor alpha-induced nuclear factor-kappa B nuclear translocation via the novel mechanism of decreasing importin α3 expression," *Molecular Biology of the Cell*, vol. 20, no. 20, pp. 4412–4423, 2009.

[28] T. Agrawal, G. K. Gupta, and D. K. Agrawal, "Calcitriol decreases expression of importin α3 and attenuates RelA translocation in human bronchial smooth muscle cells," *Journal of Clinical Immunology*, vol. 32, no. 5, pp. 1093–1103, 2012.

[29] M. Zhang, B. Hu, T. Li et al., "A CRM1-dependent nuclear export signal controls nucleocytoplasmic translocation of HSCARG, which regulates NF-κB activity," *Traffic*, vol. 13, no. 6, pp. 790–799, 2012.

[30] M. Zerfaoui, Y. Errami, A. S. Naura et al., "Poly(ADP-ribose) polymerase-1 is a determining factor in Crm1-mediated nuclear export and retention of p65 NF-κB upon TLR4 stimulation," *Journal of Immunology*, vol. 185, no. 3, pp. 1894–1902, 2010.

[31] D. Tang, R. Kang, W. Xiao, H. Wang, S. K. Calderwood, and X. Xiao, "The anti-inflammatory effects of heat shock protein 72 involve inhibition of high-mobility-group box 1 release and proinflammatory function in macrophages," *Journal of Immunology*, vol. 179, no. 2, pp. 1236–1244, 2007.

[32] E. Shaulian and M. Karin, "AP-1 in cell proliferation and survival," *Oncogene*, vol. 20, no. 19, pp. 2390–2400, 2001.

[33] J. K. Forwood, M. H. C. Lam, and D. A. Jans, "Nuclear import of Creb and AP-1 transcription factors requires importin-β1 and Ran but is independent of importin-α," *Biochemistry*, vol. 40, no. 17, pp. 5208–5217, 2001.

[34] C. Yan, L. H. Lee, and L. I. Davis, "Crm1p mediates regulated nuclear export of a yeast AP-1-like transcription factor," *EMBO Journal*, vol. 17, no. 24, pp. 7416–7429, 1998.

[35] J. Zhu, H. Yamane, J. Cote-Sierra, L. Guo, and W. E. Paul, "GATA-3 promotes Th2 responses through three different mechanisms: induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors," *Cell Research*, vol. 16, no. 1, pp. 3–10, 2006.

[36] M. Yamashita, M. Ukai-Tadenuma, T. Miyamoto et al., "Essential role of GATA3 for the maintenance of type 2 helper T (Th2) cytokine production and chromatin remodeling at the Th2 cytokine gene loci," *Journal of Biological Chemistry*, vol. 279, no. 26, pp. 26983–26990, 2004.

[37] K. Maneechotesuwan, Y. Xin, K. Ito et al., "Regulation of Th2 cytokine genes by p38 MAPK-mediated phosphorylation of GATA-3," *Journal of Immunology*, vol. 178, no. 4, pp. 2491–2498, 2007.

[38] Z. Yang, L. Gu, P. H. Romeo et al., "Human GATA-3 trans-activation, DNA-binding, and nuclear localization activities are organized into distinct structural domains," *Molecular and Cellular Biology*, vol. 14, no. 3, pp. 2201–2212, 1994.

[39] L. Chialda, M. Zhang, K. Brune, and A. Pahl, "Inhibitors of mitogen-activated protein kinases differentially regulate costimulated T cell cytokine production and mouse airway eosinophilia," *Respiratory Research*, vol. 6, article 36, 2005.

[40] D. C. Underwood, R. R. Osborn, C. J. Kotzer et al., "SB 239063, a potent p38 MAP kinase inhibitor, reduces inflammatory cytokine production, airways eosinophil infiltration, and persistence," *Journal of Pharmacology and Experimental Therapeutics*, vol. 293, no. 1, pp. 281–288, 2000.

[41] M. Akdis and C. A. Akdis, "IgE class switching and cellular memory," *Nature Immunology*, vol. 13, no. 4, pp. 312–314, 2012.

[42] H. C. Chen and N. C. Reich, "Live cell imaging reveals continuous STAT6 nuclear trafficking," *Journal of Immunology*, vol. 185, no. 1, pp. 64–70, 2010.

[43] R. Fagerlund, K. Mélen, L. Kinnunen, and I. Julkunen, "Arginine/lysine-rich nuclear localization signals mediate interactions between dimeric STATs and importin alpha 5," *Journal of Biological Chemistry*, vol. 277, no. 33, pp. 30072–30078, 2002.

[44] L. Liu, K. M. McBride, and N. C. Reich, "STAT3 nuclear import is independent of tyrosine phosphorylation and mediated by importin-α3," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 23, pp. 8150–8155, 2005.

[45] J. Ma and X. Cao, "Regulation of Stat3 nuclear import by importin α5 and importin α7 via two different functional sequence elements," *Cellular Signalling*, vol. 18, no. 8, pp. 1117–1126, 2006.

[46] B. Rost, G. Yachdav, and J. Liu, "The PredictProtein server," *Nucleic Acids Research*, vol. 32, pp. W321–W326, 2004.

[47] Y. Kurebayashi, S. Nagai, A. Ikejiri et al., "PI3K-Akt-mTORC1-S6K1/2 axis controls Th17 differentiation by regulating Gfi1 expression and nuclear translocation of RORgamma," *Cell Reports*, vol. 1, no. 4, pp. 360–373, 2012.

[48] Z. Xiao, X. Liu, Y. I. Henis, and H. F. Lodish, "A distinct nuclear localization signal in the N terminus of Smad 3 determines its ligand-induced nuclear translocation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 14, pp. 7853–7858, 2000.

[49] X. Yao, X. Chen, C. Cottonham, and L. Xu, "Preferential utilization of Imp7/8 in nuclear import of Smads," *Journal of Biological Chemistry*, vol. 283, no. 33, pp. 22867–22874, 2008.

[50] T. Agrawal, G. K. Gupta, and D. K. Agrawal, "Calcitriol decreases expression of importin α3 and attenuates RelA translocation in human bronchial smooth muscle cells," *Journal of Clinical Immunology*, vol. 32, no. 5, pp. 1093–1103, 2012.

[51] T. Agrawal, G. K. Gupta, and D. K. Agrawal, "Vitamin D deficiency decreases the expression of VDR and prohibitin in the lungs of mice with allergic airway inflammation," *Experimental and Molecular Pathology*, vol. 93, no. 1, pp. 74–81, 2012.

[52] L. G. J. Nijtmans, L. de Jong, M. A. Sanz et al., "Prohibitins act as a membrane-bound chaperone for the stabilization of mitochondrial proteins," *EMBO Journal*, vol. 19, no. 11, pp. 2444–2451, 2000.

[53] A. L. Theiss, T. S. Obertone, D. Merlin, and S. V. Sitaraman, "Interleukin-6 transcriptionally regulates prohibitin expression in intestinal epithelial cells," *Journal of Biological Chemistry*, vol. 282, no. 17, pp. 12804–12812, 2007.

[54] T. Rhen and J. A. Cidlowski, "Antiinflammatory action of glucocorticoids—new mechanisms for old drugs," *New England Journal of Medicine*, vol. 353, no. 16, pp. 1711–1658, 2005.

[55] N. D. Freedman and K. R. Yamamoto, "Importin 7 and importin α/importin β are nuclear import receptors for the glucocorticoid receptor," *Molecular Biology of the Cell*, vol. 15, no. 5, pp. 2276–2286, 2004.

[56] D. Picard and K. R. Yamamoto, "Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor," *EMBO Journal*, vol. 6, no. 11, pp. 3333–3340, 1987.

[57] T. Tao, J. Lan, G. L. Lukacs, R. J. G. Haché, and F. Kaplan, "Importin 13 regulates nuclear import of the glucocorticoid receptor in airway epithelial cells," *The American Journal of Respiratory Cell and Molecular Biology*, vol. 35, no. 6, pp. 668–680, 2006.

[58] A. Hakim, P. J. Barnes, I. M. Adcock, and O. S. Usmani, "Importin-7 mediates glucocorticoid receptor nuclear import and is impaired by oxidative stress, leading to glucocorticoid insensitivity," *FASEB Journal*, vol. 27, no. 11, pp. 4510–4519, 2013.

[59] S. Suissa and P. J. Barnes, "Inhaled corticosteroids in COPD: the case against," *European Respiratory Journal*, vol. 34, no. 1, pp. 13–16, 2009.

[60] K. Maneechotesuwan, X. Yao, K. Ito et al., "Suppression of GATA-3 nuclear import and phosphorylation: a novel mechanism of corticosteroid action in allergic disease," *PLoS Medicine*, vol. 6, no. 5, Article ID e1000076, 2009.

[61] B. A. Raby, K. van Steen, J. Lasky-Su, K. Tantisira, F. Kaplan, and S. T. Weiss, "Importin-13 genetic variation is associated with improved airway responsiveness in childhood asthma," *Respiratory Research*, vol. 10, article 67, 2009.

[62] B. W. Pickard, A. B. Hodsman, L. J. Fraher, and P. H. Watson, "Type 1 parathyroid hormone receptor (PTH1R) nuclear trafficking: association of PTH1R with importin α1 and β," *Endocrinology*, vol. 147, no. 7, pp. 3326–3332, 2006.

[63] X. Yao, H. Y. He, Y. Yang et al., "Inhibition of interleukin-13 gene expression in T cells through GATA-3 pathway by arsenic trioxide," *Chinese Medical Journal*, vol. 121, no. 22, pp. 2346–2349, 2008.

[64] S. E. Wilkie, K. J. Morris, S. S. Bhattacharya, M. J. Warren, and D. M. Hunt, "A study of the nuclear trafficking of the splicing factor protein PRPF31 linked to autosomal dominant retinitis pigmentosa (ADRP)," *Biochimica et Biophysica Acta*, vol. 1762, no. 3, pp. 304–311, 2006.

[65] F. Fazal, M. Minhajuddin, K. M. Bijli, J. L. McGrath, and A. Rahman, "Evidence for actin cytoskeleton-dependent and -independent pathways for RelA/p65 nuclear translocation in endothelial cells," *Journal of Biological Chemistry*, vol. 282, no. 6, pp. 3940–3950, 2007.

[66] S. Bhushan, C. Kuhn, A. K. Berglund, C. Roth, and E. Glaser, "The role of the N-terminal domain of chloroplast targeting peptides in organellar protein import and miss-sorting," *FEBS Letters*, vol. 580, no. 16, pp. 3966–3972, 2006.

[67] A. Argentaro, H. Sim, S. Kelly et al., "A SOX9 defect of calmodulin-dependent nuclear import in campomelic dysplasia/autosomal sex reversal," *Journal of Biological Chemistry*, vol. 278, no. 36, pp. 33839–33847, 2003.

[68] J. D. Moore, J. Yang, R. Truant, and S. Kornbluth, "Nuclear import of Cdk/cyclin complexes: identification of distinct mechanisms for import of Cdk2/cyclin E and Cdc2/cyclin B1," *Journal of Cell Biology*, vol. 144, no. 2, pp. 213–224, 1999.

[69] K. Ishiguro, T. Ando, O. Maeda, N. Ohmiya, Y. Niwa, and H. Goto, "Acetate inhibits NFAT activation in T cells via importin β1 interference," *European Journal of Immunology*, vol. 37, no. 8, pp. 2309–2316, 2007.

[70] L. G. Le Roux and J. Moroianu, "Nuclear entry of high-risk human papillomavirus type 16 E6 oncoprotein occurs via several pathways," *Journal of Virology*, vol. 77, no. 4, pp. 2330–2337, 2003.

[71] I. Waldmann, S. Wälde, and R. H. Kehlenbach, "Nuclear import of c-Jun is mediated by multiple transport receptors," *Journal of Biological Chemistry*, vol. 282, no. 38, pp. 27685–27692, 2007.

[72] L. F. Pemberton, G. Blobel, and J. S. Rosenblum, "Transport routes through the nuclear pore complex," *Current Opinion in Cell Biology*, vol. 10, no. 3, pp. 392–399, 1998.

[73] Y. Miyauchi, T. Michigami, N. Sakaguchi et al., "Importin 4 is responsible for ligand-independent nuclear translocation of vitamin D receptor," *Journal of Biological Chemistry*, vol. 280, no. 49, pp. 40901–40908, 2005.

[74] G. Chachami, E. Paraskeva, J. M. Mingot, G. G. Braliou, D. Görlich, and G. Simos, "Transport of hypoxia-inducible factor HIF-1α into the nucleus involves importins 4 and 7," *Biochemical and Biophysical Research Communications*, vol. 390, no. 2, pp. 235–240, 2009.

[75] S. Jäkel, W. Albig, U. Kutay et al., "The importin β/importin 7 heterodimer is a functional nuclear import receptor for histone H1," *EMBO Journal*, vol. 18, no. 9, pp. 2411–2423, 1999.

[76] L. Xu, C. Alarcón, S. Çöl, and J. Massagué, "Distinct domain utilization by Smad3 and Smad4 for nucleoporin interaction and nuclear import," *Journal of Biological Chemistry*, vol. 278, no. 43, pp. 42569–42577, 2003.

[77] K. A. Dean, O. Von Ahsen, D. Görlich, and H. M. Fried, "Signal recognition particle protein 19 is imported into the nucleus by importin 8 (RanBP8) and transportin," *Journal of Cell Science*, vol. 114, part 19, pp. 3479–3485, 2001.

[78] E. J. Lubert and K. D. Sarge, "Interaction between protein phosphatase 2A and members of the importin β superfamily," *Biochemical and Biophysical Research Communications*, vol. 303, no. 3, pp. 908–913, 2003.

[79] S. M. Plafker and I. G. Macara, "Ribosomal protein L12 uses a distinct nuclear import pathway mediated by importin 11," *Molecular and Cellular Biology*, vol. 22, no. 4, pp. 1266–1275, 2002.

[80] S. M. Plafker and I. G. Macara, "Importin-11, a nuclear import receptor for the ubiquitin-conjugating enzyme, UbcM2," *EMBO Journal*, vol. 19, no. 20, pp. 5502–5513, 2000.

[81] A. C. Hearps and D. A. Jans, "HIV-1 integrase is capable of targeting DNA to the nucleus via an Importin α/β-dependent mechanism," *Biochemical Journal*, vol. 398, no. 3, pp. 475–484, 2006.

[82] S. H. Liang and M. F. Clarke, "The nuclear import of p53 is determined by the presence of a basic domain and its relative position to the nuclear localization signal," *Oncogene*, vol. 18, no. 12, pp. 2163–2166, 1999.

[83] S. Benzeno and J. A. Diehl, "C-terminal sequences direct cyclin D1-CRM1 binding," *Journal of Biological Chemistry*, vol. 279, no. 53, pp. 56061–56066, 2004.

[84] S. H. Lee and M. Hannink, "Characterization of the nuclear import and export functions of IκBε," *Journal of Biological Chemistry*, vol. 277, no. 26, pp. 23358–23366, 2002.

[85] P. Macchi, A. M. Brownawell, B. Grunewald, L. DesGroseillers, I. G. Macara, and M. A. Kiebler, "The brain-specific double-stranded RNA-binding protein Staufen2. Nucleolar accumulation and isoform-specific exportin-5-dependent export," *Journal of Biological Chemistry*, vol. 279, no. 30, pp. 31440–31444, 2004.

[86] M. T. Bohnsack, T. Stüven, C. Kuhn, V. C. Cordes, and D. Görlich, "A selective block of nuclear actin export stabilizes the giant nuclei of Xenopus oocytes," *Nature Cell Biology*, vol. 8, no. 3, pp. 257–263, 2006.

[87] J. M. Mingot, M. T. Bohnsack, U. Jäkle, and D. Görlich, "Exportin 7 defines a novel general nuclear export pathway," *EMBO Journal*, vol. 23, no. 16, pp. 3227–3236, 2004.

[88] T. Kuwabara, M. Warashina, M. Sano et al., "Recognition of engineered tRNAs with an extended 3′ end byexportin-t (Xpo-t) and transport of tRNA-attached ribozymes to the cytoplasm in somatic cells," *Biomacromolecules*, vol. 2, no. 4, pp. 1229–1242, 2001.

Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2014, Article ID 826987, 9 pages
http://dx.doi.org/10.1155/2014/826987

Research Article

Splenic Stromal Cells from Aged Mice Produce Higher Levels of IL-6 Compared to Young Mice

Jihyun Park,[1,2] Takuya Miyakawa,[2] Aya Shiokawa,[1] Haruyo Nakajima-Adachi,[1] Masaru Tanokura,[2] and Satoshi Hachimura[1]

[1] *Research Center for Food Safety, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan*
[2] *Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan*

Correspondence should be addressed to Satoshi Hachimura; ahachi@mail.ecc.u-tokyo.ac.jp

Received 20 November 2013; Revised 14 January 2014; Accepted 15 January 2014; Published 4 March 2014

Academic Editor: Huiyun Zhang

Inflamm-aging indicates the chronic inflammatory state resulting from increased secretion of proinflammatory cytokines and mediators such as IL-6 in the elderly. Our principle objective was to identify cell types that were affected with aging concerning IL-6 secretion in the murine model. We compared IL-6 production in spleen cells from both young and aged mice and isolated several types of cells from spleen and investigated IL-6 mRNA expression and protein production. IL-6 protein productions in cultured stromal cells from aged mice spleen were significantly high compared to young mice upon LPS stimulation. IL-6 mRNA expression level of freshly isolated stromal cells from aged mice was high compared to young mice. Furthermore, stromal cells of aged mice highly expressed IL-6 mRNA after LPS injection in vivo. These results suggest that stromal cells play a role in producing IL-6 in aged mice and imply that they contribute to the chronic inflammatory condition in the elderly.

1. Introduction

The elderly show differences in their ability to resist disease or injury compared to the young and adults, and some of the noticeable features are alterations in the development and maintenance of immune response and cellular function [1–3]. Aging process triggers suchlike changes of immune functions and as a result of dysregulated immune functions against infection and inflammation, a number of complications and chronic medical conditions occur [3–5].

Systemic inflammatory response syndrome is induced by dysregulated immune response to trauma or infection that normally protective responses became harmful provoking shock and organ dysfunctions [3]. Inflamm-aging indicates the chronic inflammatory state resulting from increased secretion of proinflammatory cytokines and mediators in the elderly [6, 7]. Partly due to inflamm-aging, the elderly are susceptible to autoimmune diseases, bacterial pathogens, and viral exposure, and the occurrence of autoimmune diseases, infectious diseases, and cancer increases in the elderly [8, 9]. Also a number of immune alterations, such as changed cytokine profile, might facilitate allergic associated conditions in the elderly [10, 11].

Proinflammatory cytokines, such as TNF-α, IL-1, and IL-6, are significantly related to the age-associated diseases and disability, and these are important factors on inflammatory response in the elderly [12–14]. Especially, IL-6 is considered as a dependable aging parameter [15]. Aged IL-6 knockout mice show reduced mortality compared to wild type mice after injection with LPS and anti-IL-6 receptor antibodies are good formulation for rheumatoid arthritis [3]. IL-6 produced in multiple tissues during inflammation stimulates immune process, and the production of IL-6 is elevated in some tissues of aged mice compared to young adult mice such as lung, kidney, skeletal muscle, heart, fat, and spleen [3, 16]. And under stressful or depressed conditions, IL-6 levels are increased [17]. Moreover, IL-6 is detected in high levels in disease conditions including autoimmune disease,

such as rheumatoid arthritis, atherosclerosis, diabetes, and inflammatory-bowel diseases [18, 19]. It implies that IL-6 might play roles that boost the risk and mortality in inflammation in the elderly.

IL-6 is mainly produced by cells such as macrophages and dendritic cells. In secondary lymphoid organs (SLOs), where immune responses are initiated, a number of hematopoietic cells, including macrophages and dendritic cells which were mentioned above, interact with each other [20]. In addition to these immune cells, stromal cells are also present in SLOs. Stromal cells have been considered as cells that do not significantly affect the immune response but give a structural support to the lymphoid organs; however, the functions of stromal cells in immune homeostasis and immune responses have begun to emerge [21].

In this study, our principle objective was to identify cell types that were affected by aging concerning secretion of IL-6 in the murine model. To answer these questions, we compared IL-6 production in splenocytes from both young and aged mice and isolated a number of cell types from spleen and investigated IL-6 mRNA or protein expression. We found that stromal cells express IL-6 highly with aging both in noninflammatory and inflammatory conditions.

2. Materials and Methods

2.1. Animals. C57BL/6 and BALB/c female mice were obtained from CLEA Japan (Tokyo, Japan). The mice were used in the experiments at 2–4 mo for young mice and at 11–18 mo for aged mice, respectively. All procedures were performed in accordance with the guidelines for animal use and care of the University of Tokyo.

2.2. Culture Medium. Cells were cultured in RPMI 1640 medium supplemented with 5% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 3 mM L-glutamine, and 50 μM 2-mercaptoethanol.

2.3. Isolation of Cells and Cell Culture. Spleens were removed from mice and digested with collagenase type 1. After red blood cell lysis, white blood cells were counted. Five $\times$ 10^5 whole splenocytes were seeded in 96-well plates in 200 μL RPMI medium and incubated for 24 hours at 37°C and 5% CO_2. Macrophages, $CD11c^+$ dendritic cells, and $CD45^-$ stromal cells were isolated by MACS (Miltenyi Biotech, Bergisch-Gladbach, Germany). In short, cells were incubated with anti-mouse F4/80, CD11b, CD11c, and $CD45^-$ coated magnetic beads (Miltenyi Biotech), respectively, and selected on MACS separation columns (Miltenyi Biotech). Macrophages were prepared by F4/80 positive selection or CD11c negative selection and CD11b positive selection. Dendritic cells were isolated as $CD11c^+$ cells and stromal cells as $CD45^-$ cells, respectively. In certain experiments, $CD45^+$ cells were also used. MACS purified macrophages, dendritic cells, or $CD45^+$ cells were seeded in 96-well plates (5×10^5 cells/well) in 200 μL RPMI medium with or without LPS and cultured for 24 h. Five $\times$ 10^4 cells of $CD45^-$ stromal cells were cultured in the same manner as above. Since the splenic $CD45^-$ stromal cells form a dense network and FRCs, a kind of stromal cells, are connected to each other in spleen forming a three-dimensional reticulum, lower concentrations of cells were used for measurement of IL-6 protein production compared to other cell types [22, 23].

2.4. Exposure to LPS. Acute inflammation was induced by i.p. injection with LPS derived from *E. coli* (Sigma-Aldrich, St. Louis, MO, USA). LPS was dissolved in phosphate-buffered saline (PBS) and administered by i.p. with a dose of 3.3 μg/g body weight in 100 μL in in vivo experiments. In control mice, PBS was injected. Ninety minutes after LPS injection, mice were sacrificed and spleens were aseptically removed. For in vitro experiments, LPS dissolved in distilled water was diluted to 1 μg/mL and 10 μg/mL with RPMI medium. Cells were cultured with LPS for 24 h and culture supernatants were subjected to ELISA.

2.5. Measurement of IL-6. IL-6 level in the culture supernatant was assayed by enzyme-linked immunosorbent assay (ELISA). Cells were cultured with medium containing 0, 1, or 10 μg/mL LPS. Supernatants were collected after 24 h and analyzed by ELISA.

2.6. Quantitative PCR. Total RNA was isolated from purified cells by QIA shredder and RNeasy Mini kit (QIAGEN, Germantown, MD, USA) and by SV total RNA isolation system (Promega, Madison, WI, USA) from organs. Quantitative PCR reaction was performed with SYBR green PCR master mix (QIAGEN). Gene expression levels for each sample were normalized to GAPDH serving as internal standard. Sequences of the forward and reverse primers were as follows: GAPDH (*Gapdh*), 5′-TGTCCGTCGTGGATCTGAC-3′ and 5′-CCTGCTTCACCACCTTCTTG-3′; IL-6 *(Il6)*, 5′-TGGAGTCACAGAAGGAGTGGCTAAG-3′ and 5′-TCTGACCACAGTGAGGAATGTCAA-3′; IL-17A *(Il17a)*, 5′-GAAGCTCAGTGCCGCCA-3′ and 5′-TTCATGTGGTGGTCCAGCTTT-3′; IL-1β *(Il1b)*, 5′-CAGGATGAGGACATGAGCAC-3′ and 5′-CAGTTGTCTAATGGGAACGTCA-3′.

2.7. Cell Population with Flow Cytometry Analysis. Spleens were recovered from young and aged mice and spleen cells were used for flow cytometry analysis after red blood cell lysis. Cell staining for flow cytometry was performed at 4°C after Fc-block step for 15 min with the following monoclonal antibodies: FITC-conjugated anti-CD11b, FITC-conjugated anti-CD4, FITC-conjugated anti-CD45, PE-conjugated anti-F4/80, PE-conjugated anti-gp38, biotinylated anti-CD31, and PE-conjugated anti-B220 for 20 min. All data were analyzed with FlowJo.

2.8. Statistical Analysis. Results comparisons were performed by Student's t-test. A value of $P < 0.05$ was considered to be significant.

3. Results

3.1. IL-6 Production of Spleen Cells Is Higher in Aged Mice Compared to Young Mice. To examine the aging effect on IL-6 protein production levels in the spleen of young and aged mice in vitro, splenocytes of young and aged mice were incubated with or without LPS for 24 h. After incubation, we analyzed IL-6 protein concentrations in supernatant by ELISA. IL-6 protein concentrations increased in the supernatant dependent on LPS concentration in both young and aged mice splenocytes (Figure 1(a)). In the spleen cells of aged mice, stimulation of LPS led to IL-6 protein upregulation in the supernatant, while in young mice splenocytes, although the concentration of IL-6 protein increased when stimulated with LPS, this was only a modest increase. The difference of IL-6 protein levels between young and aged mice became significantly greater in LPS-concentration dependent manner. Similar results were obtained not only for C57BL/6 mice but also for BALB/c mice (data not shown). Macrophages are well known for important cells that produce the major proinflammatory cytokines such as IL-6 and TNF-α [24]. To analyze the effect of the aging process on the IL-6 secretion of splenic macrophages, macrophages were isolated from spleen using two different cell markers. F4/80$^+$ cells and CD11b$^+$CD11c$^-$ cells were prepared by using MACS. In either case, macrophages produced higher levels of IL-6 protein in young mice group than aged mice group after LPS stimulation (Figures 1(b) and 1(c)). Dendritic cells are also known as a source of IL-6 [25]. Dendritic cells from young and aged mice were isolated and IL-6 protein levels were investigated. Dendritic cells from the aged mice group produced slightly higher amounts of IL-6 proteins compared to young mice dendritic cells (Figure 1(d)). In case of the expression of IL-6 mRNA, however, the differences between young and aged mice were negligible (data not shown). Our results show that aging process does not affect the function of macrophages and dendritic cells in IL-6 secretion.

3.2. Stromal Cells from Aged Mice Produce Higher Levels of IL-6 Compared to Those from Young Mice. To identify cells that highly produce IL-6 protein with aging, we next focused on stromal cells. We isolated CD45$^-$ stromal cells from young and aged mice spleen by MACS. FACS analysis was performed and the cell population was characterized as >98% for CD45$^-$ stromal cells. The IL-6 protein production levels were high in aged mice compared to young mice in all tested LPS concentrations (0, 1, and 10 μM) (Figure 2(a)). CD45$^-$ stromal cells from young mice produced lower amounts of IL-6 protein than those from aged mice and they showed only modest changes in IL-6 protein production when stimulated by LPS. Contrary to young CD45$^-$ stromal cells, aged CD45$^-$ stromal cells yielded higher levels of IL-6 protein even in noninflammatory state, and by stimulating with LPS, IL-6 production further increased and the production was significantly high compared to those of young mice. We then extracted RNA from freshly isolated CD45$^-$ stromal cells and mRNA levels were investigated. Expression of IL-6 mRNA was significantly increased in the aged CD45$^-$ stromal cells (Figure 2(b)).

Table 1: The expression of surface markers on splenocytes of young and aged mice.

	Young	Aged
F4/80$^+$	3.87 ± 1.59	3.57 ± 0.70
CD11b$^+$	7.17 ± 2.63	5.87 ± 0.39
CD11c$^+$	0.56 ± 0.13	0.78 ± 0.05
B220$^+$	63.07 ± 2.26	61.08 ± 4.33
CD4$^+$	17.15 ± 1.62	18.63 ± 2.95
CD45$^-$	1.80 ± 2.35	2.81 ± 2.76

Splenocytes were obtained from young and aged mice (n = 4~5). Cells were stained for the surface markers F4/80, CD11b, CD11c, B220, CD4, and CD45. Values indicate the mean percentages of each subset. Data are expressed as the mean ± SD.

3.3. Cell Populations of Spleen Both of Young and Aged Mice Do Not Show Age-Dependent Differences but Those of Splenic CD45$^-$ Stromal Cells Show Different Profile. Cell populations might change with aging in spleen, and the difference in cell population may influence the overall production of IL-6 in the spleen of young and aged mice. The absolute cell number of spleen from aged mice was higher than young mice, which of young mice was $2.46 \times 10^8 \pm 0.58 \times 10^8$ and aged mice was $3.26 \times 10^8 \pm 1.07 \times 10^8$. To analyze the effect of population, we characterized the proportion of cells expressing F4/80$^+$, CD11b$^+$, CD11c$^+$, CD4$^+$, B220$^+$, and CD45$^-$ in spleen of young and aged mice by performing flow cytometric analysis. Aging process does not significantly affect cell population in the spleen and it was suggested that the difference in IL-6 production of splenocytes between young and aged mice was not due to the difference of the proportion of each cell population (Table 1). Flow cytometry analyses were performed to analyze distribution of populations within CD45$^-$ stromal cells (Figure 3). Difference between young and aged mice was observed in BEC population of CD45$^-$ stromal cells. CD45$^-$ stromal cells of aged mice show high population of BEC and low population of DNC.

3.4. IL-6 Secretion of CD45$^-$ Stromal Cells but Not CD45$^+$ Cells Is Enhanced by Aging. To investigate further whether the enhanced IL-6 secretion of splenocytes from aged mice results from IL-6 production of aged CD45$^-$ stromal cells, we cultured CD45$^-$ stromal cells and CD45$^+$ cells from aged and young mice and these cells were activated with LPS for 24 h. Five $\times$ 10^4 of CD45$^-$ stromal cells and 5 $\times$ 10^5 of CD45$^+$ cells from young and aged mice were incubated respectively and we confirmed that CD45$^-$ stromal cells of aged mice secreted almost the same amount of IL-6 protein as ten times the number of CD45$^+$ cells (Figure 4). When CD45$^+$ cells were incubated, CD45$^+$ cells from young mice secreted significantly higher IL-6 protein than those of aged mice. The results suggested that the higher IL-6 production from splenocytes of aged mice compared to young mice was

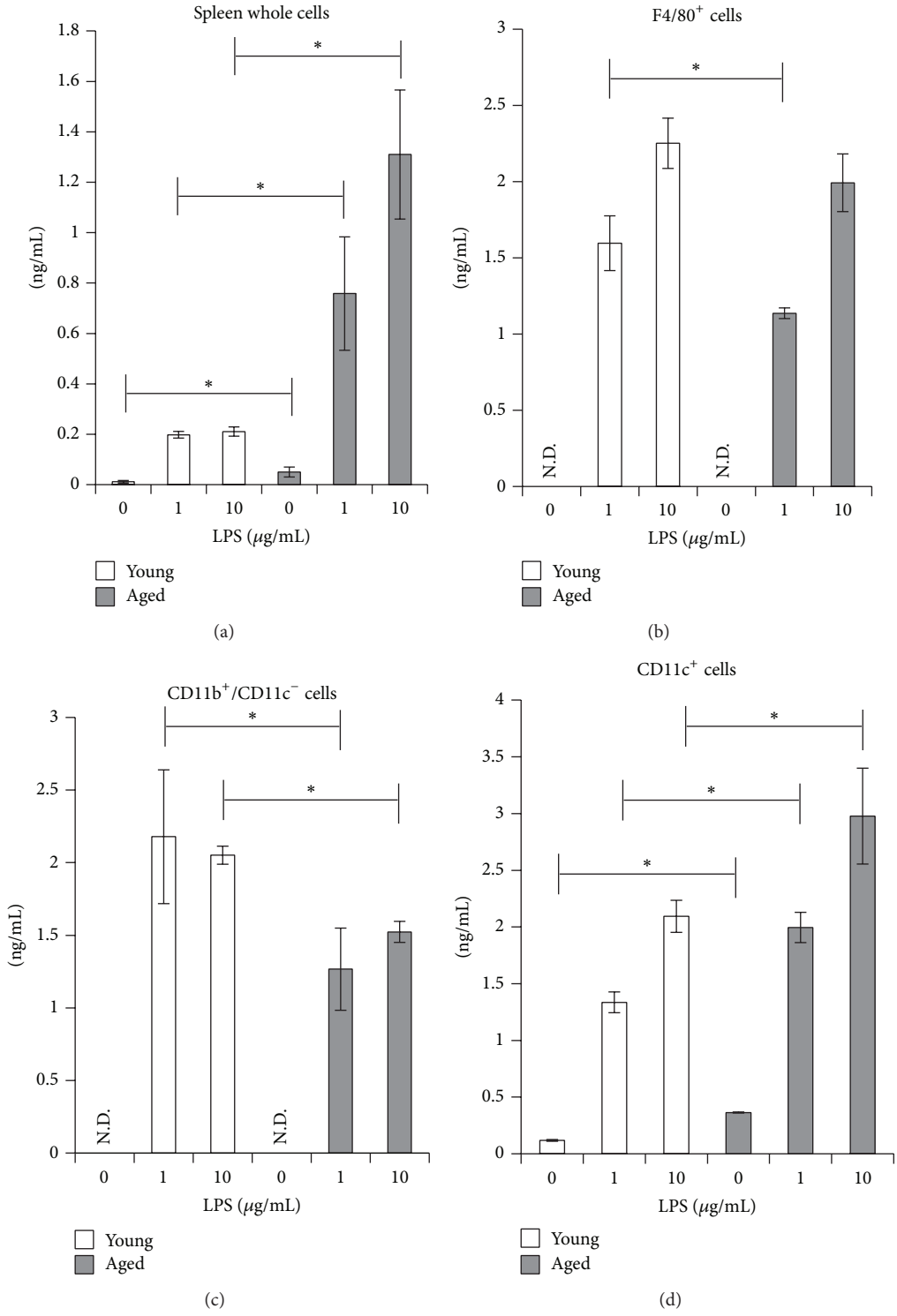


Figure 1: IL-6 protein production in splenocytes and their subpopulations from young and aged mice (n = 2~4). Whole splenocytes (a), spleen-derived F4/80$^+$ cells (b), CD11b$^+$/CD11c$^-$ cells (c), and CD11c$^+$ cells (d) from young and aged mice were cultured with 0, 1, and 10 μg/mL LPS for 24 hours. Supernatants were collected from the cell culture and IL-6 was measured by ELISA. The results are expressed as mean ± SD. The data are the representative of two similar experiments. $^*P < 0.05$.

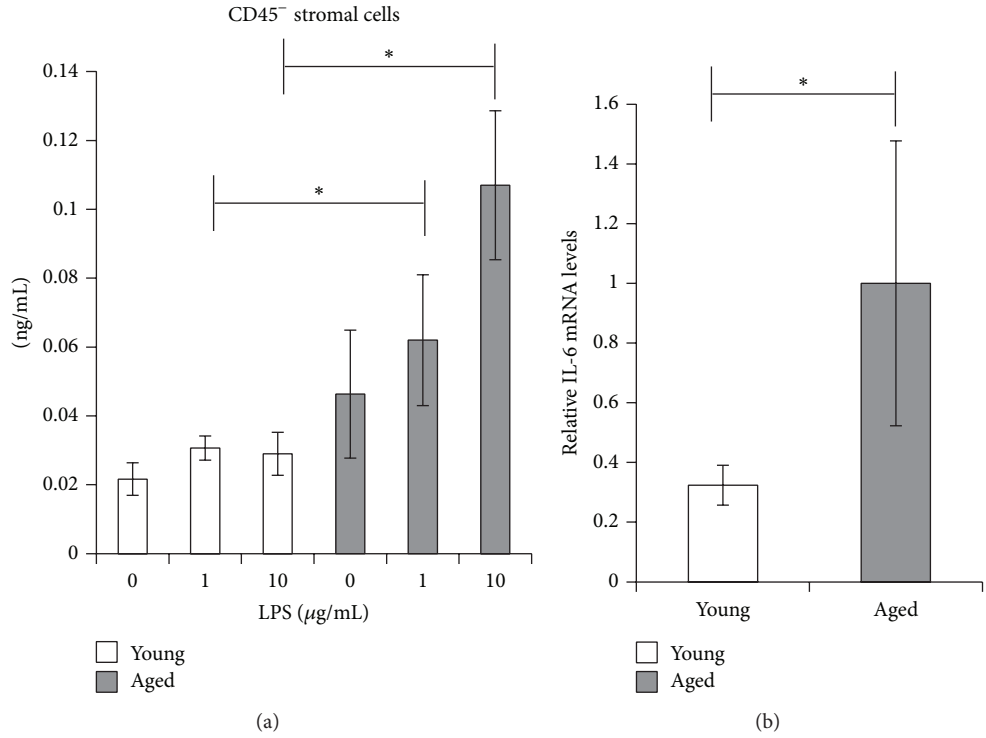


Figure 2: IL-6 protein induced by LPS in CD45⁻ stromal cells and IL-6 mRNA expression level in fresh stromal cells. (a) Splenic CD45⁻ stromal cells from young and aged mice were cultured with 0, 1, and 10 μg/mL LPS for 24 hours, and IL-6 in the supernatant was measured by ELISA. $^*P < 0.05$. The data are the representative of two similar experiments ($n = 6$). (b) IL-6 mRNA of freshly isolated splenic CD45⁻ stromal cells from young and aged mice ($n = 3$) was measured by quantitative RT-PCR. The results are expressed as mean ± SD. The average normalized IL-6 mRNA level in aged mice was set at 1.0. $^*P < 0.05$.

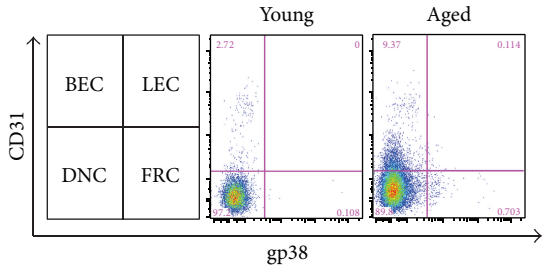


Figure 3: Splenic stromal cell populations of young and aged mice. SPL whole cells from young and aged mice were stained with anti-CD45, anti-CD31, and anti-gp38. Numbers in quadrants indicate percentage of each stromal cell subset among CD45⁻ cells. The data are the representative of three similar experiments ($n = 3$).

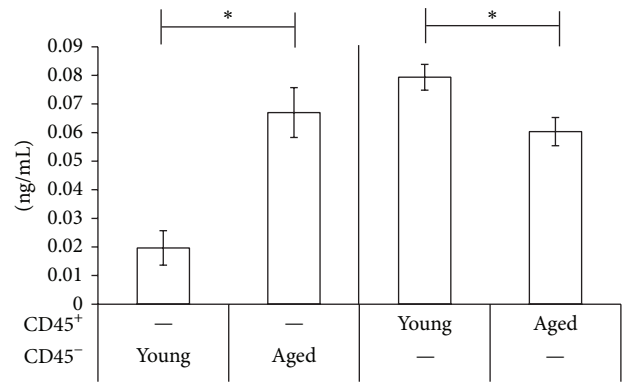


Figure 4: IL-6 protein induced by LPS in CD45⁻ or CD45⁺ cells. Spleen-derived CD45⁻ or CD45⁺ cells from young and aged mice ($n = 4$) were cultured with 10 μg/mL LPS for 24 h. Supernatants were collected from the cell culture and were analyzed for IL-6 by ELISA. The results are expressed as mean ± SD. The data are the representative of two similar experiments.

due to the enhanced production of IL-6 in stromal cells of aged mice.

3.5. IL-6 Expression Was Elevated in Splenic Stromal Cells of Aged Mice after LPS Stimulation In Vivo. To investigate age-related overexpression of IL-6 after LPS stimulation in vivo, mice were injected i.p. with LPS, and IL-6 mRNA expression of splenocytes was assessed. We found that after LPS stimulation in vivo, aged mice spleen expressed significantly higher levels of IL-6 mRNA (Figure 5). To further elucidate whether CD45⁻ stromal cells of spleen exhibited age-related differences in IL-6 mRNA levels after LPS administration in vivo, spleens were obtained from young and aged mice after LPS injection, and stromal cells were isolated. As expected, CD45⁻ stromal cells of aged mice expressed significantly

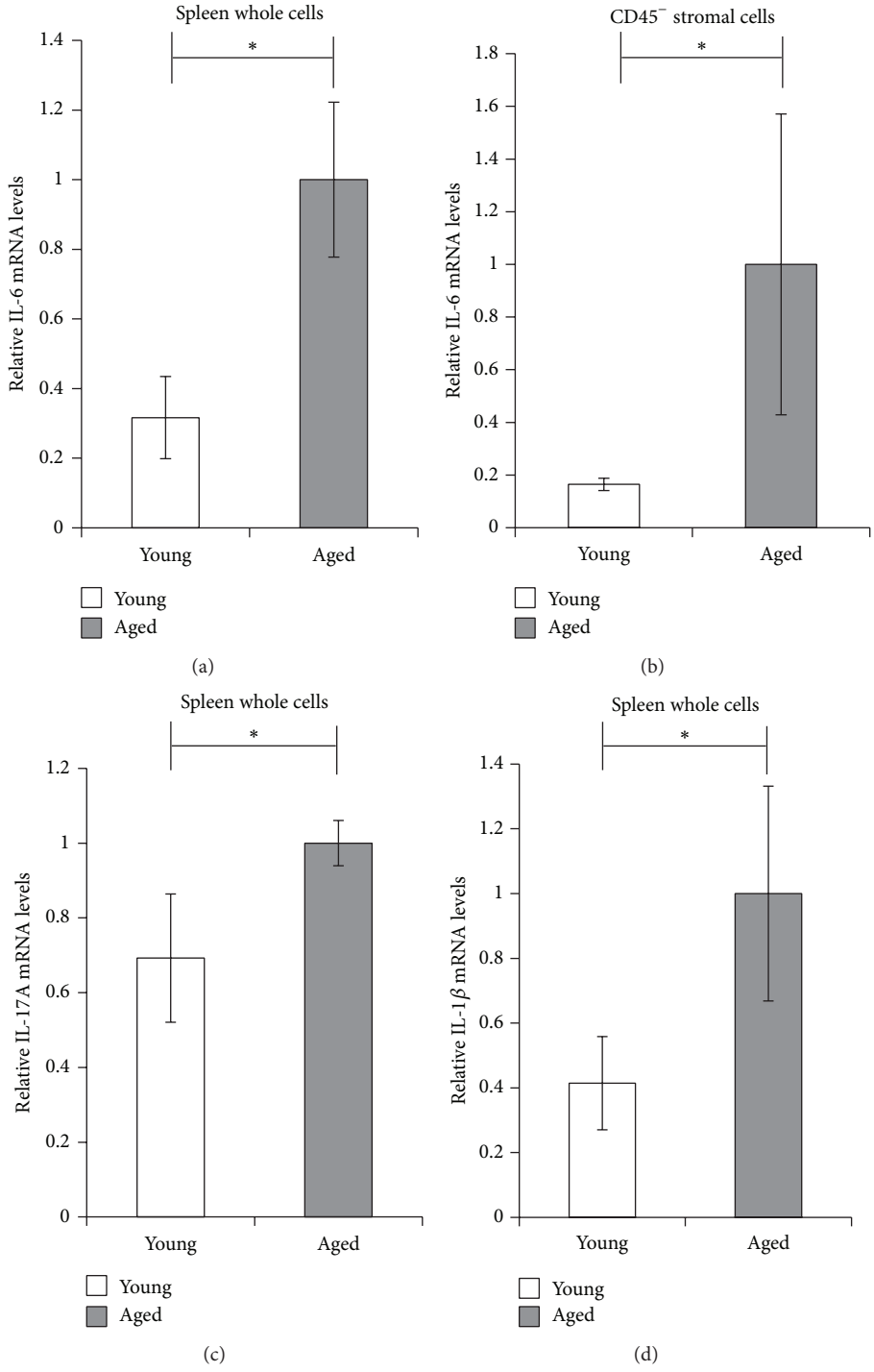


Figure 5: IL-6 and IL-17 mRNA expression levels in whole spleen cells and CD45$^-$ stromal cells in vivo after LPS intraperitoneal administration for 1.5 h. (a) IL-6 mRNA of freshly isolated whole spleen cells from young and aged mice ($n = 3$) was measured by quantitative RT-PCR after LPS injection (3.3 μg/g body weight). The average normalized IL-6 mRNA level in aged mice was set at 1.0. (b) IL-6 mRNA of splenic CD45$^-$ stromal cells from young and aged mice ($n = 5$) sacrificed 1.5 h after injection was measured by quantitative RT-PCR. The average normalized IL-6 mRNA level in aged mice was set at 1.0. $^*P < 0.05$. (c) IL-17 mRNA and (d) IL-1β of freshly isolated whole spleen cells from young and aged mice ($n = 3$) were measured by quantitative RT-PCR after LPS injection. The average normalized IL-17 mRNA level in aged mice was set at 1.0. The results are expressed as mean ± SD.

higher IL-6 mRNA after LPS stimulation in vivo (Figure 5). Since it is reported that IL-17 can enhance IL-6 production in concert with IL-6 in nonimmune cells, we also examined the expression of IL-17 mRNA [16]. The results demonstrated that IL-17 mRNA expression was also significantly higher in splenocytes of aged mice compared to young mice (Figure 5). In addition, it is reported that IL-1β also can induce higher level of IL-6 mRNA [26]; we investigated IL-1β mRNA expression. The results demonstrated that IL-1β mRNA expression was also significantly higher in splenocytes of aged mice compared to young mice (Figure 5).

4. Discussion

In this study, we examined the effect of aging process on IL-6 secretion, a proinflammatory cytokine, in several types of cells from spleen of young and aged mice. In autoimmune diseases, unbalance and dysregulation of proinflammatory cytokines are considered to play a pivotal role to develop autoimmunity [27]. Patients with elevated IL-6 show higher mortality and organ failure and anti-IL-6 receptor antibody is supposed to be effective medication for rheumatoid arthritis and Castleman's diseases [28]. It implies that IL-6 is a critical factor in autoimmune diseases, especially in rheumatoid arthritis [29–31]. There are a number of studies concerning cytokine secretions in aged mice and analyzing the age-related defects by showing changes of proinflammatory cytokines [7]. Aged mice have been shown to express increased levels of IL-6, including the serum IL-6 level after LPS treatment, and the results of our present study are consistent with these reports [3, 32–34].

In rheumatoid arthritis, it has been proposed that bacterial infections might play important roles, so the expression of proinflammatory cytokines in response to bacterial stimulation may also be important [35]. In this study, we observed that, upon LPS stimulation, splenocytes of aged mice secreted more IL-6 than those of young mice.

There are many cell types in the spleen including macrophages, dendritic cells, and other immune and nonimmune cells. We separated several types of cells from spleen and cultured these cells with or without LPS to identify cells that play roles in production of IL-6 with aging in inflammatory and noninflammatory environment with young and aged mice. Although macrophages produce IL-6 in the early phase of infectious inflammatory response [36], we found that the aging process does not affect the IL-6 production of these cells. Similarly, dendritic cells from both young and aged mice had almost similar ability to secrete IL-6. Rather, we found that the IL-6 production of $CD45^-$ stromal cells was greatly increased in aged mice. We separated $CD45^-$ stromal cells from young and aged mice spleen and cultured these cells with or without LPS for 24 h. Our results showed that $CD45^-$ stromal cells of aged mice strongly produced IL-6 even under the noninflammatory condition, and the IL-6 protein production increased upon LPS stimulation (Figure 2). In addition, IL-6 mRNA was expressed highly in freshly isolated $CD45^-$ stromal cells from aged mice compared to young mice, which alludes to the function of $CD45^-$ stromal cells in secreting IL-6 changes with aging. To further investigate the effect of $CD45^-$ stromal cells on IL-6 protein production of spleen upon LPS stimulation, we cultured $CD45^+$ cells and $CD45^-$ stromal cells under LPS stimulated condition for 24 h, respectively. $CD45^+$ cells from young mice secreted higher IL-6 protein compared to aged mice, suggesting that $CD45^+$ cells do not play a role in increased production of IL-6 with aging. This demonstrates that although hemopoietic cells other than dendritic cells may produce IL-6, the level of production did not differ between young and aged mice, and these cells did not contribute to the IL-6 secretion. And although the proportions of T cell subsets may have changed with aging, we consider that the effect on the production of IL-6 should be minimal. $CD45^-$ stromal cells from aged mice secreted almost the same amount of IL-6 protein with $CD45^+$ cells with one-tenth of $CD45^+$ cells and $CD45^-$ stromal cells from aged mice showed three times higher IL-6 protein yields than young mice. These results suggest that $CD45^-$ stromal cells are crucial to increase the IL-6 protein production after LPS stimulation in aging process. $CD45^-$ cell population can be distinguished into four groups depending on the expression of CD31 and Gp38 on the cell surface, BECs (blood endothelial cells, $CD31^+Gp38^-$), FRCs (fibroblastic reticular cells, $CD31^-Gp38^+$), LEC (lymphatic endothelial cells, $CD31^+Gp38^+$), and the double negative populations [20]. The distribution of subsets of these $CD45^-$ stromal cells changed with aging (Figure 3). The BEC population increased in $CD45^-$ stromal cells of aged mice. Although the ratio of $CD45^-$ stromal cells did not differ, the composition of $CD45^-$ stromal cells changed with aging. It implies that altered distribution of $CD45^-$ stromal cells with aging, especially increased population of BECs, might contribute to augment IL-6 production.

IL-6 is chiefly regulated by NF-κB [4]. We investigated NF-κB p65 activation by western blotting using nuclear protein of splenocytes from young and aged mice. NF-κB p65 protein content of nuclear extracts from aged mice was high compared to young mice (data not shown). It implies that, via activated NF-κB p65, IL-6 is induced and results in a characteristic proinflammatory profile in aged mice. The molecular mechanism of IL-6 secretion from stromal cells is still not clarified, but this would require a novel approach and would be a subject of future study.

Recent data showed that IL-17 stimulates stromal cells to induce production of inflammatory mediators such as IL-6 in concert with IL-6, which indicates an IL-17-mediated IL-6 amplifying system [16, 37, 38]. Under the presence of IL-17, nonimmune cells including fibroblasts can secrete increased amounts of cytokines in response to infection. IL-17 treatment alone, however, is a poor stimulus for gene expression in these cells. Nevertheless, if IL-17 cooperates with other cytokines, especially TNF-α and IL-6, synergic response would occur and therefore it might be considered as a potential mechanism that in aged mice IL-6 production can increase significantly by IL-17A-triggered positive-feedback loop mediated by IL-17 and IL-6 [39]. We investigated IL-6 and IL-17A mRNA of young and aged mice spleen after 1.5 h LPS injection in vivo and our results show that splenocytes of aged mice highly expressed IL-6 and IL-17A mRNA than

young mice spleen. Also, we confirmed that $CD45^-$ stromal cells from aged mice expressed 5-fold higher IL-6 mRNA after 1.5 h LPS injection than from young mice. Our results imply that highly produced IL-6 by stromal cells and IL-17A in aged mice trigger the IL-6 amplifier and it results in increasing IL-6 production in aged mice spleen. It has been reported that IL-1β produced in LPS-injected mice stimulates cardiac cells to produce IL-6 [26]. We also found that expression of IL-1β was higher in splenocytes of aged mice compared to young mice after LPS injection. Yet another possibility may be that IL-1β produced upon LPS stimulation enhances IL-6 secretion in aged mice.

Our observations that increased IL-6 expression in splenic $CD45^-$ stromal cells of aged mice suggest general etiological mechanisms of the chronic inflammatory condition found in the elderly, referred to as inflamm-aging. Such altered immune functions might have influence on developing diseases, susceptibility to infections, and conditions related to allergy in the elderly. In both of our in vivo and in vitro results, stromal cells play a role of causing difference in IL-6 production between young and aged mice. These results propose that increased risk with age of autoimmune diseases, especially induced by IL-6, is due partly to elevated IL-6 production by stromal cells. Additional studies are needed to make clear the mechanisms related to elevated secretion of IL-6 in stromal cells with age. And to better understand age-related IL-6 production in stromal cells, further studies concerning secretion of IL-6 in each subset of stromal cells might be necessary.

5. Conclusions

In conclusion, our results showed that, in the mouse spleen, the production of IL-6 was elevated with aging and that IL-6 production by stromal cells played a major role in this enhancement. Our results suggest the possibility that augmented production of proinflammatory cytokines such as IL-6 by stromal cells might be a cause of inflammatory diseases in the elderly.

Abbreviations

SLO: Secondary lymphoid organs.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors thank Ryutaro Kotaki for the technical assistance. This work was supported by a Grant-in-Aid for Scientific Research (S) 23228003 from the Japan Society for the Promotion of Science (JSPS).

References

[1] C. Mammucari and R. Rizzuto, "Signaling pathways in mitochondrial dysfunction and aging," *Mechanisms of Ageing and Development*, vol. 131, no. 7-8, pp. 536–543, 2010.

[2] A. L. Gruver, L. L. Hudson, and G. D. Sempowski, "Immunosenescence of ageing," *Journal of Pathology*, vol. 211, no. 2, pp. 144–156, 2007.

[3] M. E. Starr, B. M. Evers, and H. Saito, "Age-associated increase in cytokine production during systemic inflammation: adipose tissue as a major source of IL-6," *Journals of Gerontology A*, vol. 64, no. 7, pp. 723–730, 2009.

[4] M. Maggio, J. M. Guralnik, D. L. Longo, and L. Ferrucci, "Interleukin-6 in aging and chronic disease: a magnificent pathway," *Journals of Gerontology*, vol. 61, no. 6, pp. 575–584, 2006.

[5] D. Sarkar and P. B. Fisher, "Molecular mechanisms of aging-associated inflammation," *Cancer Letters*, vol. 236, no. 1, pp. 13–23, 2006.

[6] C. Franceschi, M. Bonafè, S. Valensin et al., "Inflamm-aging. An evolutionary perspective on immunosenescence," *Annals of the New York Academy of Sciences*, vol. 908, pp. 244–254, 2000.

[7] E. J. Kovacs, C. R. Gomez, J. Karavitis et al., "Interleukin-6 contributes to age-related alteration of cytokine production by macrophages," *Mediators of Inflammation*, vol. 2010, Article ID 475139, 7 pages, 2010.

[8] G. Pawelec, "Human immunosenescence: is it infectious?" *Immunological Reviews*, vol. 205, pp. 257–268, 2005.

[9] E. J. Kovacs, J. L. Palmer, C. F. Fortin, T. Fülöp Jr., D. R. Goldstein, and P.-J. Linton, "Aging and innate immunity in the mouse: impact of intrinsic and extrinsic factors," *Trends in Immunology*, vol. 30, no. 7, pp. 319–324, 2009.

[10] P. J. Busse and S. K. Mathur, "Age-related changes in immune function: effect on airway inflammation," *Journal of Allergy and Clinical Immunology*, vol. 126, no. 4, pp. 690–699, 2010.

[11] V. Cardona, M. Guilarte, O. Luengo, M. Labrador-Horrillo, A. Sala-Cunil, and T. Garriga, "Allergic diseases in the elderly," *Clinical and Translational Allergy*, vol. 1, article 11, 2011.

[12] S. Omoigui, "The Interleukin-6 inflammation pathway from cholesterol to aging—role of statins, bisphosphonates and plant polyphenols in aging and age-related diseases," *Immunity and Ageing*, vol. 4, article 1, 2007.

[13] W. E. Naugler and M. Karin, "The wolf in sheep's clothing: the role of interleukin-6 in immunity, inflammation and cancer," *Trends in Molecular Medicine*, vol. 14, no. 3, pp. 109–119, 2008.

[14] H. Huang, D. D. Patel, and K. G. Manton, "The immune system in aging: roles of cytokines, T cells and NK cells," *Frontiers in Bioscience*, vol. 10, pp. 192–215, 2005.

[15] A. Salminen, J. Huuskonen, J. Ojala, A. Kauppinen, K. Kaarniranta, and T. Suuronen, "Activation of innate immunity system during aging: NF-kB signaling is the molecular culprit of inflamm-aging," *Ageing Research Reviews*, vol. 7, no. 2, pp. 83–105, 2008.

[16] W. B. Ershler and E. T. Keller, "Age-associated increased interleukin-6 gene expression, late-life diseases, and frailty," *Annual Review of Medicine*, vol. 51, pp. 245–270, 2000.

[17] J. K. Kiecolt-Glaser, K. J. Preacher, R. C. MacCallum, C. Atkinson, W. B. Malarkey, and R. Glaser, "Chronic stress and age-related increases in the proinflammatory cytokine IL-6," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 15, pp. 9090–9095, 2003.

[18] K. E. Wellen and G. S. Hotamisligil, "Inflammation, stress, and diabetes," *Journal of Clinical Investigation*, vol. 115, no. 5, pp. 1111–1119, 2005.

[19] H. Ogura, M. Murakami, Y. Okuyama et al., "Interleukin-17 promotes autoimmunity by triggering a positive-feedback loop via interleukin-6 induction," *Immunity*, vol. 29, no. 4, pp. 628–636, 2008.

[20] J. G. Cyster, "Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs," *Annual Review of Immunology*, vol. 23, pp. 127–159, 2005.

[21] R. Roozendaal and R. E. Mebius, "Stromal cell-immune cell interactions," *Annual Review of Immunology*, vol. 29, pp. 23–43, 2011.

[22] A. Link, T. K. Vogt, S. Favre et al., "Fibroblastic reticular cells in lymph nodes regulate the homeostasis of naive T cells," *Nature Immunology*, vol. 8, no. 11, pp. 1255–1265, 2007.

[23] J. M. den Haan, R. E. Mebius, and G. Kraal, "Stromal cells of the mouse spleen," *Frontiers in Immunology*, vol. 3, pp. 1–5, 2012.

[24] E. D. Boehmer, J. Goral, D. E. Faunce, and E. J. Kovacs, "Age-dependent decrease in Toll-like receptor 4-mediated proinflammatory cytokine production and mitogen-activated protein kinase expression," *Journal of Leukocyte Biology*, vol. 75, no. 2, pp. 342–349, 2004.

[25] K. Shortman and S. H. Naik, "Steady-state and inflammatory dendritic-cell development," *Nature Reviews Immunology*, vol. 7, no. 1, pp. 19–30, 2007.

[26] H. Saito, C. Patterson, Z. Hu et al., "Expression and self-regulatory function of cardiac interleukin-6 during endotoxemia," *American Journal of Physiology: Heart and Circulatory Physiology*, vol. 279, no. 5, pp. H2241–H2248, 2000.

[27] J. J. O'Shea, A. Ma, and P. Lipsky, "Cytokines and autoimmunity," *Nature Reviews Immunology*, vol. 2, no. 1, pp. 37–45, 2002.

[28] S. Q. Latifi, M. A. O'Riordan, A. D. Levine, and A. Stallion, "Persistent elevation of serum interleukin-6 in intraabdominal sepsis identifies those with prolonged length of stay," *Journal of Pediatric Surgery*, vol. 39, no. 10, pp. 1548–1552, 2004.

[29] K. Ishihara and T. Hirano, "IL-6 in autoimmune disease and chronic inflammatory proliferative disease," *Cytokine and Growth Factor Reviews*, vol. 13, no. 4-5, pp. 357–368, 2002.

[30] M. Murakami, Y. Okuyama, H. Ogura et al., "Local microbleeding facilitates IL-6-and IL-17-dependent arthritis in the absence of tissue antigen recognition by activated T cells," *Journal of Experimental Medicine*, vol. 208, no. 1, pp. 103–114, 2011.

[31] N. Komatsu and H. Takayanagi, "Inflammation and bone destruction in arthritis: synergistic activity of immune and mesenchymal cells in joints," *Frontiers in Immunology*, vol. 3, pp. 1–11, 2012.

[32] N. Kumagai, Y. Chiba, M. Hosono et al., "Involvement of pro-inflammatory cytokines and microglia in an age-associated neurodegeneration model, the SAMP10 mouse," *Brain Research*, vol. 1185, no. 1, pp. 75–85, 2007.

[33] B. M. Meador, C. P. Krzyszton, R. W. Johnson, and K. A. Huey, "Effects of IL-10 and age on IL-6, IL-1β, and TNF-α responses in mouse skeletal and cardiac muscle to an acute inflammatory insult," *Journal of Applied Physiology*, vol. 104, no. 4, pp. 991–997, 2008.

[34] C. R. Gomez, J. Goral, L. Ramirez, M. Kopf, and E. J. Kovacs, "Aberrant acute-phase response in aged interleukin-6 knockout mice," *Shock*, vol. 25, no. 6, pp. 581–585, 2006.

[35] S. Gajanant, "Bacterial infections and the pathogenesis of autoimmune conditions," *British Journal of Medical Practitioners*, vol. 2, pp. 6–13, 2009.

[36] K. Tanaka and T. Kishimoto, "Targeting interleukin-6: all the way to treat autoimmune and inflammatory diseases," *International Journal of Biological Sciences*, vol. 8, pp. 1227–1236, 2012.

[37] J. Wolf, B. Weinberger, C. R. Arnold, A. B. Maier, R. G. Westendorp, and B. Grubeck-Loebenstein, "The effect of chronological age on the inflammatory response of human fibroblasts," *Experimental Gerontology*, vol. 47, pp. 749–753, 2012.

[38] Y. Katz, O. Nadiv, and Y. Beer, "Interleukin-17 enhances tumor necrosis factor alpha-induced synthesis of interleukins 1, 6, and 8 in skin and synovial fibroblasts: a possible role as a "fine-tuning cytokine" in inflammation processes," *Arthritis Rheumatism*, vol. 44, pp. 2176–2184, 2001.

[39] J. Hartupee, C. Liu, M. Novotny, X. Li, and T. Hamilton, "IL-17 enhances chemokine gene expression through mRNA stabilization," *Journal of Immunology*, vol. 179, no. 6, pp. 4135–4141, 2007.

Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2014, Article ID 102160, 13 pages
http://dx.doi.org/10.1155/2014/102160

Research Article

5-Lipoxygenase-Dependent Recruitment of Neutrophils and Macrophages by Eotaxin-Stimulated Murine Eosinophils

Ricardo Alves Luz,[1,2] Pedro Xavier-Elsas,[1] Bianca de Luca,[2] Daniela Masid-de-Brito,[1] Priscila Soares Cauduro,[1] Luiz Carlos Gondar Arcanjo,[1] Ana Carolina Cordeiro Faria dos Santos,[1] Ivi Cristina Maria de Oliveira,[3] and Maria Ignez Capella Gaspar-Elsas[2]

[1] *Department of Immunology, IMPG, Universidade Federal do Rio de Janeiro, CCS, Bloco I, Room I-2-066, 21941-590 Rio de Janeiro, Brazil*
[2] *Department of Pediatrics, IFF, FIOCRUZ, 22250-020 Rio de Janeiro, Brazil*
[3] *Department of Medical Microbiology, IMPG, Universidade Federal do Rio de Janeiro, 21941-590 Rio de Janeiro, Brazil*

Correspondence should be addressed to Pedro Xavier-Elsas; pxelsas@yahoo.com.br

Received 14 November 2013; Revised 14 January 2014; Accepted 15 January 2014; Published 25 February 2014

Academic Editor: Shaoheng He

The roles of eosinophils in antimicrobial defense remain incompletely understood. In ovalbumin-sensitized mice, eosinophils are selectively recruited to the peritoneal cavity by antigen, eotaxin, or leukotriene(LT)B4, a 5-lipoxygenase (5-LO) metabolite. 5-LO blockade prevents responses to both antigen and eotaxin. We examined responses to eotaxin in the absence of sensitization and their dependence on 5-LO. BALB/c or PAS mice and their mutants (5-LO-deficient ALOX; eosinophil-deficient GATA-1) were injected i.p. with eotaxin, eosinophils, or both, and leukocyte accumulation was quantified up to 24 h. Significant recruitment of eosinophils by eotaxin in BALB/c, up to 24 h, was accompanied by much larger numbers of recruited neutrophils and monocytes/macrophages. These effects were abolished by eotaxin neutralization and 5-LO-activating protein inhibitor MK886. In ALOX (but not PAS) mice, eotaxin recruitment was abolished for eosinophils and halved for neutrophils. In GATA-1 mutants, eotaxin recruited neither neutrophils nor macrophages. Transfer of eosinophils cultured from bone-marrow of BALB/c donors, or from ALOX donors, into GATA-1 mutant recipients, i.p., restored eotaxin recruitment of neutrophils and showed that the critical step dependent on 5-LO is the initial recruitment of eosinophils by eotaxin, not the secondary neutrophil accumulation. Eosinophil-dependent recruitment of neutrophils in naive BALB/c mice was associated with increased binding of bacteria.

1. Introduction

Eosinophils are a minority granulocyte population, which contributes to the pathophysiology of allergic inflammation, hypereosinophilic syndromes, and some malignancies [1–4]. A role for eosinophils in resistance to *multicellular* (helminth) parasites has long been proposed, based on the strong association of blood and tissue eosinophilia with worm infections and on the evidence that eosinophils can damage or kill helminths, in specific experimental conditions [5, 6]. Nevertheless, a generally protective in vivo role for eosinophils against worm infections remains elusive [4], partly because host responses to multicellular parasites represent a compromise between the competing needs to reduce parasite burden and to limit immune-mediated tissue damage, to which eosinophils significantly contribute [7, 8].

Alternatively, mechanisms through which eosinophils may directly fight infection by various classes of *microbial* (bacterial, fungal, protozoal, or viral) pathogens include secretion of antimicrobial defensin-like proteins [9]; release of sticky cellular contents that capture pathogens, closely resembling neutrophil extracellular traps [10]; secretion of halogen microbicidal derivatives [11]; release of enzymes with antiviral activity and other roles in innate immunity

[12, 13]; and secretion of a wide array of immunoregulatory cytokines [14]. While the contribution of eosinophils to immunity as directly antimicrobial effector cells is likely limited by their scarcity, they could be helpful in conditions in which neutrophil access or macrophage function would be reduced: for neutrophils, tissue entry is restricted in normal conditions [15]; regarding macrophages, their microbicidal effector function is highly dependent on appropriate activating signals, including cytokines [16]. By contrast, eosinophils are far more numerous in normal tissues than in blood and home to mucosal interfaces with the environment [2–4], which represent potential gateways for microbial infection. They are a source of numerous immunoregulatory cytokines [13] and lipid mediators [17], which might play a role in recruitment/activation of other leukocyte subtypes.

Because of the scarcity of eosinophils, many important observations were made in conditions in which their numbers are already increased, due to allergic sensitization or experimental helminth infection, such as the discovery of eotaxin (CCL11), a chemoattractant that induces eosinophil accumulation in the skin of sensitized (i.e., eosinophilic) guinea pigs [2, 3, 18]. While other potent eosinophil chemoattractants, such as PGD2 [19, 20] and oxo-ETE [21], have also been characterized, many factors reinforce the current understanding of eotaxin as a specialized chemoattractant which acts primarily on granulocyte subtypes relevant to allergy and worm infections [2–4]. These factors include the reported selectivity of eotaxin for the eosinophil [4, 17, 22, 23] and basophil [20, 24, 25] lineages, and its interaction with hematopoietic cytokines, such as IL-5 [26, 27] and GM-CSF [27], which promotes eosinophil production in bone marrow [27] and extramedullary sites [26], ultimately inducing blood and tissue eosinophilia [4]. The alternative view, namely, that eotaxin is part of a broader regulatory network comprising multiple cell populations in addition to eosinophils and basophils, is also suggested by observations of a wide variety of eotaxin effects, including its ability to attract neutrophils and macrophages [28] and smooth muscle cells [29]. Eotaxin, also produced by fibroblasts [30, 31], has been associated with fibrotic processes in several settings [32, 33].

Within this wider framework, we have reexamined whether, in a nonsensitized host, eotaxin would recruit other leukocyte populations besides eosinophils and basophils and further examined whether its effects were dependent on 5-lipoxygenase (5-LO), the key enzyme in the leukotriene production by eosinophils [17, 27]. The evaluation of both aspects was prompted by observations in mice which develop eosinophilia in response to subcutaneously implanted insoluble antigen pellets [34]. While i.p. challenge of implant recipients with soluble allergen selectively recruited eosinophils to the peritoneal cavity, this effect was blocked by the 5-LO-activating protein inhibitor, MK886, and duplicated by the 5-LO product, LTB4, neither of which is eosinophil-selective. Importantly, eotaxin, which duplicated the effects of allergen, was equally blocked by MK886. Equally unexpected was the failure of LTB4, a potent neutrophil chemoattractant, to recruit neutrophils, while it effectively attracted eosinophils in this allergic model. These observations raised the possibility that the eosinophil selective effect of both chemoattractants (eotaxin and LTB4) observed in vivo was dependent on the host being sensitized. We tested this hypothesis for eotaxin first, by examining its effects in a naïve host, as well as the effect of 5-LO blockade on the effectiveness of eotaxin. We report that eotaxin recruits a mixed leukocyte population to the peritoneal cavity of naïve mice and provide evidence of essential roles for both 5-LO and eosinophils in the accumulation and functional activation of neutrophils in this model.

2. Materials and Methods

2.1. Reagents. RPMI 1640 medium (SH30011.01) and fetal calf serum (SH30088.03) were from Hyclone (Logan, UT); Penicillin 100 U/mL (PEN-B), Streptomycin 100 mg/mL (S9137), Ovalbumin (grade II and grade IV), isotonic Percoll, and Histopaque density 1.083 solution from Sigma-Aldrich (St. Louis, MO); recombinant murine Eotaxin (250-01) from PeproTech (Rocky Hill, NJ); and recombinant murine IL-5 from R&D; MK-886 (475889) 1 mg/kg from Cayman Chemicals (Ann Arbor, MI), dissolved in 0.1% methylcellulose, was given as an intragastric bolus in a 0.2 mL volume [33]. Rat anti-murine eotaxin monoclonal neutralizing antibody (clone 42285) and rat anti-murine IgG2a control monoclonal antibody of matched isotype (clone 54447) were from R&D (Minneapolis, MN).

2.2. Animals and Animal Handling. Inbred mice, male and female, aged 8–10 weeks, provided in SPF condition by CECAL-FIOCRUZ (Rio de Janeiro), were of the following strains: BALB/c; ALOX (5-LO-deficient) and PAS-129 (wild-type control of the same background) [27]; and BALB/c mutants lacking an enhancer element in the promoter region of gene coding for the GATA-1 transcription factor [35], required for eosinophil lineage determination (GATA-1 mice, for short). Animal housing, care, and handling followed institutionally approved (CEUA number L-010/04, CEUA number L-002/09) protocols. Naive animals received eotaxin i.p., in 0.2 mL of RPMI 1640 medium with Penicillin/Streptomycin. Controls received medium (RPMI). After the indicated times, animals were killed in a CO_2 chamber, and peritoneal lavage was carried out with 10 mL chilled RPMI. For sensitized animals, see Section 2.6.

2.3. Neutralization of Eotaxin Activity. 50 ng eotaxin was incubated with 5 μg anti-eotaxin neutralizing antibody or 5 μg isotype-matched anti-IgG2a antibody, in a final volume of 200 μL, for 30 minutes, before injection into each BALB/c recipient. 4 h later, peritoneal lavage fluid was collected from the injected mice and handled as detailed above.

2.4. Collection, Enumeration, and Staining of Peritoneal Leukocytes. Peritoneal lavage cells were washed at 500 ×g and resuspended in 2 mL RPMI. Total counts were carried out in a hemocytometer after a 1 : 10 dilution in Turk's solution. Differential counts were done on Giemsa-stained (ice-cold methanol-fixed, air-dried, and Giemsa-stained for 5 minutes) cytocentrifuge smears (500 rpm, 8 minutes in a Cytospin 3,

Thermo Scientific, Waltham, MA), by counting at least 300 cells in 1000x magnification under oil.

2.5. Bacteria and Phagocytosis Assay. We used nonpathogenic *Escherichia coli* bacteria (clone DH5, provided by Dr. Z. Vasconcelos, from INCA and FIOCRUZ, Rio de Janeiro) genetically altered to constitutively express the gene for green fluorescent protein (GFP), grown in LB broth. The cells obtained in the peritoneal lavage of BALB/c mice, induced by eotaxin or RPMI, were subjected to total cell count as well as differential neutrophil counts as previously described. Then 5×10^5 neutrophils were incubated for 30 minutes, in the dark at room temperature, with the bacteria in a 1 : 400 proportion. The cells were then washed and the resulting cell suspension was run in a FACScalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA), with the acquisition of at least 50.000 events, and analyzed with the help of Summit 4.3 software (Dako Cytomation, UK).

2.6. Eosinophil Procedures. For eosinophil transfer studies, where indicated, BALB/C, ALOX or PAS mice were sensitized (100 μg ovalbumin grade IV and 1,6 mg alum in a final volume of 400 μL saline per animal, two s. c. injections in the dorsum, at days 0 and 7) and challenged (ovalbumin grade IV, 1 μg in 400 μL saline i.p. at day 14) according to Ebihara and colleagues [36]. Bone marrow was collected 48 h after i.p. challenge, examined, and cultured as previously described elsewhere [37]. Briefly, bone-marrow cultures were established for 5 days at 37°C in 95% air/5% CO2, in RPMI1640, with 10% FBS and 5 ng/mL IL-5, at a culture density of 1×10^6 cells/mL. The nonadherent cells were then collected and loaded on top of 3 mL of a Histopaque-1083 solution, followed by centrifugation at 400 ×g, 20°C, 35 minutes, without brakes. The mononuclear cell ring and the supernatant were discarded; the granulocyte-rich pellet was collected, washed and resuspended in 3 mL RPMI, and used for total and differential counts as above. The suspension contained ≥80% eosinophils, with no neutrophils, and the minor contaminant population consisted of macrophages alone, which do not interfere with the interpretation of transfer experiments. Where indicated, naive GATA-1, ALOX, or PAS recipient mice were injected with 1×10^6 eosinophils from the appropriate donors (see below) i.p., followed by eotaxin 50 ng/mL, and leukocyte accumulation was monitored in the peritoneal lavage fluid 4 h after eotaxin injection, as above.

For flow cytometric studies of CCR3 expression, the following modification of this protocol was adopted, for it yielded eosinophils of higher purity: sensitized mice were challenged twice, initially by aerosol exposure (1 h, Ovalbumin grade II, 2.5%, w/v, at day 14) and 7 h later with soluble ovalbumin i.p. (grade IV, 1 μg in 400 μL saline). Bone marrow was collected 24 h after aerosol challenge and cultured as above, after separation on a Percoll gradient (75%/60%/45% isotonic Percoll, 100 ×g, 20 min, room temperature). The hematopoietic cells from the 45%/60% interface [38] were cultured at a lower IL-5 concentration (2.5 ng/mL) for twice as long (10 days), yielding a population containing at least 95% eosinophils, with mature morphology. Contaminants at day 10 were degenerating (nonviable) mononuclear and stromal cells.

2.7. Statistical Analyses. All data were analyzed with Systat for Windows 5.04 (Systat, Inc. Everston, IL, USA), using the two-tailed t-test for pairwise comparisons. Where indicated, ANOVA was also used for multiple comparisons, with the Tukey HSD correction and the Bonferroni correction for groups of equal and unequal size, respectively.

3. Results

3.1. Mixed Leukocyte Migration Induced by Eotaxin. We initially examined whether i.p. injection of eotaxin in various doses would recruit eosinophils in a relatively short period (4 h) and whether eosinophil accumulation would be selective, as previously observed in sensitized mice, or accompanied by migration of other leukocyte populations. As shown in Figure 1(a), leukocytes accumulated in response to 50 and 100 ng/cavity eotaxin, in amounts that were significantly different from the RPMI controls (0 ng/cavity) as well as from lower doses of eotaxin (10 and 25 ng/cavity). These leukocytes included variable numbers of eosinophils (Figure 1(b)), monocytes/macrophages [39, 40] (Figure 1(c)), and neutrophils (Figure 1(d)). The morphology of all three leukocyte populations was recognizable without ambiguity, as shown in a representative photomicrograph (supplementary Figure 1 available online at http://dx.doi.org/10.1155/2014/102160). Lymphocyte and basophil migration was not significant in any of these doses (not shown). Importantly, neutrophils and macrophages greatly outnumbered eosinophils, with counts, respectively, 8.2- and 9.9-fold greater in the experiment shown. For all three leukocyte populations, the dose-response relationships were identical, and in subsequent experiments 50 ng/mL was used as the standard stimulus, since no improvement was observed at a higher dose.

Despite the heterogeneity of the recruited leukocyte population, neutralization of eotaxin with specific monoclonal antibody brought leukocyte accumulation to negative control levels (Figure 2; compare with Figure 1(a) for the 0–25 ng eotaxin dose range), while control antibody of the same isotype with irrelevant specificity had no effect. This confirms that the stimulus for recruitment of all three leukocyte populations is eotaxin itself, not any unidentified contaminant, which by definition would not be neutralized by specific antibody.

The kinetics of recruitment of this mixed leukocyte population by eotaxin in naive BALB/c mice shows significant accumulation as early as 2 h, with a maximum at 4 h, thereafter decreasing but remaining significant at 12 and 24 h (Figure 3(a)). We can observe very early arrival of eosinophils (significant from 2 h and remaining so at 12 and 24 h, Figure 3(b)). By contrast, accumulation of both monocytes/macrophages (Figure 3(c)) and neutrophils (Figure 3(d)) became significant only at 4 h. Significant accumulation was also observed at 12 and 24 h for monocytes/macrophages and 12 h for neutrophils. Hence, monocyte/macrophage and neutrophil accumulation followed

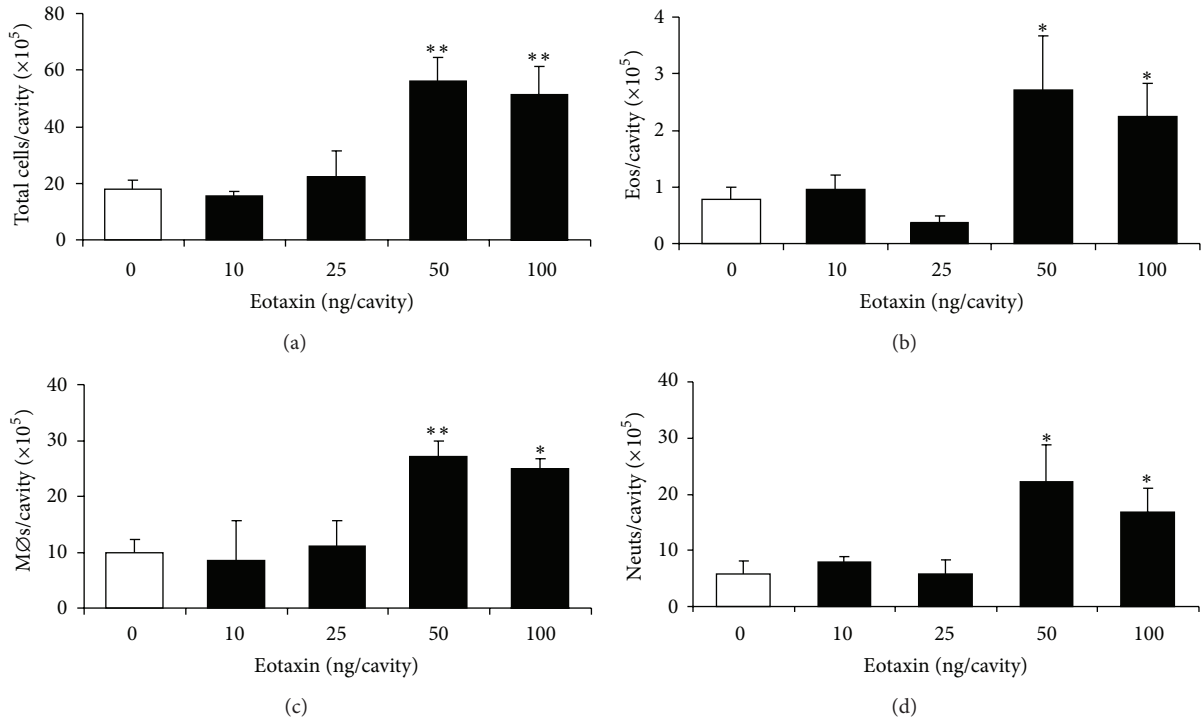


Figure 1: Accumulation of different leukocyte subtypes induced by eotaxin in naive mice: dose-response relationship. BALB/c mice were injected with the indicated doses of eotaxin (black bars), and the peritoneal lavage fluid collected 4 h later was used for quantitation of total leukocytes (a), eosinophils (b), macrophages (c), and neutrophils (d). Data are mean ± SEM. *, $P \leq 0,05$; **, $P \leq 0,01$, for the differences relative to the negative (RPMI) control (0 ng/mL eotaxin, open bars). (a) Data from 3–18 experiments. (b)–(d) Data from 6–11 experiments.

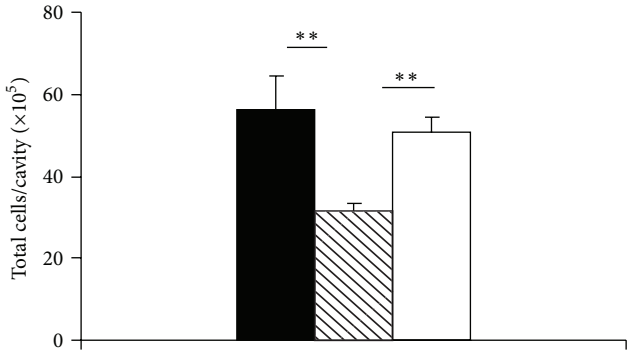


Figure 2: Accumulation of different leukocyte subtypes induced by eotaxin: effect of specific antibody neutralization. Eotaxin was preincubated with specific neutralizing monoclonal antibody (hatched bar), or with irrelevant isotype-matched monoclonal antibody (open bar), before i.p. injection in BALB/c mice. Controls (black bar) received eotaxin but no antibody. Peritoneal lavage fluid, collected 4 h later, was used for total leukocyte quantitation. Data are mean ± SEM. **, $P \leq 0,01$, for the differences relative to the positive (eotaxin) and specificity (irrelevant antibody) controls. Data from 5–18 experiments.

eosinophil entry. Eosinophils outlasted neutrophils, but not monocytes/macrophages, in the observation period. For subsequent experiments, the 4 h observation time was chosen, because it showed significant accumulation of eosinophils, monocytes/macrophages, and neutrophils in naive BALB/c mice.

3.2. Relationship to 5-LO. We first evaluated the effect of eotaxin in naive BALB/c mice pretreated with FLAP inhibitor MK886 or vehicle. MK886 abolished mixed leukocyte recruitment by eotaxin (Figure 4(a)). By contrast, vehicle-pretreated control animals showed significant leukocyte recruitment. MK886 was very effective in preventing eosinophil accumulation (Figure 4(b)). BALB/c mice responded to eotaxin with significant monocyte/macrophage accumulation by 4 h, which was abolished by MK886 (Figure 4(c)). MK886-pretreated BALB/c mice showed no neutrophil migration in response to eotaxin, while migration was significant in vehicle-treated controls (Figure 4(d)).

Next, we evaluated the effect of eotaxin in naïve ALOX mice, which lack 5-LO, and wild-type PAS controls. In ALOX mice, eotaxin had no significant effect on total leukocyte numbers. By contrast, significant recruitment was observed in PAS controls (Figure 5(a)). Importantly, ALOX mice, unlike PAS controls, showed no significant eosinophil recruitment (Figure 5(b)). In this genetic background, unlike BALB/c, no significant monocyte/macrophage recruitment by eotaxin was observed at this time point (4 h; Figure 5(c)), regardless of whether mice were 5-LO-deficient or wild-type; furthermore, monocyte/macrophage numbers were higher in ALOX than in PAS mice. By contrast, neutrophil recruitment was significant in PAS controls and inhibited by ≈55% in ALOX mice, although residual neutrophil recruitment remained significant (Figure 5(d)). Together, these observations show that, in this genetic background, eosinophils and neutrophils differ in their requirements for 5-LO to migrate

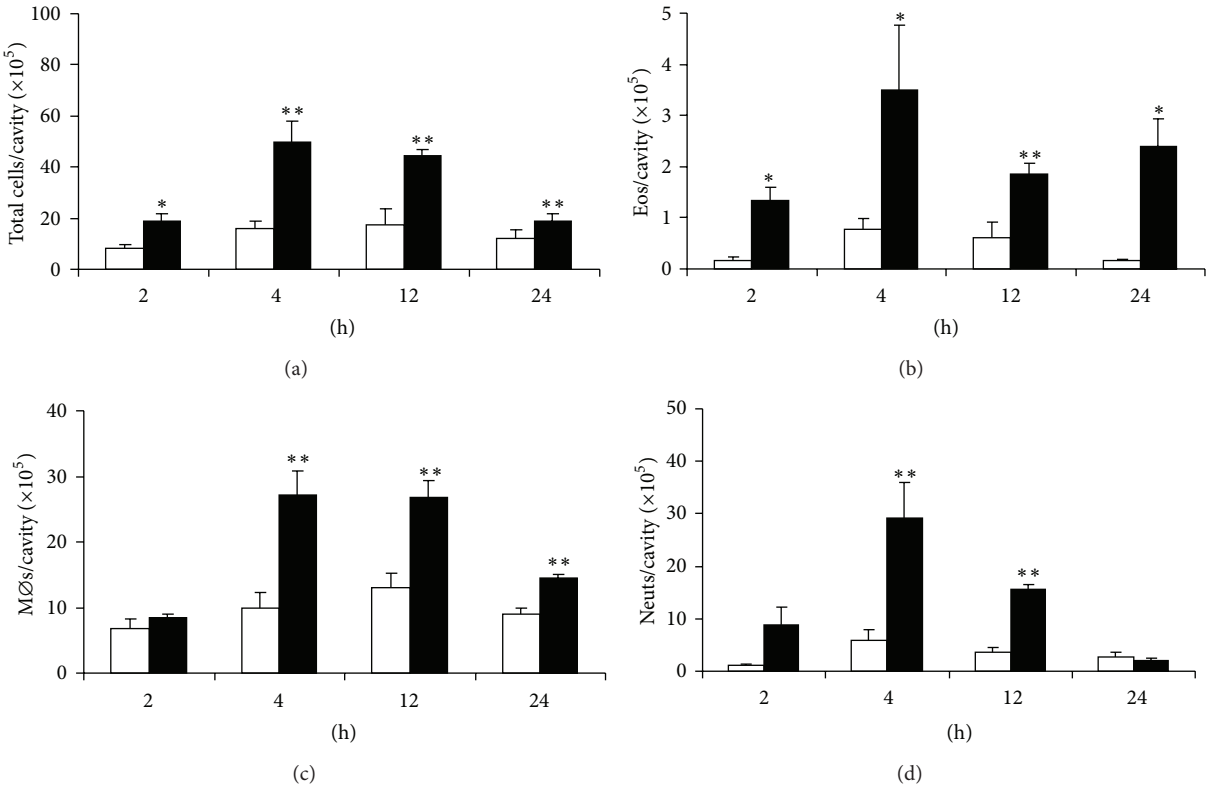


Figure 3: Accumulation of different leukocyte subtypes induced by eotaxin: kinetics. BALB/c mice were injected with 50 ng eotaxin i.p. (black bars), and the peritoneal lavage fluid collected after the indicated periods was used for quantitation of total leukocytes (a), eosinophils (b), macrophages (c), and neutrophils (d). Data are mean ± SEM. *, $P \leq 0,05$; **, $P \leq 0,01$, for the differences relative to the respective negative (RPMI) controls (open bars). Data from 3–11 experiments.

in response to eotaxin, which are total for the former but only partial for the latter.

3.3. Eosinophil-Dependent Neutrophil and Monocyte/Macrophage Migration. The kinetics of mixed leukocyte recruitment in naive BALB/c mice raised the issue of whether eotaxin-stimulated eosinophils recruit other leukocyte types. If so, neutrophil and/or monocyte/macrophage migration in response to eotaxin would be decreased in the absence of eosinophils. Since naive mice carrying a mutation in the high-affinity GATA-1 binding site of the promoter from the gene coding for the GATA-1 transcription factor lack eosinophils [4], we evaluated the effect of eotaxin on leukocyte numbers 4 h after i.p. injection in GATA-1 mutant mice and BALB/c wild-type controls. In GATA-1 mice, unlike BALB/c controls, leukocyte numbers in the peritoneal cavity were not significantly increased by eotaxin (Figure 6(a)). As expected, eosinophils were undetectable in GATA-1 mice,and effectively recruited by eotaxin in BALB/c controls (Figure 6(b)). In both RPMI-treated and eotaxin-treated GATA-1 mice, monocyte/macrophages (which were the predominant resident leukocyte population) were about twice as numerous as in RPMI-treated BALB/c controls (Figure 6(c)), reaching counts comparable to those in eotaxin-treated BALB/c. Importantly, neutrophil numbers were not significantly increased by eotaxin (Figure 6(d)) in GATA-1 mice, unlike BALB/c controls, suggesting that neutrophil recruitment by eotaxin is eosinophil-dependent. To rule out the possibility that neutrophil migration is somehow defective in this strain, separate control GATA-1 mice were injected with thioglycollate broth, which induces an intense neutrophil accumulation in a 4 h period. GATA-1 and BALB/c mice responded equally well to thioglycollate (not shown), indicating that failure of neutrophil recruitment in GATA-1 mice is a feature of their eotaxin response, not evidence of a general defect in neutrophil migration.

We further explored this issue by reconstituting a peritoneal eosinophil population in GATA-1 mice by transfer of purified (90%) BALB/c eosinophils, devoid of neutrophil contamination. Total leukocyte counts were not significantly different between GATA-1 mice given eotaxin alone, eosinophils alone, or eotaxin plus eosinophils (Figure 6(e)), and this was closely paralleled by monocyte/macrophage counts, which account for most leukocytes in all groups (Figure 6(f)). As expected, eosinophils could be recovered from GATA-1 recipients of eosinophils, and eotaxin did not significantly increase their numbers, as the recipients produce no eosinophils of their own (Figure 6(g)). Importantly, neutrophil numbers were significantly increased by eosinophil transfer and further significantly increased by the association of eosinophil transfer and eotaxin (Figure 6(h)). Together,

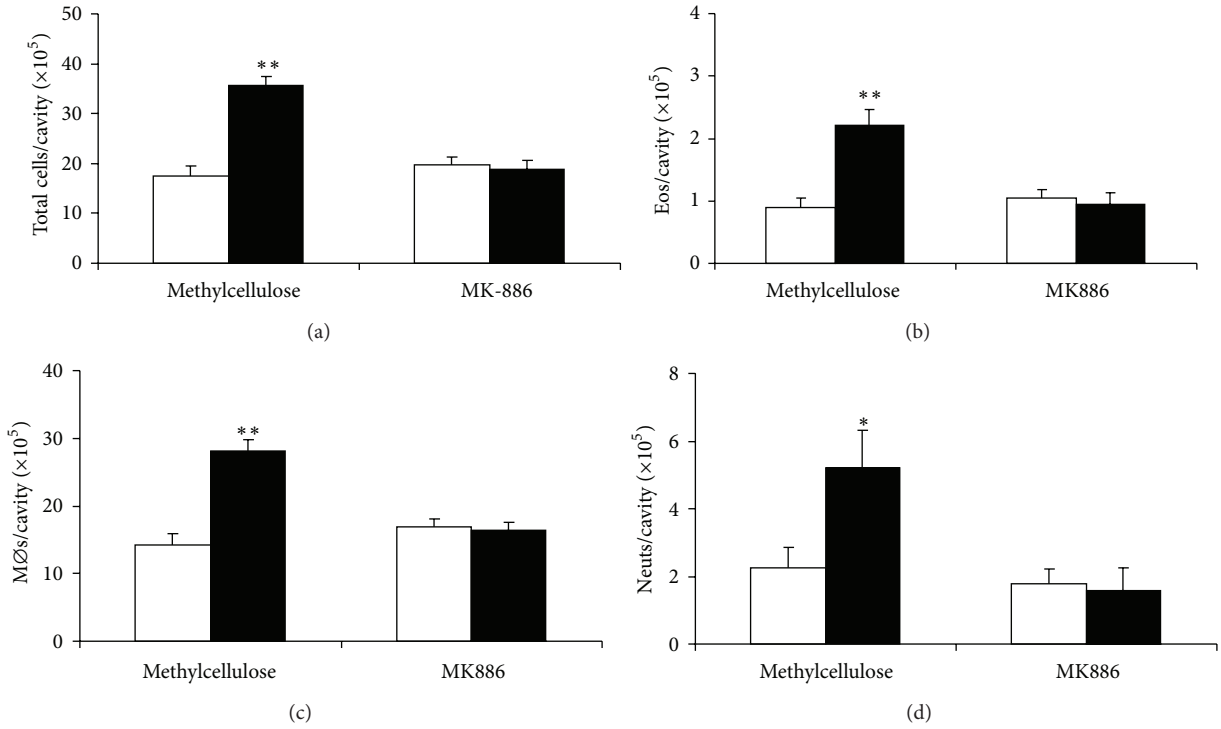


Figure 4: Accumulation of different leukocyte types induced by eotaxin: effect of MK886. BALB/c mice were pretreated with vehicle (methylcellulose) or MK886 and injected with RPMI medium (negative control, open bars) or eotaxin, 50 ng/cavity (black bars). Peritoneal lavage fluid collected after 4 h was used for quantitation of total leukocytes (a), eosinophils (b), macrophages (c), and neutrophils (d). Data are mean ± SEM. *, $P \leq 0,05$; **, $P \leq 0,01$, for the differences relative to the respective negative control in each group. Data from 6 experiments.

these data suggest that in naive mice eosinophils mediate the accumulation of neutrophils induced by eotaxin.

If, as suggested by the preceding results, neutrophils and monocyte/macrophages accumulate in GATA-1 mice as a result of eosinophil activation, not of direct exposure to eotaxin, one should expect the leukocytes harvested from the peritoneal cavity of GATA-1 mice to show little or no expression of CCR3, unlike eosinophils. We have therefore compared the expression of CCR3 in peritoneal lavage leukocytes from BALB/c and GATA-1 mice collected 4 h after eotaxin injection (Figure 6(i)). Mean fluorescence intensity was monitored in the granulocyte region, since our transfer protocol reconstitutes migration of neutrophils, not monocytes/macrophages (see above). No eotaxin-induced recruitment of CCR3+ granulocytes was observed in GATA-1 mice (dotted line), unlike BALB/c mice (thin line). To make sure that CCR3+ cells would be detectable, if present in a suspension of GATA-1 granulocytes, we also added purified BALB/c eosinophils to GATA-1 leukocytes as a control (Figure 6(i), thick line). Exogenously added CCR3+ cells were easily detectable in these conditions.

We took advantage of the effectiveness of eosinophil transfer to examine the relationship of 5-LO to the migration of eosinophils, as well as to the secondary recruitment of neutrophils and monocytes/macrophages. A mixed leukocyte population accumulated in the peritoneal cavity of ALOX recipients of PAS eosinophils (Figure 7(a)), 4 h following administration of eotaxin. No significant improvement was observed in PAS recipients of PAS eosinophils in the same conditions, showing that recruitment is as effective in the ALOX recipients as in the wild-type recipients. The recruited leukocyte population from ALOX recipients included eosinophils (Figure 7(b)), comprising both the transferred eosinophils and those recruited by eotaxin administration to the recipients, again reaching levels comparable to those of PAS recipients of PAS eosinophils. Secondary recruitment was observed for both macrophages (Figure 7(c)) and neutrophils (Figure 7(d)), with similar effectiveness in comparison to the PAS into PAS transfers.

We next examined whether the critical step requiring 5-LO in this model is the initial eosinophil accumulation, rather than the secondary recruitment of neutrophils by eosinophils. If so, one would predict that direct transfer of ALOX eosinophils into eosinophil-deficient GATA-1 recipients should restore neutrophil accumulation in response to eotaxin. When purified eosinophils from ALOX bone-marrow cultures were transferred to GATA-1 recipients (Figure 7(e)), recruitment of neutrophils was very effective. This rules out the possibility that the step critically dependent on 5-LO is the generation by eosinophils of a neutrophil chemoattractant. On the other hand, as shown above for BALB/c eosinophil transfer into GATA-1 recipients, monocytes/macrophages were not increased by ALOX eosinophil transfer at this time point.

3.4. Impact on Granulocyte Interaction with Bacteria. We further examined whether eosinophil-mediated responses to

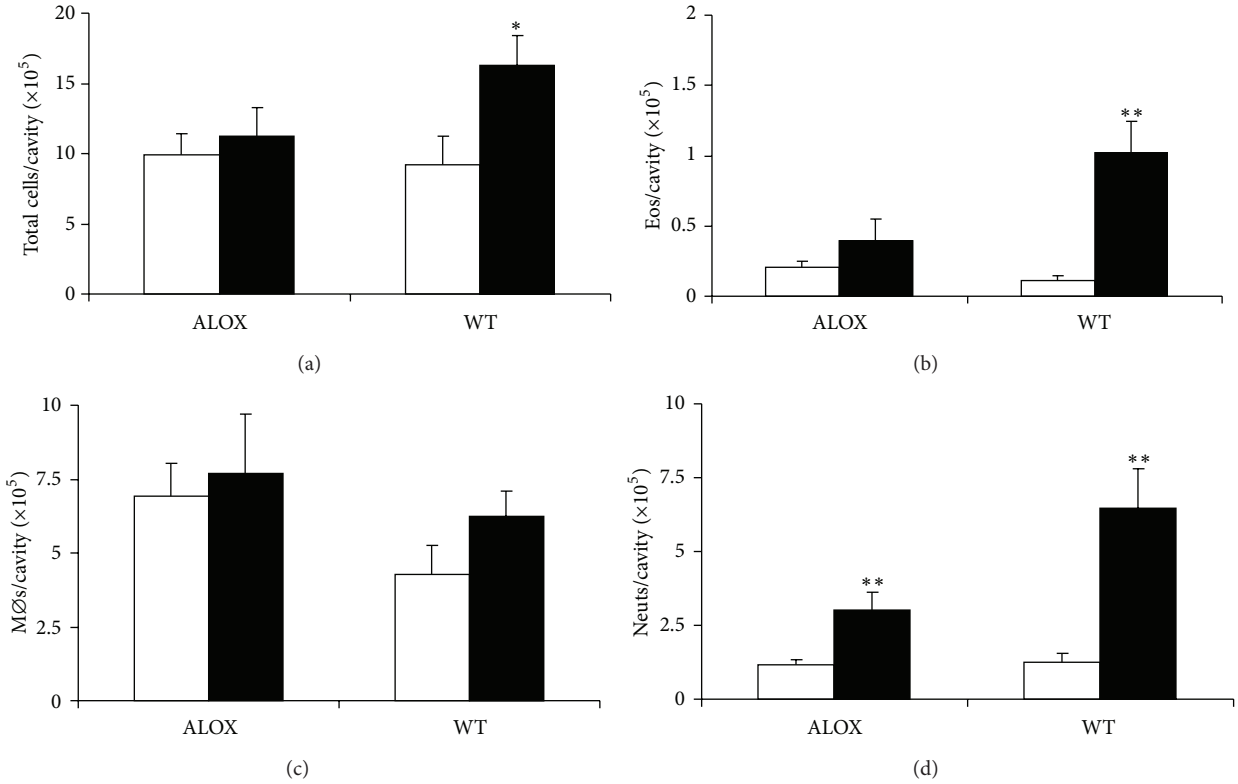


Figure 5: Mixed leukocyte accumulation induced by eotaxin in ALOX and PAS mice. 5-LO-deficient mutant (ALOX) and wild-type (WT) control PAS mice were injected with RPMI (open bars) or eotaxin, 50 ng/cavity (black bars). The peritoneal lavage fluid collected after 4 h was used for quantitation of total leukocytes (a), eosinophils (b), macrophages (c), and neutrophils (d). Data are mean + SEM. *, $P \leq 0,05$; **, $P \leq 0,01$. Data from 10 experiments.

eotaxin in this model had an effect on the ability of the secondarily recruited neutrophils and their bacterial targets. To do so, mixed leukocyte populations induced by eotaxin (RPMI in controls) were collected from naive BALB/c mice at 4 h after injection, counted, and mixed for 30 minutes with GFP-expressing *E. coli* at a bacteria/leukocyte ratio adjusted to 400 : 1, before analysis by flow cytometry. Cells gated in the granulocyte region on the basis of size and complexity were examined for green fluorescence, resulting from both binding and internalization of bacteria. Figure 8(a) shows that eotaxin-stimulated granulocytes bind/internalize fluorescent *E. coli* bacteria more effectively than those collected from RPMI-injected control mice. This increase in effectiveness is detectable as an increased fraction of granulocytes binding bacteria (Figure 8(b)) and an increased mean fluorescent intensity (Figure 8(c)). This suggests that eosinophil-mediated recruitment of neutrophils is accompanied by an increased capacity to bind and/or ingest bacteria.

4. Discussion

We describe here a mixed leukocyte accumulation occurring in the peritoneal cavity of naive mice injected with eotaxin. This is, to our knowledge, the first experimental evidence that *recruitment of neutrophils and macrophages by eotaxin in nonsensitized animals is mediated by eosinophils*. For neutrophils, recruitment was associated with an increased ability to bind/ingest bacteria and therefore might have an impact on antimicrobial defenses in specific conditions. Because the ability of eosinophils to act as effective antimicrobial defenses is limited by their scarcity, these findings also highlight conditions in which, by recruiting much larger numbers of cells with well-characterized microbicidal function, eosinophils actually overcome this theoretical disadvantage.

We will below address a number of specific points which are important for putting our observations in a proper perspective.

4.1. Roles of Eotaxin, Eotaxin Receptors, and Eosinophils. Migration of all three leukocyte types in BALB/c mice was induced by eotaxin, as shown by identical dose-response relationships and overlapping kinetics, as well as by identical effects of neutralizing eotaxin with specific antibodies. The relationship of this migration to the expression of CCR3, by contrast, is more complex. Lymphocytes, some of which have been shown by others to express CCR3 [41, 42], were not attracted by eotaxin to the peritoneal cavity of naive mice in significant numbers. On the other hand, despite the commonly held view that CCR3 expression is restricted to eosinophils [4, 18, 20, 22, 23], basophils [24, 25], eosinophil

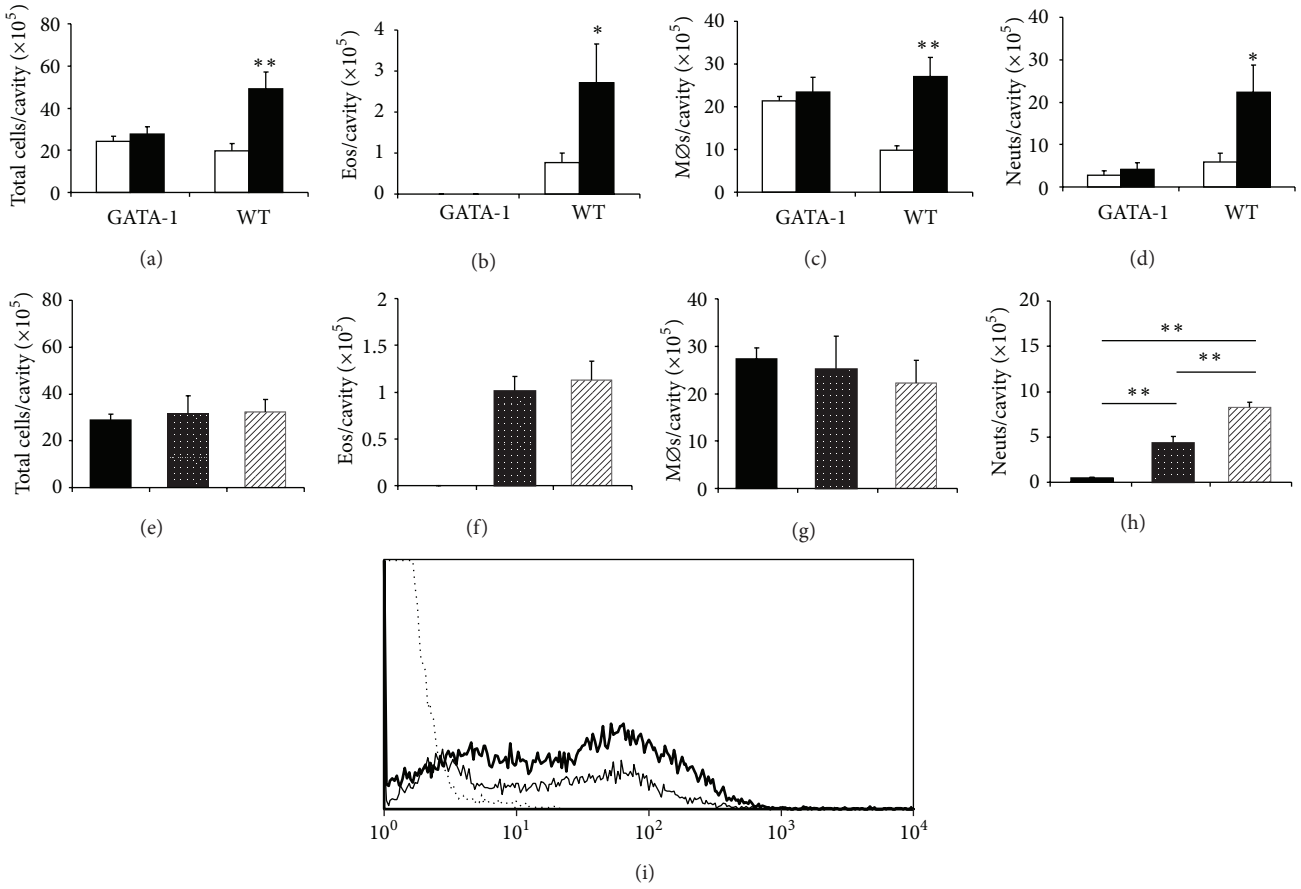


Figure 6: Effect of eosinophil transfer into GATA-1 recipients on neutrophil accumulation. (a)–(d) Eosinophil-deficient GATA-1 mice and wild-type (WT) controls (BALB/c) were injected with RPMI (open bars) or eotaxin, 50 ng/cavity (black bars). The peritoneal lavage fluid collected after 4 h was used for quantitation of total leukocytes (a), eosinophils (b), macrophages (c), and neutrophils (d). E-H, GATA-1 mice received eotaxin (black bars), BALB/c eosinophils (stippled bars), or BALB/c eosinophils followed by eotaxin administration 30 minutes later (hatched bars). Peritoneal lavage fluid collected after 4 h of eotaxin administration was used for quantitation of total leukocytes (e), eosinophils (f), macrophages (g), and neutrophils (h). Data are mean ± SEM. *, $P \leq 0{,}05$; **, $P \leq 0{,}01$, for the indicated differences. Data from 3–11 experiments. (i) Intensity of CCR3 expression in granulocytes. Cells were collected 4 h after eotaxin injection from the peritoneal cavity of GATA-1 and BALB/c donors and stained for CCR3. Representative MFI profiles for the granulocyte gate are shown. Dotted line, GATA-1. Thin line, BALB/c. Thick line, GATA-1 sample to which purified BALB/c eosinophils were added in vitro (up to 20% of total cells).

and basophil progenitors/precursors [26, 27], T cell subsets [41, 42], and smooth muscle cells [29], several studies have suggested that human and murine neutrophils and macrophages can also express CCR3, at least in specific experimental settings [28, 32], as suggested by studies in neutrophils [43]. This would imply that all three leukocyte populations shown to be recruited in our study in wild-type (BALB/c, PAS) mice could be simply responding to eotaxin binding to CCR3 at the individual cell level, with no contribution from cellular interactions involving eosinophils. If so, there should be no decrease in neutrophil or macrophage accumulation by eliminating eosinophils, but one should expect neutrophils and macrophages to express CCR3 at significant levels even in eosinophil-deficient GATA-1 mice. This possibility, however, has been directly ruled out by the demonstration that eotaxin in GATA-1 mutant mice does not recruit neutrophils nor macrophages. The evidence for cellular interactions in the neutrophil response to eotaxin is reinforced by experiments using the same strain, which show neutrophil recruitment following transfer of highly purified BALB/c eosinophils. Finally, we observed no significant accumulation of CCR3+ granulocytes in eotaxin-injected GATA-1 mice.

By contrast, GATA-1 mice had constitutively increased macrophage numbers in the peritoneal cavity, which were unaffected by 4 h of eotaxin administration, both with and without eosinophil transfer. It is possible that the GATA-1 mutation affects the cellular function, tissue distribution, and/or turnover of monocytes/macrophages so as to prevent responses to eotaxin, regardless of whether these are mediated or not by eosinophils. Therefore, we cannot conclude from our present observations in GATA-1 mice alone that eosinophils also recruit monocytes/macrophages. Direct evidence for eosinophil recruitment of monocytes/macrophages was, however, obtained through transfer of eosinophils from PAS donors into ALOX mice. Importantly, in the

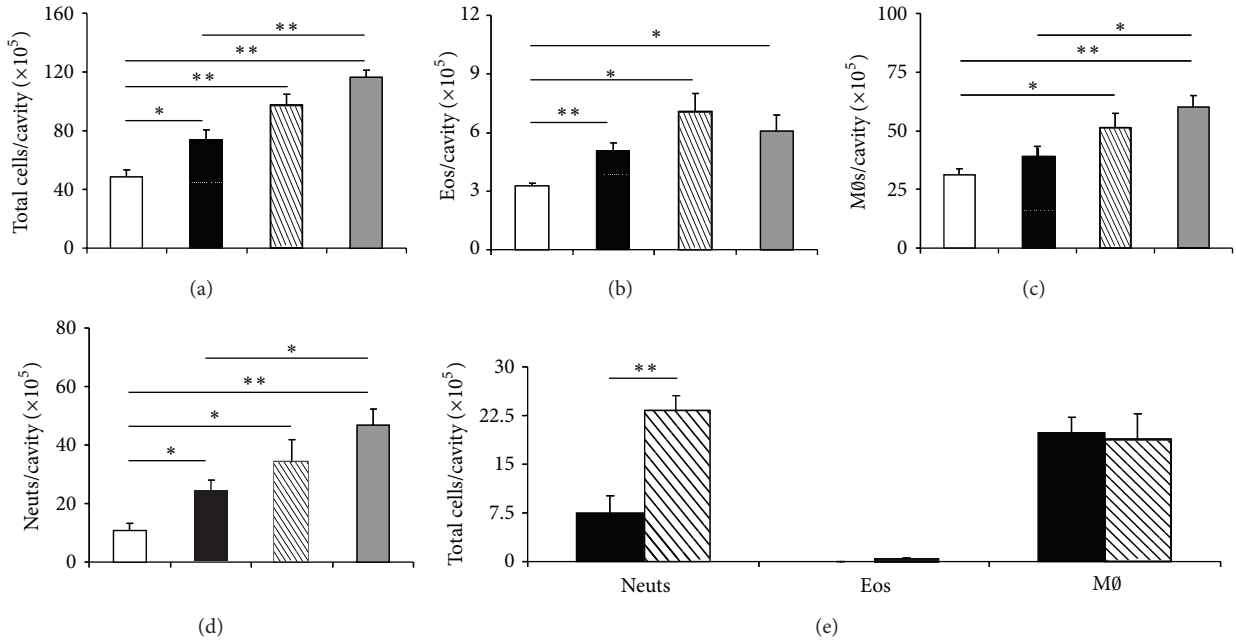


Figure 7: Effect of transfer of PAS or ALOX eosinophils on neutrophil accumulation in the peritoneal cavity of ALOX, PAS, and GATA-1 recipients. (a)–(d) ALOX mice received RPMI (open bars), PAS eosinophils (stippled bars), PAS eosinophils followed by eotaxin, 50 ng/cavity, 30 minutes later (hatched bars). As positive controls, PAS mice received PAS eosinophils followed by eotaxin (gray bars). Peritoneal lavage fluid collected 4 h after eotaxin injection was used for quantitation of total leukocytes (a), eosinophils (b), macrophages (c), and neutrophils (d). (e) GATA-1 mice received eotaxin (black bars), or ALOX eosinophils, followed by eotaxin, 30 minutes later (hatched bars). Peritoneal lavage fluid collected 4 h after eotaxin administration was used for quantitation of neutrophils (Neuts), eosinophils (Eos), and macrophages (Mϕ). Data are mean ± SEM. *, $P \leq 0,05$; **, $P \leq 0,01$, for the indicated differences. Data from 3–5 experiments.

absence of eosinophil transfer, ALOX mice showed neither eosinophil nor monocyte/macrophage recruitment by eotaxin. Interestingly, although in the direct stimulation protocol ALOX mice resembled GATA-1 mice, in their absence of monocyte/macrophage accumulation by 4 h, it is likely that different mechanisms underlie these similar outcomes, since (a) a similar failure to respond to eotaxin with monocyte/macrophage accumulation was observed in wild-type PAS controls and cannot therefore be ascribed to the absence of active 5-LO; (b) transfer experiments show that eosinophil transfer from PAS donors allows a significant recruitment of monocytes/macrophages by eotaxin in ALOX recipients. These observations suggest that an active 5-LO is not required for monocytes/macrophages (or neutrophils) to respond to eotaxin, provided eosinophils are present.

Overall, the data indicate that eotaxin recruits a mixed leukocyte population in naive mice through a mechanism dependent on eosinophils. Evidence that eosinophils play an active role is provided by the observation that full effect in eosinophil transfer experiments requires both eosinophils and eotaxin, which would not be expected if eosinophils played a merely passive or permissive role. On the other hand, in transfer experiments about 10% of the transferred eosinophils were recovered by 4 h eotaxin stimulation of the recipients. This raises the issue of whether the remaining transferred eosinophils underwent changes such as degranulation [4] or release of extracellular traps [10], which might represent a significant difference relative to the direct (nontransfer) protocol used in the initial experiments.

4.2. Role of 5-LO. Mixed leukocyte recruitment by eotaxin in naive mice shows the same dependence on 5-LO that was observed for selective eosinophil recruitment in sensitized mice. Hence, it is likely that eosinophil accumulation itself, the shared feature in both models, is the 5-LO-dependent step. This is consistent with the observation that ALOX eosinophils, when directly transplanted to the peritoneal cavity of GATA-1 recipients (which are unable to respond to eotaxin by accumulation of neutrophils), are able to mediate the neutrophil recruitment induced by eotaxin. Importantly, further recruitment of eosinophils occurs in eotaxin-stimulated ALOX recipients of PAS eosinophils, where the only cells bearing a functional 5-LO are the transferred eosinophils. This suggests that eosinophils can be a source as well as a target for a 5-LO pathway product, such as LTB4. LTB4 was previously shown to selectively attract eosinophils, in a model in which eotaxin duplicated the effect of antigen in a 5-LO-dependent manner [33]. Furthermore, there is significant evidence that recruitment involves interactions between cytokines and lipid mediators [44]. In neutrophil migration, LTB4 represents a signaling relay, raising the possibility that it acts similarly in eosinophils [45]. Whatever mechanism is involved, eosinophil generation of a 5-LO-derived neutrophil chemoattractant is not required for the eosinophil-dependent secondary recruitment of neutrophils in eotaxin-injected naïve mice. While in previous studies of sensitized mice, LTB4, as did antigen and eotaxin, selectively recruited eosinophils [34], it remains to be determined whether it accounts for the rapid eosinophil recruitment

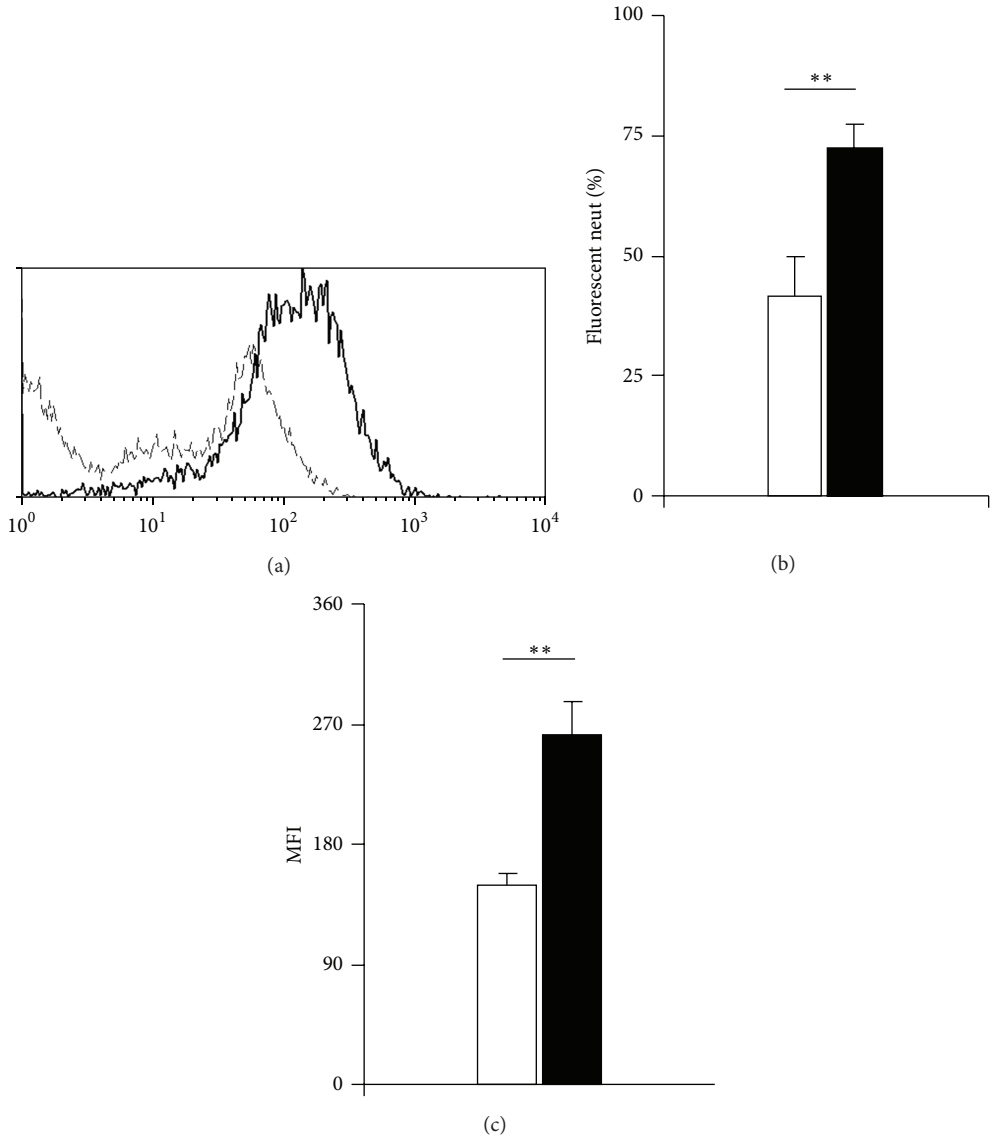


Figure 8: Flow cytometric analyses of peritoneal lavage leukocytes. (a)–(c) Interaction between fluorescent bacteria and neutrophils from eotaxin-injected and control mice. BALB/c mice were injected with eotaxin 50 ng/cavity i.p. Controls received RPMI. After 4 h, peritoneal lavage fluid was collected from both groups. After counting neutrophils, fluorescent, viable *E. coli* bacteria were mixed with leukocytes at a 400 : 1 bacteria/neutrophil ratio and further incubated for 30 min before washing to eliminate unbound bacteria, and analysis of neutrophil-associated fluorescence by flow cytometry. (a) Representative profiles of eotaxin-induced (thick, continuous line) and RPMI-induced (thin, interrupted line) neutrophil-associated fluorescence. (b) Fraction of the neutrophils positive for fluorescent bacteria in RPMI-induced (open bar) and eotaxin-induced (black bar) peritoneal leukocyte populations (mean ± SEM); (c) mean fluorescent intensity of neutrophils in the same samples (mean ± SEM). Data from 4-5 experiments.

to the peritoneal cavity of the eotaxin-injected nonsensitized mice in the present study. A related issue for further investigation is whether 5-LO is required for the increased effectiveness of bacterial binding that was detected in BALB/c leukocytes, as LTB4 is known to activate as well as attract neutrophils [45].

4.3. Relationship to Innate and Acquired Immunity. Despite a common requirement for 5-LO, leukocyte recruitment by eotaxin differs in several aspects between naive versus sensitized mice, especially the lack of eosinophil selectivity in the former, as opposed to the latter. In sensitized mice, selective eosinophil recruitment was observed with widely different chemical stimuli (allergen, eotaxin, or LTB4). It is unlikely, therefore, that such selectivity reflects some features of eotaxin signaling and even less of LTB4 signaling (which should be very effective in mice having normal neutrophil numbers). Alternatively, the failure of eotaxin and LTB4 to recruit neutrophils and monocytes/macrophages in sensitized mice could involve changes in the expression of adhesion proteins at endothelial surfaces, which would

prevent their emigration from blood vessels to peritoneal cavity, regardless of whether the chemoattractant is LTB4 or eotaxin. We have not examined this possibility, since our current observations, which are centered on responses from nonsensitized animals, do not depend on clarifying mechanisms that were not applicable to the present conditions.

We view our findings as manifestations of innate immunity, because of (a) the very fast kinetics of eosinophil and neutrophil accumulation; (b) the recruitment of neutrophils and macrophages in the absence of significant lymphocyte accumulation; (c) the detectable increase in granulocyte binding of live extracellular bacteria in the absence of antibodies. On the other hand, the fast recruitment of monocytes/macrophages by eotaxin-exposed eosinophils raises the issue of whether eosinophils could also enhance protection from more specialized pathogens, such as the intracellular mycobacteria and protozoa that cause chronic infections, which are usually handled by monocytes/macrophages. Relatively little attention has been paid to the possibility that eosinophils play a role in fighting microbial pathogens with the help of other leukocyte types. Our observations suggest that small numbers of eosinophils might recruit a large neutrophil and/or macrophage infiltrate. *While this would make eosinophils surprisingly effective players in innate immunity, this might paradoxically obscure their contribution, if their contribution were to be taken as commensurate with their numbers in inflammatory infiltrates, where they would often amount to no more than one-tenth of total leukocytes*. It is therefore fortunate that, in transfer experiments of wild-type and mutant eosinophils into eosinophil-null GATA-1 mice, eosinophil recruitment of neutrophils can be unequivocally demonstrated. In view of the differences between naive and sensitized models in this respect, it is of interest to determine whether this eosinophil functional capacity is modified by allergen sensitization of the host and whether such a change in innate immune functions can be duplicated by passively or actively sensitizing the host.

4.4. Possible Cellular Mechanisms Underlying the Effect of Eosinophils. Several, but not all, of the observations reported here are consistent with those of previous studies, carried out by other groups in different experimental models. Eotaxin recruitment of a mixed leukocyte population, including neutrophils and macrophages, was described in human subjects [28]; Das and colleagues [46] reported that eotaxin was effective when injected in the peritoneal cavity of mice but not in a dorsal air pouch, drawing attention to the important differences between challenge sites responding to the same chemically defined stimulus. Responses in the air pouch occurred after local inoculation of mast cell-containing peritoneal cell populations, but allergen sensitization was essential to local responses to eotaxin in this transfer model. In addition, neutrophil migration accompanied recruitment of eosinophils in specific conditions. Harris and colleagues [47] confirmed that mast cells were important for full responses to eotaxin and further showed that eotaxin responses were blocked by 5-LO inhibitors. Together, these studies suggest that eotaxin effectiveness is constrained in vivo by several factors that may be absent from in vitro (e. g., migration chamber or flow cytometric) studies. These constraints include mast cells and 5-LO. None of these published studies, however, evaluated the contribution of the recruited eosinophils themselves.

We suggest that a cytokine, rather than a 5-LO derivative, is released by eosinophils in the peritoneal cavity, once they have been recruited by eotaxin in the presence of an active 5-LO, or, alternatively, directly inoculated in the cavity through a transfer protocol. Candidate cytokines would include TNF-α and TGF-β1, both potent neutrophil chemoattractants. One hypothesis that could reconcile our observations with those of Das and Harris and their colleagues [46, 47] would involve the amplification of the role of eosinophils through interactions with resident peritoneal mast cells, since mast cells are an important source of neutrophil chemoattractants, including TNF-α [48].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This paper is supported by fellowships from CNPq to ACCFS (Undergraduate fellowship, PIBIC-UFRJ), Ricardo Alves Luz (Ph.D. candidate fellowship), and Pedro Xavier-Elsas and Maria Ignez Capella Gaspar-Elsas (Research Productivity Fellowships) and by research Grants from CNPq (no. 470377/2011-9 Edital Universal) and FAPERJ (E-26/103.138/2011 Cientista do Nosso Estado) and E-26-112.694-2012 Collaborative Network on Immunomodulators to Pedro Xavier-Elsas. Due to their equally essential contributions to the execution and design of experiments, respectively, Ricardo Alves Luz and Pedro Xavier-Elsas should be considered as joint first authors. The authors wish to express their indebtedness to Dr. Sergeijs Berdnikovs, University of Chicago, for helpful comments and suggestions, as well as steadfast encouragement in this line of research.

References

[1] H. Kita, "Eosinophils: multifaceted biological properties and roles in health and disease," *Immunological Reviews*, vol. 242, no. 1, pp. 161–177, 2011.

[2] G. M. Walsh, Ed., *Eosinophils: Structure, Functional Properties and Role in Disease*, Nova Science, New York, NY, USA, 2012.

[3] M. E. Rothenberg and S. P. Hogan, "The eosinophil," *Annual Review of Immunology*, vol. 24, pp. 147–174, 2006.

[4] H. F. Rosenberg, S. Phipps, and P. S. Foster, "Eosinophil trafficking in allergy and asthma," *Journal of Allergy and Clinical Immunology*, vol. 119, no. 6, pp. 1303–1310, 2007.

[5] A. D. Klion and T. B. Nutman, "The role of eosinophils in host defense against helminth parasites," *Journal of Allergy and Clinical Immunology*, vol. 113, no. 1, pp. 30–37, 2004.

[6] A. E. O'Connell, J. A. Hess, G. A. Santiago et al., "Major basic protein from eosinophils and myeloperoxidase from neutrophils are required for protective immunity to Strongyloides

stercoralis in mice," *Infection and Immunity*, vol. 79, no. 7, pp. 2770–2778, 2011.

[7] F. D. Finkelman and J. F. Urban Jr., "The other side of the coin: the protective role of the TH2 cytokines," *Journal of Allergy and Clinical Immunology*, vol. 107, no. 5, pp. 772–780, 2001.

[8] M. H. Shin, Y. A. Lee, and D.-Y. Min, "Eosinophil-mediated tissue inflammatory responses in helminth infection," *Korean Journal of Parasitology*, vol. 47, pp. S125–S131, 2009.

[9] D. Yang, A. Biragyn, D. M. Hoover, J. Lubkowski, and J. J. Oppenheim, "Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense," *Annual Review of Immunology*, vol. 22, pp. 181–215, 2004.

[10] S. Yousefi, J. A. Gold, N. Andina et al., "Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense," *Nature Medicine*, vol. 14, no. 9, pp. 949–953, 2008.

[11] A. N. Mayeno, A. J. Curran, R. L. Roberts, and C. S. Foote, "Eosinophils preferentially use bromide to generate halogenating agents," *Journal of Biological Chemistry*, vol. 264, no. 10, pp. 5660–5668, 1989.

[12] J. Bystrom, K. Amin, and D. Bishop-Bailey, "Analysing the eosinophil cationic protein—a clue to the function of the eosinophil granulocyte," *Respiratory Research*, vol. 12, article 10, 2011.

[13] H. F. Rosenberg, "Eosinophil-derived neurotoxin/RNase 2: connecting the past, the present and the future," *Current Pharmaceutical Biotechnology*, vol. 9, no. 3, pp. 135–140, 2008.

[14] R. C. N. Melo, L. Liu, J. J. Xenakis, and L. A. Spencer, "Eosinophil-derived cytokines in health and disease: unraveling novel mechanisms of selective secretion," *Allergy*, vol. 68, pp. 274–284, 2013.

[15] M. J. Christopher and D. C. Link, "Regulation of neutrophil homeostasis," *Current Opinion in Hematology*, vol. 14, pp. 3–8, 2007.

[16] L. Cassetta, E. Cassol, and G. Poli, "Macrophage polarization in health and disease," *TheScientificWorldJournal*, vol. 11, pp. 2391–2402, 2011.

[17] M. A. Giembycz and M. A. Lindsay, "Pharmacology of the eosinophil," *Pharmacological Reviews*, vol. 51, no. 2, pp. 213–339, 1999.

[18] P. J. Jose, D. A. Griffiths-Johnson, P. D. Collins et al., "Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea pig model of allergic airways inflammation," *Journal of Experimental Medicine*, vol. 179, no. 3, pp. 881–887, 1994.

[19] F. G. Gervais, R. P. G. Cruz, A. Chateauneuf et al., "Selective modulation of chemokinesis, degranulation, and apoptosis in eosinophils through the PGD2 receptors CRTH2 and DP," *Journal of Allergy and Clinical Immunology*, vol. 108, no. 6, pp. 982–988, 2001.

[20] V. E. L. Stubbs, P. Schratl, A. Hartnell et al., "Indomethacin causes prostaglandin D2-like and eotaxin-like selective responses in eosinophils and basophils," *Journal of Biological Chemistry*, vol. 277, no. 29, pp. 26012–26020, 2002.

[21] G. E. Grant, J. Rokach, and W. S. Powell, "5-Oxo-ETE and the OXE receptor," *Prostaglandins and Other Lipid Mediators*, vol. 89, no. 3-4, pp. 98–104, 2009.

[22] M. E. Rothenberg, R. Ownbey, P. D. Mehlhop et al., "Eotaxin triggers eosinophil-selective chemotaxis and calcium flux via a distinct receptor and induces pulmonary eosinophilia in the presence of interleukin 5 in mice," *Molecular Medicine*, vol. 2, no. 3, pp. 334–348, 1996.

[23] J.-A. Gonzalo, G.-Q. Jia, V. Aguirre et al., "Mouse eotaxin expression parallels eosinophil accumulation during lung allergic inflammation but it is not restricted to a Th2-type response," *Immunity*, vol. 4, no. 1, pp. 1–14, 1996.

[24] U. Forssmann, M. Uguccioni, P. Loetscher et al., "Eotaxin-2, a novel CC chemokine that is selective for the chemokine receptor CCR3, and acts like eotaxin on human eosinophil and basophil leukocytes," *Journal of Experimental Medicine*, vol. 185, no. 12, pp. 2171–2176, 1997.

[25] H. Yamada, K. Hirai, M. Miyamasu et al., "Eotaxin is a potent chemotaxin for human basophils," *Biochemical and Biophysical Research Communications*, vol. 231, no. 2, pp. 365–368, 1997.

[26] R. T. Palframan, P. D. Collins, T. J. Williams, and S. M. Rankin, "Eotaxin induces a rapid release of eosinophils and their progenitors from the bone marrow," *Blood*, vol. 91, no. 7, pp. 2240–2248, 1998.

[27] T. Queto, M. I. Gaspar-Elsas, D. Masid-de-Brito et al., "Cysteinyl-leukotriene type 1 receptors transduce a critical signal for the up-regulation of eosinophilopoiesis by interleukin-13 and eotaxin in murine bone marrow," *Journal of Leukocyte Biology*, vol. 87, no. 5, pp. 885–893, 2010.

[28] A. Menzies-Gow, S. Ying, I. Sabroe et al., "Eotaxin (CCL11) and eotaxin-2 (CCL24) induce recruitment of eosinophils, basophils, neutrophils, and macrophages as well as features of early- and late-phase allergic reactions following cutaneous injection in human atopic and nonatopic volunteers," *Journal of Immunology*, vol. 169, no. 5, pp. 2712–2718, 2002.

[29] R. B. Kodali, W. J. H. Kim, I. I. Galaria et al., "CCL11 (Eotaxin) induces CCR3-dependent smooth muscle cell migration," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 24, no. 7, pp. 1211–1216, 2004.

[30] F. Jundt, I. Anagnostopoulos, K. Bommert et al., "Hodgkin/Reed-Sternberg cells induce fibroblasts to secrete eotaxin, a potent chemoattractant for T cells and eosinophils," *Blood*, vol. 94, no. 6, pp. 2065–2071, 1999.

[31] M. Miyamasu, T. Nakajima, Y. Misaki et al., "Dermal fibroblasts represent a potent major source of human eotaxin: in vitro production and cytokine-mediated regulation," *Cytokine*, vol. 11, no. 10, pp. 751–758, 1999.

[32] D. Mangieri, D. Corradi, D. Martorana et al., "Eotaxin/CCL11 in idiopathic retroperitoneal fibrosis," *Nephrology Dialysis Transplantation*, vol. 27, pp. 3875–3884, 2012.

[33] F. Huaux, M. Gharaee-Kermani, T. Liu et al., "Role of eotaxin-1 (CCL11) and CC chemokine receptor 3 (CCR3) in bleomycin-induced lung injury and fibrosis," *American Journal of Pathology*, vol. 167, no. 6, pp. 1485–1496, 2005.

[34] A. B. Cheraim, P. Xavier-Elsas, S. H. P. de Oliveira et al., "Leukotriene B4 is essential for selective eosinophil recruitment following allergen challenge of CD4+ cells in a model of chronic eosinophilic inflammation," *Life Sciences*, vol. 83, no. 5-6, pp. 214–222, 2008.

[35] C. Yu, A. B. Cantor, H. Yang et al., "Targeted deletion of a high-affinity GATA-binding site in the GATA-1 promoter leads to selective loss of the eosinophil lineage in vivo," *Journal of Experimental Medicine*, vol. 195, no. 11, pp. 1387–1395, 2002.

[36] S. Ebihara, H. Kurachi, and Y. Watanabe, "A simple preparation method for mouse eosinophils and their responses to anti-allergic drugs," *Inflammation Research*, vol. 56, no. 3, pp. 112–117, 2007.

[37] M. I. C. Gaspar Elsas, D. Joseph, P. X. Elsas, and B. B. Vargaftig, "Rapid increase in bone-marrow eosinophil production and

responses to eosinopoietic interleukins triggered by intranasal allergen challenge," *American Journal of Respiratory Cell and Molecular Biology*, vol. 17, no. 4, pp. 404–413, 1997.

[38] M. I. C. Gaspar Elsas, P. Xavier Elsas, D. Joseph et al., "Stimulation of early eosinophil progenitors by a heat stable alveolar macrophage product from ovalbumin-sensitized and non-sensitized guinea pigs," *Clinical and Experimental Allergy*, vol. 27, no. 2, pp. 208–217, 1997.

[39] G. Majno and I. Joris, *Cells, Tissues and Disease*, Oxford University Press, New York, NY, USA, 2nd edition.

[40] B. Desai, J. Mattson, H. Paintal et al., "Differential expression of monocyte/macrophage-selective markers in human idiopathic pulmonary fibrosis," *Experimental Lung Research*, vol. 37, no. 4, pp. 227–238, 2011.

[41] A. Saxena, A. Panigrahi, S. Gupta et al., "Frequency of T cell expressing Th1 and Th2 associated chemokine receptor in patients with renal allograft dysfunction," *Transplantation Proceedings*, vol. 44, no. 1, pp. 290–295, 2012.

[42] P. Lopez, I. Gonzalez-Rodriguez, B. Sanchez et al., "Interaction of Bifidobacterium bifidum LMG13195 with HT29 cells influences regulatory-T-cell-associated chemokine receptor expression," *Applied and Environmental Microbiology*, vol. 78, pp. 2850–2857, 2012.

[43] D. Hartl, S. Krauss-Etschmann, B. Koller et al., "Infiltrated neutrophils acquire novel chemokine receptor expression and chemokine responsiveness in chronic inflammatory lung diseases," *Journal of Immunology*, vol. 181, no. 11, pp. 8053–8067, 2008.

[44] C. D. Sadik and A. D. Luster, "Lipid-cytokine-chemokine cascades orchestrate leukocyte recruitment in inflammation," *Journal of Leukocyte Biology*, vol. 91, no. 2, pp. 207–215, 2012.

[45] P. V. Afonso, M. Janka-Junttila, Y. J. Lee et al., "LTB4 Is a signal-relay molecule during neutrophil chemotaxis," *Developmental Cell*, vol. 22, pp. 1079–1091, 2012.

[46] A. M. Das, R. J. Flower, and M. Perretti, "Resident mast cells are important for eotaxin-induced eosinophil accumulation in vivo," *Journal of Leukocyte Biology*, vol. 64, no. 2, pp. 156–162, 1998.

[47] R. R. Harris, V. A. Komater, R. A. Marett, D. M. Wilcox, and R. L. Bell, "Effect of mast cell deficiency and leukotriene inhibition on the influx of eosinophils induced by eotaxin," *Journal of Leukocyte Biology*, vol. 62, no. 5, pp. 688–691, 1997.

[48] K. D. Stone, C. Prussin, and D. D. Metcalfe, "IgE, mast cells, basophils, and eosinophils," *Journal of Allergy and Clinical Immunology*, vol. 125, no. 2, pp. S73–S80, 2010.

Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2014, Article ID 591468, 10 pages
http://dx.doi.org/10.1155/2014/591468

Research Article

Preparation and Identification of Per a 5 as a Novel American Cockroach Allergen

Ji-Fu Wei,[1,2] Haiwei Yang,[3] Dongning Li,[1] Peisong Gao,[4] and Shaoheng He[1,2]

[1] *Allergy and Clinical Immunology Research Centre, The First Affiliated Hospital of Liaoning Medical University, Jinzhou, Liaoning 121001, China*
[2] *Research Division of Clinical Pharmacology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu 210029, China*
[3] *Department of Urology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu 210029, China*
[4] *Johns Hopkins Asthma & Allergy Center, Baltimore, MD 21224, USA*

Correspondence should be addressed to Shaoheng He; shoahenghe@hotmail.com

Received 24 November 2013; Revised 15 January 2014; Accepted 15 January 2014; Published 23 February 2014

Academic Editor: Huiyun Zhang

Glutathione S-transferase (GST) from various arthropods can elicit allergic reactions. In the present study, Per a 5, a GST, was cloned from American cockroach (CR) and expressed in both baculovirus-infected insect cell (iPer a 5) and *E. coli* expression (bPer a 5) systems. The secondary structures were predicted to be 45.93 and 8.69% of α-helix β-sheets in iPer a 5 and 42.54 and 8.49% of α-helix and β-sheets in bPer a 5, respectively. It is found that 4 out of 16 (25%) sera from American CR allergy patients reacted to both bPer a 9 and iPer a 9 as assessed by ELISA and Western blotting analysis, confirming that Per a 5 is not a major allergen of American CR. Induction of upregulated expression of CD63 and CCR3 on passively sensitized human basophils (sera from American CR allergy patients) by approximately up to 4.5- and 3.2-fold indicates that iPer a 5 and bPer a 5 are functionally active. Recombinant Per a 5 (rPer a 5) should be a useful tool for studying and understanding the role of Per a 5 in CR allergy.

1. Introduction

CR allergy has been recognized as important IgE-mediated type I hypersensitivity since 1964 [1]. It is associated with the development of asthma and recognized as a risk factor for emergency room admission of asthmatic patients, especially among inner city children living in low-income houses infested with cockroaches [2]. In China, totally 25.7% of allergy patients are skin prick test (SPT) positive to the American CR (*Periplaneta americana*, Per a) and 18.7% SPT positive to the German CR (*Blattella germanica*, Bla g) [3].

American CR, German CR, and smoky brown CR (*Periplaneta fuliginosa*) are the dominant indoor CR species which cause allergy among human population worldwide [2]. Twenty-two immunoglobulin E (IgE) binding components including the proteins of 23, 28, 35, 38, 40, 49, 72, 78, and 97 kDa were identified as major allergens in American CR [4], but only Per a 1 [5], Per a 2 [6], Per a 3 [7], Per a 4 [8], Per a 7 [9], Per a 9 [10], and Per a 10 [11] are characterized. For example, Per a 1 is an isoallergen with 5 isoforms reported so far, that is, Per a 1.0101–Per a 1.0105. Per a 1 is a major American CR allergen as it binds to IgE in the sera of 90–100% of CR allergic subjects [4]. Per a 2 is an inactive aspartic protease found in the American CR digestive tract and feces [5]. Per a 5 belongs to GSTs, but the allergenicity of the GST homologues in American CR has not been reported. Since the GST of German CR (Bla g 5) is a major cockroach allergen, which induces specific IgE expression in 30 to 71% of CR allergy [12–14], we anticipate that Per a 5 is likely a major allergen of American CR and investigate the potential allergenicity of it in the present study.

Two GST homologue (Per a 5) genes are available in the Genbank (Accession: AY792949 and AEV23867), which provides the possibility of producing recombinant Per a 5 (rPer a 5) in large amounts to study its role in allergic reactions. Many, but not all allergens expressed from cDNA have shown a considerable IgE binding reactivity that seems to be comparable to their natural counterparts. The majority

of these recombinant allergens are produced in *E. coli*, but, unfortunately, the amount and/or reactivity is sometimes reduced when the allergen is purified and subjected to immunological and biochemical assays [15]. To overcome some of these problems, eukaryotic expression systems such as yeast and baculovirus in insect cells have been used [16]. The aim of the present study is to generate rPer a 5 by using eukaryotic (baculovirus-infected insect cells) and prokaryotic (*E. coli*) expression systems and characterize its biochemical and immunologic properties.

2. Materials and Methods

2.1. Ethics Statement. The study protocol was approved by the Ethical Committee of the First Affiliated Hospital of Nanjing Medical University. Written informed consent for the use of blood samples was obtained from all participants before study entry according to the declaration of Helsinki.

2.2. Patients and Samples. A total of 16 allergic rhinitis patients with positive SPT (allergens were supplied by ALK-Abelló, Inc., Denmark) and positive serum IgE test to American CR extract (by using Immuno-CAP assay (Pharmacia Diagnostics AB, Uppsala, Sweden)) and 6 healthy controls (HC) were recruited in the study. Serum (4 mL) from peripheral venous blood was collected from each patient and HC for Western blot analysis.

2.3. Cloning of cDNAs Encoding Full Length of Per a 5 Gene. Total RNA was isolated from adult female CR reared at our institute by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was quantified by measuring absorbance ratios at 260/280 nm. The cDNA was prepared by reverse transcription using a commercial RNA-PCR kit according to the manufacturer's instruction (TaKaRa Biotech Co. Ltd., Dalian, China). For each reaction, 1 μg of total RNA was reversely transcribed using oligo-d (T). The cDNAs encoding Per a 5 were amplified by PCR using primers based nucleotide sequence of Per a 5 gene (AY792949 and AEV23867; forward: 5′-ATGACCATCGACTTCTACTA-3′; reverse: 5′-TCACTTCTTGGCGAGGTTAT-3′). PCR condition was 95°C/5 min (one cycle), 95°C/1 min, 52°C/1 min and 72°C/1 min (30 cycles), and 72°C/5 min (one cycle). The purified PCR product was cloned into apMD18-T vector (TaKaRa Biotech Co. Ltd., Dalian, China) before being transformed into *Escherichia coli* strain DH5α. The inserts were sequenced on an ABIprism 377 DNA sequencer (Applied Biosystems, Foster, CA, USA). DNA sequence data were translated to amino acid sequence by Show Translation tool in SMS software package (http://www.bioinformatics.org/SMS/). The glycosylation motifs of Per a 5 were predicted by using NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc).

2.4. Expression and Purification of Per a 5 in Baculovirus-Infected Insect Cells. The Per a 5 gene was subcloned into pFastBac1 vector (Novagen, Madison, WI, USA) using *EcoR* I and *Sal I* sites and the resulted construct was transformed into *E. coli* strain DH10Bac to generate recombinant bacmid. The positive colonies were selected and followed by PCR identification. The recombinant bacmid was transfected into Sf-9 cells by using Cellfectin (Invitrogen Corporation, Carlsbad, USA) and incubated in SF-900II liquid medium (Invitrogen Corporation, Carlsbad, USA) for 5 days at 27°C until the cells got swollen. The supernatant was collected as P1 viral stock. P2 viruses were amplified for later infection. A total of 500 mL of Sf-9 cells were infected by P2 viruses and harvested at 72 h. The cells were lysed against 50 mM Tris-HCl with 300 mM NaCl and 5% glycerol. The supernatant was loaded on Ni-NTA column (Genscript, Nanjing, China), washed with running buffer containing 50 mM Tris-HCl, 300 mM NaCl, and 5% glycerol (pH 8.0) and eluted with elution buffer containing 50 mM Tris-HCl, 300 mM NaCl, 250 mM imidazole, and 5% glycerol (pH 8.0). The eluted fractions were obtained and identified as Per a 5 (iPer a 5). The purified iPer a 5 was dialyzed in carbonate-bicarbonate buffer (0.05 M, pH 9.6) for further investigation. The concentration of iPer a 5 was determined by using a Coomassie Plus assay kit with BSA as standard (Thermo Scientific Pierce, Rockford, IL, USA).

2.5. Expression and Purification of Per a 5 in E. Coli. The Per a 5 gene was subcloned into pCold II vector (TaKaRa Biotech Co. Ltd., Dalian, China) using *Nde I* and *Xba I* sites and verified by DNA sequencing. The recombinant pCold II-Per a 5 plasmid was transformed into *E. coli* Origami host strain. A colony of the selected transformed *E. coli* Origami on an overnight LB-ampicillin agar plate was inoculated into 5 mL of LB-ampicillin broth and incubated at 37°C overnight. For IPTG optimization, the overnight culture was added into fresh LB media in a ratio of 1 : 100. Once cell density reached the optical density at $A_{600\,\mathrm{nm}}$ (OD_{600}), the cells were incubated with 0.1, 0.5, and 1 mM IPTG, respectively, at 15°C overnight. Expression of the target protein was analyzed by SDS-PAGE. For scale-up expression, 40 mL of the culture was inoculated into 2 L of fresh LB-ampicillin broth and incubated at 37°C until OD_{600} reached 0.6. IPTG was added to the final concentration of 0.5 mM and the culture was incubated overnight. The bacterial cells were harvested by centrifugation at 4,000 ×g at 4°C for 20 min and were lysed in a lysis buffer by sonication at 20 kHz, 2 min pulse-on, 3 min pulse-off. Cell debris was removed by centrifugation at 12,000 ×g at 4°C for 20 min. The supernatant was loaded on the Nickel column (Genscript, Nanjing, China) as described above, and the eluted fractions were obtained and identified as Per a 5 (bPer a 5). The purified bPer a 5 was dialyzed in carbonate-bicarbonate buffer (0.05 M, pH 9.6) for further investigation. The concentration of bPer a 5 was determined by using a Coomassie Plus assay kit with BSA as standard.

2.6. CD Analysis of rPer a 5 Expressed in E. Coli and Insect Cells. Far UV CD spectra of bPer a 5 and iPer a 5 were collected on a Jasco J-810 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) using a 1 mm path length quartz cuvette at protein concentrations of 0.1 mg/mL. Spectra were measured from 240 to 190 nm, with a 0.5 nm resolution at a scanning speed of 50 nm/min, and resulted from averaging

of three scans. All measurements were performed in 10 mM Na_2HPO_4, pH 7.0. The final spectra were baseline corrected by subtracting the corresponding buffer spectrum. Results were expressed as the mean residue ellipticity (y) at a given wavelength. The secondary structure content of bPer a 5 and iPer a 5 was calculated by using the secondary structure estimation program K2D2 [17].

2.7. Immunoreactivity of Human Sera with rPer a 5. A 96-well plate was coated with purified bPer a 5 and iPer a 5 at 10 μg/mL in carbonate-bicarbonate buffer (0.05 M, pH 9.6) overnight at 4°C, 100 μL per well. Human serum samples (1 : 20 dilution in PBS-Tween with 2% BSA) were then added to the plates for 2 h at room temperature. After IgE binding, plates were incubated with horseradish peroxidase-labeled goat anti-human IgE (1 : 2500 dilution) (KPL, Inc., MD, USA), and the color was developed with tetramethylbenzidine peroxidase substrate (Thermo Scientific Pierce, Rockford, IL, USA). The plates were read on a microplate reader at absorbance of 405 nm. The cutoff of the ELISA was calculated as the mean of the negative controls plus 2 SDs.

For competitive ELISA test, a 96-well plate was coated with American cockroach extract (10 μg/mL) in carbonate-bicarbonate buffer (0.05 M, pH 9.6) overnight at 4°C. The 1 : 20-diluted pooled serum alone or preincubated with various quantities of the crude extract, iPer a 5 and bPer a 5, for 2 h was added to the plates. The color development and plate reading procedure was the same as the previous section. Crude extract of cockroaches were prepared according to the methods described previously [18] with few modifications. Briefly, 30 g of cockroaches was pulverized in liquid nitrogen. The sample was defatted in 200 mL of ethyl ether and ethyl acetate (1 : 1, by volume) and extracted with slow overhead stirring in carbonate-bicarbonate buffer (0.05 M, pH 9.6), containing 6 mM 2-mercaptoethanol and 1/1,000 volume of protease inhibitor cocktail (Shenggong, Shanghai, China) at 4°C overnight. The extract was then centrifuged at 10,000 ×g for 30 min at 4°C, and the supernatant was filtered through a 0.22 μm-pore-size filter (Millipore, Bedford, USA) before use.

2.8. Immunoblot Analysis of IgE Reactivity. Immunoblots for detection of serum specific IgE were performed with bPer a 5 and iPer a 5 as described previously [19, 20]. bPer a 5 and iPer a 5 (5 μg) were added to a SDS-PAGE (gel concentration of 15%) under reducing conditions and then transferred to nitrocellulose membranes. The nitrocellulose membranes were incubated with the sera from the patients with American CR allergy (1 : 5 in PBS-Tween with 1% BSA, 10% normal goat serum) for 90 min. Following rinsing with PBS, the membranes were incubated with peroxidase-labeled anti-human IgE monoclonal antibody. The positive protein bands were visualized by incubating the membranes with tetramethylbenzidine peroxidase substrate. Sera from 2 nonatopic subjects were used as negative controls.

2.9. Basophil Activation Test. Expression of CD63 and CCR3 on basophil surface has been considered as the indicator of basophil activation [21, 22]. Briefly, peripheral blood mononucleated cells (PBMC) from 20 mL blood donated by 4 healthy volunteers were separated by Ficoll-Paque density gradient and treated with 10 mL LS (a solution containing 1.3 M NaCl, 0.005 M KCl, and 0.01 lactic acid, at pH 3.9) for 2 min at 8°C. After neutralization with 12% Tris (pH 10.9), nonspecific IgE on basophils was stripped off and cells were passively sensitized with sera of the patients with American CR allergy or HC (n = 4, 1 in 10 dilution, 2 h at 37°C) as described previously [23]. The cells were then challenged with various concentrations of bPer a 5 and iPer a 5 for 15 min at 37°C. A goat anti-human IgE antibody (Serotec, Kidlington, UK) was used as a positive control. Anti-human CCR3-PE antibody (eBioscience Inc. San Diego, CA, USA) and anti-human CD63-FITC antibody (Invitrogen Corporation, Camarillo, CA, USA) were added to cells for 15 min at 37°C in the dark. Flow cytometry analysis of surface markers was performed at 488 nm on a FACSAria flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed by FACSDiva software. Basophils were gated in the lymphocyte region of the SSC or FSC pattern, and identified as a single population of cells that stained positively for CCR3-PE antibody. Upregulation of CD63 expression was determined by an increase in fluorescence in the FL-1 channel. Acquisition was terminated after 300 basophil target events. Responses were quantified as percentages of CD63 expressing basophils in a higher FL-1 region, which had been adjusted to contain 4% of basophils.

2.10. Statistics. Data are expressed as mean ± SEM for the indicated number of independently performed duplicated experiments. Statistical significance between means was analyzed by one-way ANOVA or the Student's t-test utilizing the SPSS 13.0 version. $P < 0.05$ was taken as statistically significant.

3. Results

3.1. Cloning of cDNAs Encoding Full Length of Per a 5 Sequence. The cDNAs encoding Per a 5 were amplified by PCR using primers based on the nucleotide sequence of Per a 5 gene. It is a 645 bp gene and encoded 215 amino acids protein (Figure 1). The sequence identity of Per a 5 to cDNAs deposited in Genbank (Accession no. AY563004) was 100% (215/215) at protein level. Per a 5 shows 81, 15, and 13% sequence similarity to German CR allergen BGGSTD1, Der p 8, and Bla g 5 (Figure 2). One motif (198NHSG201) was predicted to be the glycosylation motif of Per a 5.

3.2. Expression and Purification of Per a 5 in E. Coli. The Per a 5 gene was subcloned into pCold II vector and transformed into *E. coli* Origami host strain. The optimal induction condition for Per a 5 was 0.5 mM IPTG (Figure 3(a)), the concentration chosen as the final condition throughout the study. The Per a 5 protein was purified by Ni column. More than 6 mg bPer a 5 was obtained from 2 L cell culture medium. The purity of the purified Per a 5 was identified by SDS-PAGE. It showed single band with an apparent molecular weight of 25 kDa (Figure 3(b)).

```
  1 M T I D F Y Y L P G S A P C R S V L L A
  1 ATGACCATCGACTTCTACTACCTGCCCGGCAGCGCACCATGCCGTTCAGTTCTCCTGGCT
 21 A K A I G V D L N L K V T N L M A G E H
 61 GCCAAGGCCATCGGCGTGGATCTGAACCTCAAAGTGACGAACCTCATGGCTGGCGAACAT
 41 L T P E F L K M N P Q H T I P T L N D N
121 CTCACGCCTGAATTTCTTAAGATGAATCCTCAACATACGATCCCAACCCTCAACGACAAC
 61 G F C L W E S R A I L S Y L A D Q Y G K
181 GGTTTCTGTTTGTGGGAGAGCCGAGCCATTCTCAGTTACCTGGCTGACCAGTATGGCAAG
 81 D D S L Y P K D A K K R A L V D Q R L Y
241 GACGACTCGCTGTACCCCAAGGACGCCAAGAAGCGAGCTCTTGTGGACCAGAGACTGTAC
101 F D I G T L Y H R F G E Y Y Y P I Y F A
301 TTCGATATTGGAACCCTGTACCACAGATTCGGAGAATACTATTATCCAATCTATTTCGCA
121 K Q A A D P E K M K K L E E A F E F L N
361 AAACAAGCTGCAGACCCTGAAAAGATGAAGAAACTGGAGGAGGCCTTCGAGTTCTTGAAT
141 K F L E S Q E F V A G N K L T I A D L A
421 AAGTTCCTGGAATCGCAAGAGTTTGTGGCAGGAAATAAGCTCACCATTGCGGACCTGGCA
161 I V S S V S T A D I M G F D V S K Y S N
481 ATTGTCTCCTCTGTCTCCACTGCTGACATCATGGGCTTTGATGTAAGCAAATACTCAAAC
181 V A K W F E K C K K I V P G Y E E L N H
541 GTCGCCAAATGGTTCGAGAAATGCAAGAAGATTGTTCCAGGCTATGAGGAACTGAATCAC
201 S G C L K F K E M C D N L A K K *
601 TCCGGATGCTTGAAGTTCAAGGAGATGTGCGATAACCTCGCCAAGAAGTGA
```

Figure 1: cDNA sequence encoding Per a 5 and deduced amino acid sequence. The first three bases ATG represent start codon. The last three bases indicate the stop codon.

3.3. Expression and Purification of Per a 5 in Baculovirus-Infected Insect Cells. The Per a 5 encoding gene was subcloned into pFastBac1 vector and transformed into *E. coli* strain DH10Bac to generate recombinant bacmid. The recombinant bacmid was then transfected into Sf-9 cells to generate the baculovirus. The Per a 5 protein was expressed in Sf-9 cells and purified by Ni column. About 16 mg of iPer a 5 was obtained from 2 L cell culture medium. The purity of the purified Per a 5 was identified by SDS-PAGE, which showed major band with an apparent molecular weight of 25 kDa (Figure 4).

3.4. CD Analysis of rPer a 5 Expressed in Insect Cells and E. Coli. The far UV CD spectra of iPer a 5 and bPer a 5 showed similar curves with two minima at 220 and 209.5 nm and a large maximum at 192 nm, which represent characteristics of proteins with predominantly α-helical structure (Figure 5). Calculation of the secondary structure using the program K2D2 resulted in predicted 45.93 and 8.69% of α-helix and β-sheets in iPer a 5 and 42.54 and 8.49% of α-helix and β-sheets in bPer a 5, respectively.

3.5. Immunoreactivity to IgE. In order to determine the allergenicity of Per a 5, we examined the ability of Per a 5 to bind IgE in the sera of American CR allergy by a direct ELISA technique. Patients serum including patients 5, 9, 10, and 14 showed positive IgE reactivity to both bPer a 5 and iPer a 5. The results showed that 4 out of 16 (25%) sera from these patients reacted to both bPer a 5 and iPer a 5 (Figure 6(a)). The IgE reactivity of bPer a 5 and iPer a 5 in the sera from the Per a 5 positive patients was increased by 5.0- and 7.9-fold, respectively in comparison with the sera from HC. Moreover, competitive ELISA showed that bPer a 5 and iPer a 5 inhibited the IgE reactivity to the cockroach extract by approximately 25.4 and 35.5%, respectively (Figure 6(b)). IgE binding activity of Per a 5 in a representative group of 3 patients and two HC was assessed by Western blot and was illustrated in Figure 6(c). IgE binding bands appeared clearer

```
Per a 5       ---TIDFYYLPGSA-PCRSVLLAAKAIG-VDLNLKVT---NLMAGEHLTPEFLKMNPQHT
BGGSTD1       ---TIDFYYLPGSA-PCRSVLLAAKAFG-VDLNLKVT---NLMAGEHLTPEFLKMNPQHT
Der p 8       SQPILGYWDIRGYAQPIR-LLLTYSGVDFVDKRYQIGPAPDFDRSEWLNEKFNLGLDFPN
Blag a 5      --YKLTYCPVKALGEPIR-FLLSYGEKDFEDYRFQEG--------DWPN--LKPSMPFGK
                        * *  **      *

Per a 5       IPTLNDNGFCLWESRAILSYLADQYGKDDSLYPKDAKKRALVDQRLYFDIGTLYHRFGEY
BGGSTD1       IPTLNDNGFCLWESRAILSYLADQYGKDDSLYPKDPKKRALVDQRLYFDLGTLYQRFGDY
Der p 8       LPYYIDGDMKMTQTFAILRYLGRKYKLNGSND-HEEIRISMAEQQTEDMMAAMI-R---V
Blag a 5      TPVLEIDGKQTHQSVAISRYLGKQFGLSGKDD-WENLEIDMIVDTISDFRAAIAN--YHY
               *              ** **

Per a 5       YYPIYFAKQAADPEKMKKLEEAFEFLNKFLESQE-FVAGNKLTIADLAIVSSVSTADIMG
BGGSTD1       YYPIMFAKASPDAEKMKKLEEAYQFLDKFLEGQK-FVAGNSLTIADIATIASVSTAAILG
Der p 8       CYDANCDKLKPD--YLKSLPDCLKLMSKFVGEHA-FIAGANISYVDFNLYEYLCHVKVMV
Blag a 5      DADENSKQKKWDPLKKETIPYYTKKFDEVVKANGGYLAAGKLTWADFYFVAILDYLNHMA
                        *                          *     *

Per a 5       -FDVS-KYSNVAKWFEKCKKIVPGYEELNHSGCLKFKEMCDNLAKK--  100%
BGGSTD1       -FDIT-RYPNVNKWFENAKKVIPGYDELNHSGCLEFKKMWDNLTQK--  81%
Der p 8       -PEVFGQFENLKRYVERMESLPRVSDYIKKQQPKTFNAPTSKWNASYA  15%
Blag a 5      KEDLVANQPNLKALREKVLGLPAIKAWVAKRPPTDL------------  13%
                        *     *
```

Figure 2: Alignment of Per a 5 with other GST allergen. Deduced amino acid sequence of Per a 5 is aligned with GST allergens. Per a 5 was cloned and sequenced in the present study. BGGSTD1 [29] and Bla g 5 [13] are two GST allergens identified in German CR, and Der p 8 is a GST allergen identified from *Dermatophagoides pteronyssinus* [30]. The identical residues in all four sequences are marked with asterisk (∗).

with iPer a 5 than with bPer a 5. Both iPer a 5 and bPer a 5 did not react to the sera from the HC.

3.6. Induction of Basophil Activation by rPer a 5. iPer a 5 and bPer a 5 at 1.0 μg/mL induced approximately up to 4.5- and 3.2-fold increase in the expression of CD63 and CCR3 in CD63 and CCR3 double positive cells when incubating with passively sensitized basophils (by sera from American CR allergy). Both iPer a 5 and bPer a 5 had no effect on the basophils sensitized by the sera from HC (Figure 7).

4. Discussion

Aerosolized proteins derived from saliva, fecal material, secretions, cast skins, debris, and dead bodies of cockroaches induce IgE-mediated hypersensitivity [24]. To better understand the Per a 5 mediated CR allergies and promise to improve diagnosis and treatment of CR allergies, we prepared biologically active and highly pure American CR allergen Per a 5 in relatively large amount in the present study. We have identified Per a 5 as a novel American CR allergen, which is recognized by 25% of the subjects with American CR allergy.

Several allergens from German and American CR have been identified and their IgE cross-reactivity has been described. These include Bla g 1 and Per a 1 (food intake and digestion); Bla g 2 and Per a 2 (inactive aspartic protease); Bla g 3 and Per a 3 (arylphorin-like storage protein); Bla g 4 and Per a 4 (male pheromone transport lipocalin); and Bla g 7 and Per a 7 (tropomyosin) [25]. Among CR allergens, 3 GSTs were identified from male adults of German CR by glutathione-agarose affinity chromatography including one in which N-terminal amino acid sequence is identical to that of Bla g 5 [26]. Two IgE-reactive GSTs were detected from 25 IgE-reactive spots by proteomic analysis, and one of them was found to be Bla g 5 [27]. Recently, a Delta class GST (BgGSTD1), which has 15% amino acid sequence identity with Bla g 5, was purified from the German CR [28]. However, the allergenicity and IgE cross-reactivity of the GST homologues in American CR have not been reported. As a member of the Delta class GSTs, Per a 5 shows 81% sequence similarity to BGGSTD1, but only 13% similarity to Bla g 5, a Sigma class GST identified from German CR. In the present study, only 25% IgE reactivity to rPer a 5 was observed when sera from cockroach-sensitive patients was incubated with rPer a 5, suggesting that Per a 5 is not likely a major allergen in American CR. The IgE reactivity rate of Per a 5 seems lower than that of Bla g 5 (30 to 71%) [12–14], but higher than that of BGGSTD1 (17.9%) [28]. It has previously been reported that American CR extracts could not inhibit IgE antibody binding to Bla g 5 using an RIA assay, suggesting that there was no significant IgE cross-reactivity between GSTs from these two species [12]. However, Huang et al. found that GST(s) in American CR is(are) allergenic and is(are) pan-allergens in

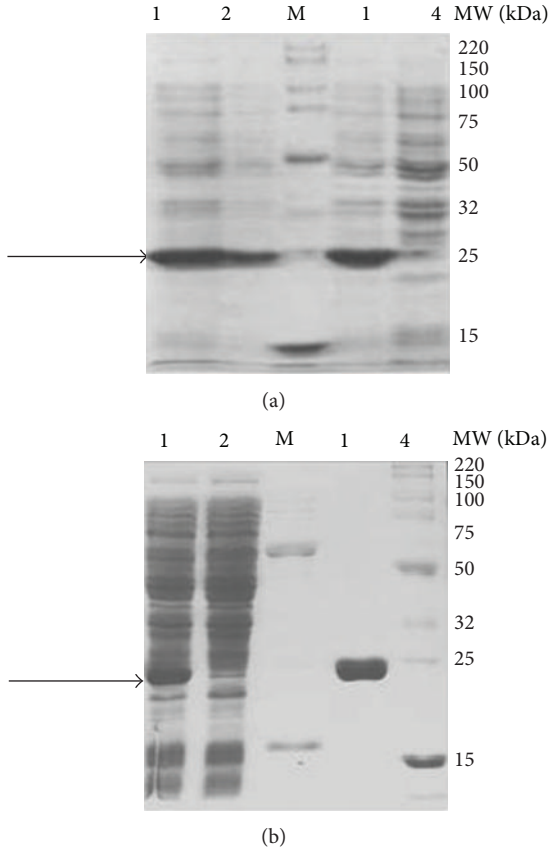


Figure 3: Expression and purification of Per a 5 in *E. coli*. (a) Per a 5 was induced by 0.1, 0.5, and 1 mM IPTG, respectively, and was analyzed by SDS-PAGE. Lane M: Smart Broad-Range Protein Standard (Genscript, Nanjing, China); lanes 1, 2, 3, and 4 represent lysates of cells induced with 0.5, 0.1, 1.0, or 0 mM IPTG, respectively. The arrow indicates Per a 5 protein. (b) SDS-PAGE analysis of purified Per a 5 expressed in *E. coli*. Lane M: protein standard; lane 1: the supernatant of cells lysate after centrifugation; lane 2: run through; lane 3: wash with 50 mM imidazole; lane 4: wash with 250 mM imidazole. The arrow indicates recombinant Per a 5 protein.

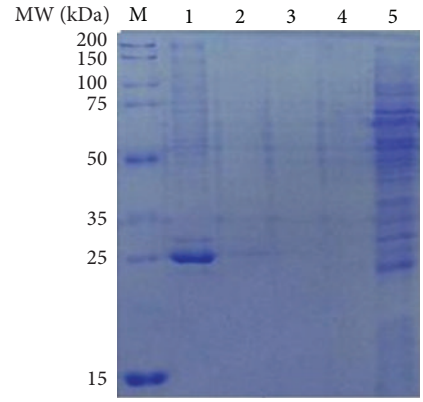


Figure 4: SDS-PAGE analysis of purification of Per a 5 in baculovirus-infected insect cells through Ni column. Lane M: protein standard; lane 1: elute with the elution buffer containing 250 mM imidazole; lane 2: elute with the elution buffer containing 20 mM imidazole; lane 3: elute with the elution buffer containing 50 mM imidazole; lane 4: run through; lane 5: the cytosol.

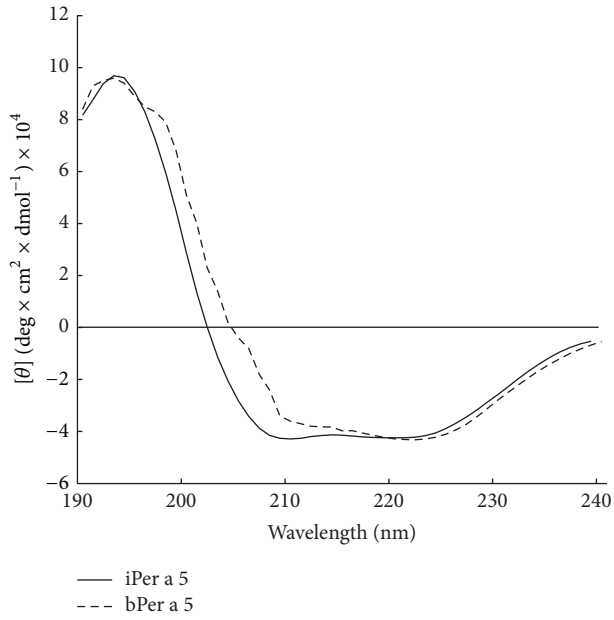


Figure 5: Circular dichroism analysis of recombinant Per a 5 generated from baculovirus-infected insect cells (iPer a 5) and *E. coli* (bPer a 5). The molecular ellipticities (y) (y-axes) at different wavelengths (190–240 nm, x-axis) are displayed for iPer a 5 and bPer a 5.

CR and mites [29]. The finding that Per a 5 reacts with specific IgE of American CR allergy in the present study suggests that GST in American CR is allergenic.

We prepared rPer a 5 by using two different expression systems in the present study and found that as little as 2 L of *E. coli* and Sf-9 cell culture medium was able to produce 8 and 16 mg of highly pure rPer a 5, respectively, which is enough for functional study of Per a 5. The *E. coli* system is a well-established system offering many advantages: easy handling of the bacteria cells and selection of a large variety of vectors using different promoters. Among the disadvantages, overexpressed proteins can be incorrectly folded and may require chemical refolding procedures to obtain the protein in a native, fully active, biological form. In the present study, we chose pCold II vector and *E. coli* Origami host strain to produce bPer a 5 in a soluble form without any reconstitution process. The eukaryotic baculovirus expression system is characterized by an extensive array of posttranslational processing, typical for higher eukaryotic cells. The production of recombinant proteins in this system offers the advantage that secreted proteins are often glycosylated and disulphide-bonded correctly leading to a biologically active conformation. Because of the advantage of baculovirus-infected insect cell expression system, we employed it to express iPer a 5 and managed to obtain substantial quantity of iPer a 5 in the present study. Numerous insect allergens such as Api m 1 [30], Api m 2 [31] from honeybee venom, Sol i 3 [32] from ant venom, Dol m 5 [33] from bald-faced hornet (*Dolichovespula maculate*), Cul s 1 [34] from

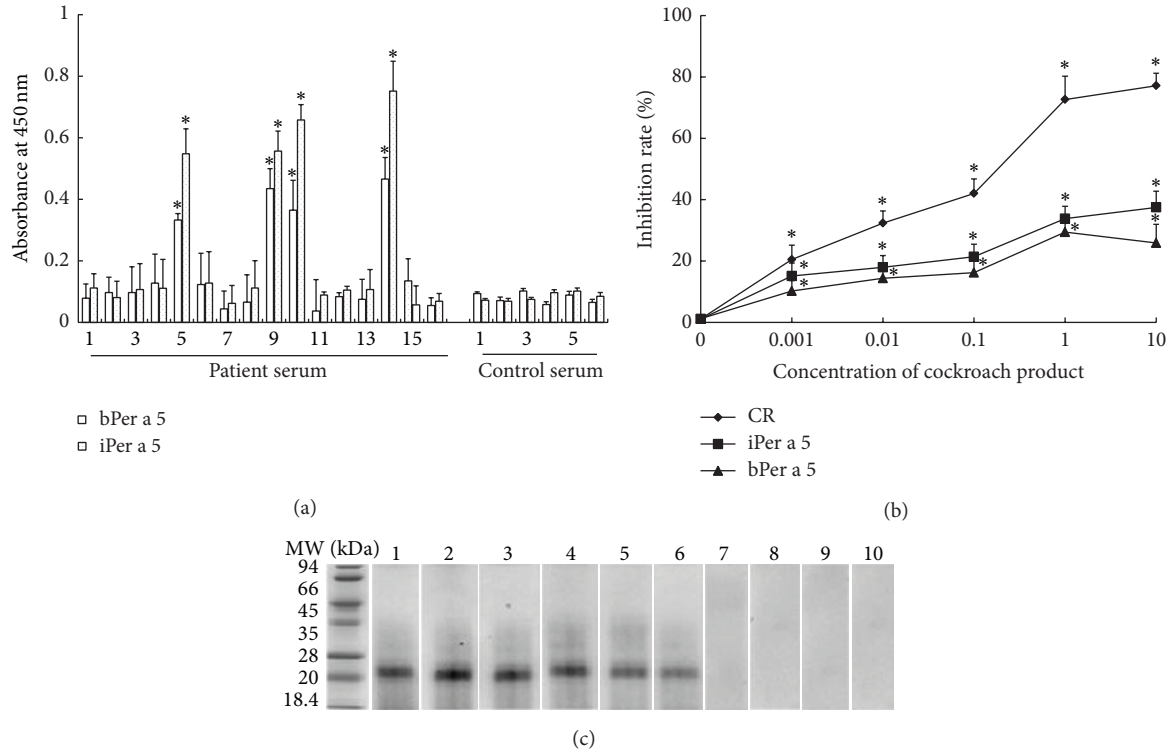


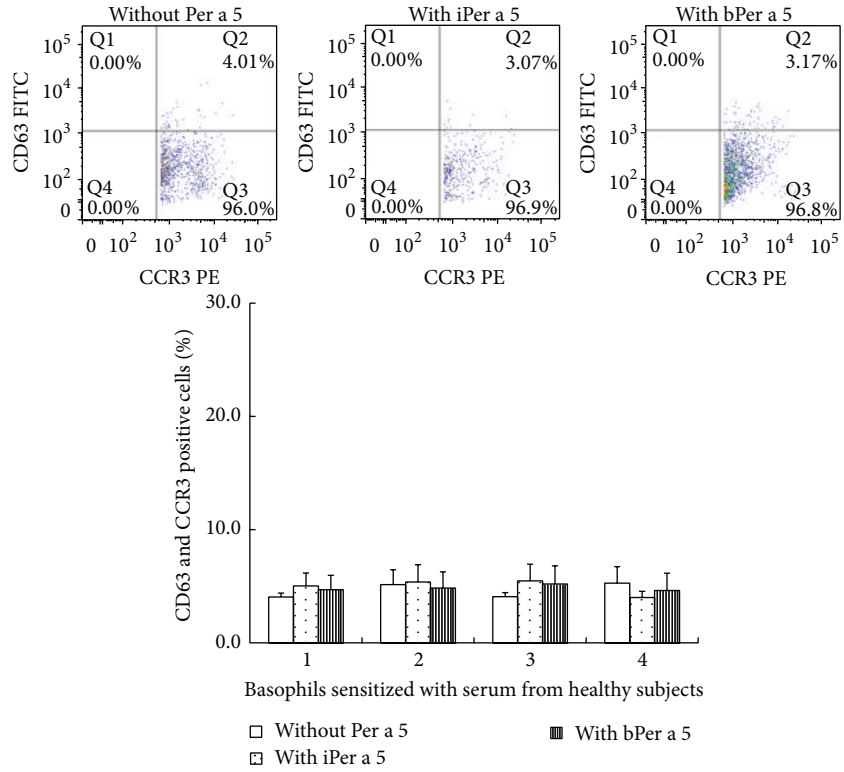
Figure 6: (a) Analysis of specific IgE reactivity of recombinant bPer a 5 and iPer a 5 by direct ELISA. The sera were collected from the patients with American CR allergy and healthy control subjects. The values shown are mean ± SEM for the triplicate experiments. $^{*}P < 0.05$ in comparison with those of healthy control subjects. (b) Inhibition of the IgE reactivity to the cockroach extract by bPer a 5 and iPer a 5. CR = American cockroach extract. The values shown are mean ± SEM for the triplicate experiments. $^{*}P < 0.05$ in comparison with the uninhibited control. (c) Western blot analysis of IgE reactivity to bPer a 5 and iPer a 5 in the sera from the patients with American CR allergy. Lanes 1–3: iPer a 5 reacted with the serum from patients 5, 9, and 10. Lanes 4–6: bPer a 5 reacted with the serum from patients 5, 9, and 10. Lanes 7-8: iPer a 5 reacted with the serum from controls 1 and 2. Lanes 9-10: bPer a 5 reacted with the serum from controls 1 and 2.

the North American midge (*Culicoides sonorensis*), Der f 1 [35] from house dust mite, Blo t 11 [36] from dust mite (*Blomia tropicalis*), Lep d 2 [15] from dust mite (*Lepidoglyphus destructor*), and Aed a 1 and Aed a 2 [37] from mosquito (*Aedes aegypti*) have been successfully expressed in insect cells using a baculovirus expression system. They are reported to possess similar structural and biological activities to their natural forms [38].

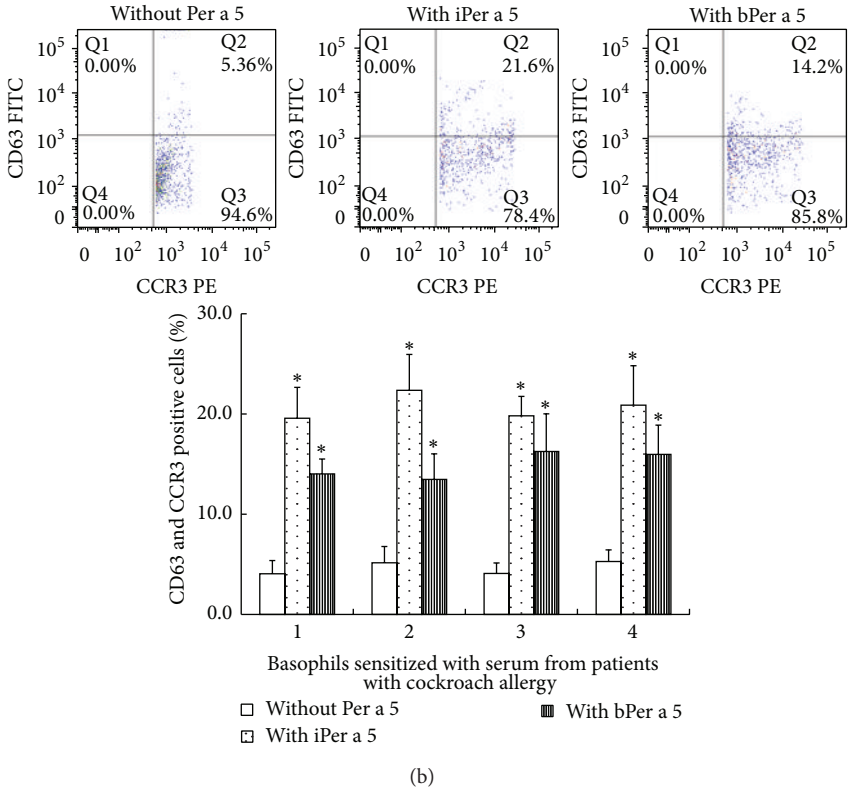
Regarding the immunoreactivity, rPer a 5 produced in the two systems showed IgE-binding activities towards the sera from the American CR allergic patients. iPer a 5 seems to have better binding activity than bPer a 5, implicating that the IgE-binding activity of rPer a 5 is likely to associate with its glycosylation sites and tertiary conformation. One motif (198NHSG201) was predicted to be the glycosylation motif in Per a 5, but we are not sure of the numbers of α-Gals in it, as most of the carbohydrate epitopes are known to lead false-positive reactions except α-Gal [39]. The far UV CD spectra of recombinant iPer a 5 and bPer a 5 showed similar secondary structures, which consist of predominantly α-helical structure. But iPer a 5 appears to have more α-helical structures than bPer a 5. IgE-binding epitopes recognized by IgE antibodies are either linear or conformational and are located on the surface of the molecule accessible to antibodies [40]. Conformational epitope comprises amino acids that are close in space in the folded molecule, despite being noncontiguous in the amino acid sequence. They are dependent on the 3-dimensional structure of the protein. For globular inhaled allergens, conformational epitopes play a very important role in allergenicity [41, 42]. Therefore, it seems likely that extra conformational IgE-binding epitopes may exist in iPer a 5, as a result of more α-helical structures in its molecular structure.

The basophil activation test we employed herein is a more advanced technique for determination of allergenicity of a given compound. We confirm that Per a 5 is an active allergen of CR as it is able to activate CR-sensitized basophils. Similar to IgE-binding activity, iPer a 5 seems more potent in activation of basophils compared with bPer a 5, suggesting that the basophil-activating activity of rPer a 5 is likely to associate with its glycosylation sites and tertiary conformation. Obviously, further work is required to confirm our anticipation.

The availability of recombinant allergens has increased our understanding of IgE-mediated allergies and promises to improve diagnosis and treatment of these diseases [43]. In our case, rPer a 5 should be a useful tool for functional and clinical study. These observations also confirm that



(a)



(b)

Figure 7: Induction of basophil activation by iPer a 5 and bPer a 5. After nonspecific IgE on basophils being stripped off, cells from each donor were passively sensitized with sera from 4 different healthy subjects (a) or from 4 different patients with American CR allergy (b) and were then challenged with iPer a 5 and bPer a 5 at 1.0 μg/mL. The values shown are mean ± SEM for the sera from 4 different subjects. $^{*}P < 0.05$ in comparison with the corresponding carrier alone control.

baculovirus-infected insect cell expression system is more suitable for the production of more active Per a 5 allergen than *E. coli* expression system. In conclusion, we have cloned and prepared two rPer a 5 allergens by using a eukaryotic and a prokaryotic expression system. We confirm that there is GST allergen (Per a 5) in American CR, though it is not a major allergen. rPer a 5 should be a useful tool for studying and understanding the role of Per a 5 in CR allergy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Ji-Fu Wei and Haiwei Yang contributed to this paper equally as the first author.

Acknowledgments

This project was sponsored by the Grants from the Major State Basic Research Program of China (973 Program) (no. 2013CB530501), the National Natural Science Foundation of China (31340073, 81273274, 81172836, 30972822, 81001329, and 81030054), Jiangsu Province's Key Provincial Talents Program (RC201170), The Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), National "Twelfth Five-Year" Plan for Science & Technology Support Project (2014BAI07B02), the Innovation team project of Education Department of Liaoning Province (LT2013017), the higher Education Climb Scholars Program of Liaoning Province, China (no. LJ2013222), and the Liaoning Province Translational Medicine Research Center for Allergy (no. LK2013041).

References

[1] H. S. Bernton and H. Brown, "Insect allergy-preliminary studies of the cockroach," *Journal of Allergy*, vol. 35, no. 6, pp. 506–513, 1964.

[2] L. K. Arruda, L. D. Vailes, V. P. L. Ferriani, A. B. R. Santos, A. Pomés, and M. D. Chapman, "Cockroach allergens and asthma," *Journal of Allergy and Clinical Immunology*, vol. 107, no. 3, pp. 419–428, 2001.

[3] B.-Q. Sun, X.-X. Lai, B. Gjesing, M. D. Spangfort, and N.-S. Zhong, "Prevalence of sensitivity to cockroach allergens and IgE cross-reactivity between cockroach and house dust mite allergens in Chinese patients with allergic rhinitis and asthma," *Chinese Medical Journal*, vol. 123, no. 24, pp. 3540–3544, 2010.

[4] V. T. Sudha, N. Arora, S. Sridhara, S. N. Gaur, and B. P. Singh, "Biopotency and identification of allergenic proteins in *Periplaneta americana* extract for clinical applications," *Biologicals*, vol. 35, no. 2, pp. 131–137, 2007.

[5] S. He, Z. Zhang, H. Zhang et al., "Analysis of properties and proinflammatory functions of cockroach allergens per a 1.01s," *Scandinavian Journal of Immunology*, vol. 74, no. 3, pp. 288–295, 2011.

[6] Q. Pan, S. Wang, H. Shang, and F. Chew, "Identification and characterization of Per a 2, the Bla g 2 allergen homologue from American cockroach (*Periplaneta americana*)," *Journal of Allergy and Clinical Immunology*, vol. 117, no. 2, p. S115, 2006.

[7] B. Mindykowski, E. Jaenicke, S. Tenzer et al., "Cockroach allergens Per a 3 are oligomers," *Developmental and Comparative Immunology*, vol. 34, no. 7, pp. 722–733, 2010.

[8] Y. W. Tan, S. L. Chan, T. C. Ong et al., "Structures of two major allergens, Bla g 4 and Per a 4, from cockroaches and their IgE binding epitopes," *Journal of Biological Chemistry*, vol. 284, no. 5, pp. 3148–3157, 2009.

[9] J. A. Asturias, N. Gómez-Bayón, M. C. Arilla et al., "Molecular characterization of American cockroach tropomyosin (*Periplaneta americana* allergen 7), a cross-reactive allergen," *Journal of Immunology*, vol. 162, no. 7, pp. 4342–4348, 1999.

[10] N. Sookrung, W. Chaicumpa, A. Tungtrongchitr et al., "*Periplaneta americana* arginine kinase as a major cockroach allergen among the patients with major cockroach allergies," *Environmental Health Perspectives*, vol. 114, no. 6, pp. 875–880, 2006.

[11] V. T. Sudha, N. Arora, S. N. Gaur, S. Pasha, and B. P. Singh, "Identification of a serine protease as a major allergen (Per a 10) of *Periplaneta americana*," *Allergy*, vol. 63, no. 6, pp. 768–776, 2008.

[12] L. K. Arruda, L. D. Vailes, T. A. E. Platts-Mills, M. L. Hayden, and M. D. Chapman, "Induction of IgE antibody responses by glutathione S-Transferase from the German cockroach (*Blattella germanica*)," *Journal of Biological Chemistry*, vol. 272, no. 33, pp. 20907–20912, 1997.

[13] K. Y. Jeong, K.-J. Jeong, M.-H. Yi, H. Lee, C.-S. Hong, and T.-S. Yong, "Allergenicity of sigma and delta class glutathione s-transferases from the german cockroach," *International Archives of Allergy and Immunology*, vol. 148, no. 1, pp. 59–64, 2008.

[14] S. M. Satinover, A. J. Reefer, A. Pomes, M. D. Chapman, T. A. E. Platts-Mills, and J. A. Woodfolk, "Specific IgE and IgG antibody-binding patterns to recombinant cockroach allergens," *Journal of Allergy and Clinical Immunology*, vol. 115, no. 4, pp. 803–809, 2005.

[15] S. Olsson, M. Van Hage-Hamsten, P. Whitley et al., "Expression of two isoforms of Lep d 2, the major allergen of *Lepidoglyphus destructor*, in both prokaryotic and eukaryotic systems," *Clinical and Experimental Allergy*, vol. 28, no. 8, pp. 984–991, 1998.

[16] D. R. O'Reilly, L. K. Miller, and V. A. Luckow, *Baculovirus Expression Vectors: A Laboratory Manual*, Oxford University Press, New York, NY, USA, 1992.

[17] C. Perez-Iratxeta and M. A. Andrade-Navarro, "K2D2: estimation of protein secondary structure from circular dichroism spectra," *BMC Structural Biology*, vol. 8, article 25, 2008.

[18] K. Y. Jeong, H. Hwang, J. Lee et al., "Allergenic characterization of tropomyosin from the dusky brown cockroach, *Periplaneta fuliginosa*," *Clinical and Diagnostical Laboratory Immunology*, vol. 11, no. 4, pp. 680–685, 2004.

[19] S. An, L. Chen, J.-F. Wei et al., "Purification and characterization of two new allergens from the venom of *Vespa magnifica*," *PLoS One*, vol. 7, no. 2, Article ID e31920, 2012.

[20] S. An, D. Ma, J. F. Wei et al., "A novel allergen Tab y 1 with inhibitory activity of platelet aggregation from salivary glands of horseflies," *Allergy*, vol. 66, no. 11, pp. 1420–1427, 2011.

[21] M. L. Sanz, P. M. Gamboa, I. Antépara et al., "Flow cytometric basophil activation test by detection of CD63 expression in

patients with immediate—type reactions to betalactam antibiotics," *Clinical and Experimental Allergy*, vol. 32, no. 2, pp. 277–286, 2002.

[22] J. Sainte-Laudy, C. Vallon, and J.-C. Guerin, "Analysis of the CD63 membrane expression by human basophil activation. Applications to allergologic diagnosis," *Allergie et Immunologie*, vol. 26, no. 6, pp. 211–214, 1994.

[23] J. J. Pruzansky, L. C. Grammer, R. Patterson, and M. Roberts, "Dissociation of IgE from receptors on human basophils. I. Enhanced passive sensitization for histamine release," *The Journal of Immunology*, vol. 131, no. 4, pp. 1949–1953, 1983.

[24] P. A. Eggleston, "Cockroach allergen abatement: the good, the bad, and the ugly," *Journal of Allergy and Clinical Immunology*, vol. 112, no. 2, pp. 265–267, 2003.

[25] J. C. Gore and C. Schal, "Cockroach allergen biology and mitigation in the indoor environment," *Annual Review of Entomology*, vol. 52, pp. 439–463, 2007.

[26] S. J. Yu and S. W. Huang, "Purification and characterization of glutathione S-transferases from the German cockroach, *Blattella germanica* (L.)," *Pesticide Biochemistry and Physiology*, vol. 67, no. 1, pp. 36–45, 2000.

[27] P. T. Duong and F. N. Chang, "A simple method for assigning multiple immunogens to their protein on a two-dimensional blot and its application to asthma-causing allergens," *Electrophoresis*, vol. 22, no. 10, pp. 2098–2102, 2001.

[28] B. Ma and F. N. Chang, "Purification and cloning of a delta class glutathione S-transferase displaying high peroxidase activity isolated from the German cockroach *Blattella germanica*," *The FEBS Journal*, vol. 274, no. 7, pp. 1793–1803, 2007.

[29] C. H. Huang, L. M. Liew, K. W. Mah, I. C. Kuo, B. W. Lee, and K. Y. Chua, "Characterization of glutathione S-transferase from dust mite, Der p 8 and its immunoglobulin E cross-reactivity with cockroach glutathione S-transferase," *Clinical and Experimental Allergy*, vol. 36, no. 3, pp. 369–376, 2006.

[30] S. Blank, Y. Michel, H. Seismann et al., "Evaluation of different glycoforms of honeybee venom major allergen phospholipase A2 (Api m 1) produced in insect cells," *Protein & Peptide Letters*, vol. 18, no. 4, pp. 415–422, 2011.

[31] L. N. Soldatova, C. Tsai, E. Dobrovolskaia, Z. Marković-Housley, and J. E. Slater, "Characterization of the N-glycans of recombinant bee venom hyaluronidase (Api m 2) expressed in insect cells," *Allergy and Asthma Proceedings*, vol. 28, no. 2, pp. 210–215, 2007.

[32] A. S. Borer, P. Wassmann, M. Schmidt et al., "Crystal structure of Sol i 2: a major allergen from fire ant venom," *Journal of Molecular Biology*, vol. 415, no. 4, pp. 635–648, 2012.

[33] M. D. Tomalski, T. P. King, and L. K. Miller, "Expression of hornet genes encoding venom allergen antigen 5 in insects," *Archives of insect biochemistry and physiology*, vol. 22, no. 3-4, pp. 303–313, 1993.

[34] K. F. A. Langner, D. L. Jarvis, M. Nimtz et al., "Identification, expression and characterisation of a major salivary allergen (Cul s 1) of the biting midge *Culicoides sonorensis* relevant for summer eczema in horses," *International Journal for Parasitology*, vol. 39, no. 2, pp. 243–250, 2009.

[35] H. Shoji, M. Hanawa, I. Shibuya et al., "Production of recombinant mite allergen der fI in insect cells and characterization of products: removal of pro-sequence is essential to IgE-binding activity," *Bioscience, Biotechnology and Biochemistry*, vol. 60, no. 4, pp. 621–625, 1996.

[36] A. S. M. Teo, J. D. A. Ramos, B. W. Lee, N. Cheong, and K. Y. Chua, "Expression of the *Blomia tropicalis* paramyosin Blo t 11 and its immunodominant peptide in insect cells," *Biotechnology and Applied Biochemistry*, vol. 45, no. 1, pp. 17–24, 2006.

[37] Z. Peng, W. Xu, H. Lam, L. Cheng, A. A. James, and F. E. R. Simons, "A new recombinant mosquito salivary allergen, rAed a 2: allergenicity, clinical relevance, and cross-reactivity," *Allergy*, vol. 61, no. 4, pp. 485–490, 2006.

[38] M. D. Chapman, A. M. Smith, L. D. Vailes, L. K. Arruda, V. Dhanaraj, and A. Pomés, "Recombinant allergens for diagnosis and therapy of allergic disease," *Journal of Allergy and Clinical Immunology*, vol. 106, no. 3, pp. 409–418, 2000.

[39] H. Malandain, "IgE-reactive carbohydrate epitopes—classification, cross-reactivity, and clinical impact," *European Annals of Allergy and Clinical Immunology*, vol. 37, no. 4, pp. 122–128, 2005.

[40] A. Pomés, "Relevant B cell epitopes in allergic disease," *International Archives of Allergy and Immunology*, vol. 152, no. 1, pp. 1–11, 2010.

[41] T. Midoro-Horiuti, V. Mathura, C. H. Schein et al., "Major linear IgE epitopes of mountain cedar pollen allergen Jun a 1 map to the pectate lyase catalytic site," *Molecular Immunology*, vol. 40, no. 8, pp. 555–562, 2003.

[42] S. J. Maleki, R. A. Kopper, D. S. Shin et al., "Structure of the major peanut allergen Ara h 1 may protect IgE-binding epitopes from degradation," *Journal of Immunology*, vol. 164, no. 11, pp. 5844–5849, 2000.

[43] M. Schmidt and D. R. Hoffman, "Expression systems for production of recombinant allergens," *International Archives of Allergy and Immunology*, vol. 128, no. 4, pp. 264–270, 2002.