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Immune Mechanisms of Childhood Asthma

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1. Introduction

Asthma is the syndrome defined with chronic airway inflammation and hypersensitivity. Asthma is classified into two phenotypes, atopic with IgE antibodies for specific allergens and nonatopic without IgE antibodies. Unlike adults, 90-95% of pediatric asthma patients exhibit an atopic phenotype (Japanese Society of Pediatric Allergy and Clinical Immunology [JSPACI], 2008). In addition, there are several significant differences between adult and childhood asthma such as duration of disease, extent of lung and immunological development, and duration of inhaled corticosteroid (ICS) use.

The phenotype of airway inflammation is caused by a complex network of various immunocytes; such as T helper 2 (Th2) cells, T helper 17 (Th17) cells, eosinophils, basophils etc (Broide et al., 2011). Typically, it is known that Th2 cells can promote eosinophil activation and IgE production by B cells, while recently Th17 cells have been thought to play a part in exacerbation of asthma, due to their ability to recruit neutrophils following neutrophil activation at an inflammatory site (Molet et al., 2001; Barczyk et al., 2003; Zhao et al., 2008).

Regulatory $CD4^+CD25^+$ T (Treg) cells, which are characterized by their anergy and immune-regulatory functions, can control allergic responses such as airway eosinophilia and airway hypersensitivity. To date, several reports have indicated that reduced numbers of Treg cells or functionally impaired Treg cells are implicated in asthma, rheumatoid arthritis and Kawasaki disease, among others (de Kleer et at., 2004; Furuno et al., 2004; Karlsson et al., 2004; Haddeland et al., 2005., Orihara et al., 2007; Schaub et al., 2008; Ly et al., 2009). Consistent with these reports, our own data suggested that Treg cells from childhood asthma patients were impaired in their suppressive functions (Yamamoto et al., 2011).

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Coincident with the reduced regulatory functions of Treg cells, the ratio of Th17 cells also increased in childhood asthma patient (Yamamoto et al., 2010). Furthermore, single nucleotide polymorphisms of *FOXP3* have been associated with childhood allergy (Bottema et al., 2009).

Treg cells are anergic in both the resting state and after activation by TCR stimulation. Murine Tregs showed a low Ca^{2+} level accompanying their anergic state (Gavin et al., 2002) and our recent human Treg data imply that it the same low Ca^{2+} level accompanies their anergy (Yamamoto et al., 2011). The Ca^{2+} channel on the cell surface of T cells that responds to TCR stimulation is called the calcium release-activated Ca^{2+} (CRAC) channel. We have hypothesized that, in contrast to naïve T cells, the CRAC channel in resting Treg cells may not open easily in response to TCR stimulation and thus the regulation of the CRAC channel may be impaired in Treg cells from asthma patients. This impaired Ca^{2+} regulation in Treg cells may then partly contribute to reduce their regulatory functions.

2. Immune and inflammatory pathology in childhood asthma

Airway inflammation plays a critical role in the pathogenesis of asthma in both adults and during childhood (Warner et al., 1998; Wenzel, 2006; Broide et al., 2011). The immune mechanisms underlying adult asthma derive from the infiltration and activation of immune cells such as eosinophils, mast cells, T cells, basophils and neutrophils, and the activation of parenchymal cells like epithelial cells. It is also generally known that there are different inflammatory phenotypes in adult asthma such as those with a neutrophilic or an eosinophilic predominance (Wenzel, 2006). The airway inflammatory pattern of eosinophilic asthma is characterized by mast cell activation and increasing numbers of activated eosinopils and T cells. Neutrophilic asthma, which is dominanted by neutrophil infiltration and activation in the airways, is related to the severity of adult asthma and steroid-resistant disease (Wenzel et al., 1997). Almost all pediatric asthma has a similar basis to chronic asthma in adults. However, broncho-alveolar lavage cell profiles and induced sputum in childhood asthma revealed increasing numbers of eosinophils and neutrophils compared with controls (Warner et al., 1998). The number of neutrophils in childhood asthma was correlated with the frequency of symptoms and with positive bacterial cultures from the alveolar lavage. In the case of childhood asthma, the increasing neutrophil infiltration appears not to be an exacerbating factor related to disease severity, in contrast to such findings in adult asthma.

Human lung development can be broken down into four prenatal phases including *the embryonic phase* (up to the sixth week of gestation), *pseudoglandular phase* (from the seventh to the sixteenth week of gestation), *canalicular phase* (from the 16th to the 26th week of gestation), and *saccular phase* (from the 24th to the 26th week of gestation) (DiFiore & Wilson, 1994; Jeffery et al., 1998; Bolt et al., 2001). The development persists postnatally as *the alveolar phase,* during the formation of alveoli by 2yrs of age and the further development until adulthood, i.e., suggesting that the respiratory system in children is immature (Schittny et al., 1998; Bolt et al., 2001).

Likewise, development of the immune system is very important in early childhood and a significant body of evidence suggests that antigen reactivity could be initiated by the fetal immune system after approximately 22 weeks of gestation (Jones et al., 1996; Szépfalusi et

al., 2000). Immunotoxin exposure during pregnancy through causes such as maternal smoking, folate intake, heavy metals, antibiotics and environmental estrogens etc is a particular concern at a period from mid-gestation until 2 years after birth (Dietert & Zelikoff, 2008). Furthermore, maternal exposure to allergens can induce the fetus to respond specifically to the allergens at birth (Prescott et al., 1998, 1999); implying that allergen sensitization could be determined prenatally. Both innate and acquired immune responses are still immature in infancy, for instances poor T cell responses due to defective functions of antigen presenting cells (APC) (Delespesse et al., 1998; Levy et al., 2004; Maródi, 2006; Lappalainen et al., 2009). Interestingly, the germ-free status of intrauterine environment favors Th2 responses, and a Th2-skewed response at birth in the human has been demonstrated (Prescott et al., 1998).

Asthma is an inflammatory disease that features a Th2 type immune response caused by inhaled allergens. The immune response is characterized by Th2 type cytokines such as IL-4, IL-13 and allergen-specific IgE. IL-4 and IL-13 play a role in class switching of B cell to produce allergen-specific IgE antibodies that bind to specific receptors on mast cells and basophils. IL-4 also promotes differentiation of naïve T cells into Th2 cells (Robinson et al., 1992; Constant et al., 2000).

Recently, Th17 cells, which are considered to be developmentally distinct from Th1 and Th2 cells, were found to be a subset of Th cells closely connected with the increased prevalence of allergies and asthma (Molet et al., 2001; Laan et al., 2002; Barczyk et al., 2003; Oboki et al., 2008). IL-17 gives rise to production of IL-6, IL-8 and CXCL1 from bronchial fibroblasts or epithelial cells, consequently inducing a positive neutrophil chemotaxis followed by chronic airway inflammation (Kawaguchi et al., 2001; Molet et al., 2001). In some cases, Th17 cells were able to secrete both Th2 and Th17 type cytokines and the cells increased in the peripheral blood from asthma patients (Cosmi et al., 2010). In addition, human eosinophils constantly expressed IL-17- and IL-23-receptors and IL-23 stimulated eosinophils to produce both chemokines (CXCL1, CXCL8, and CCL4) and cytokines (IL-1β, IL-6 and IL17/IL-23) (Cheung et al., 2008).

Our preliminary data have suggested that pediatric asthma patients exhibited a higher frequency of Th17 cells within the peripheral $CD4^+$ T cell population (Yamamoto et al., 2010). Th17 cells appeared during an early stage at the onset of child asthma and the increased frequency of Th17 cells in the peripheral blood could reflect the presence of their symptoms of asthma, but it could not be connected with the severity of asthma. The data implies that early neutrophil infiltration in the airways of children with asthma may be attributed to the high frequency of Th17 cells. In line with our results, polymorphisms of the *IL-17A* gene were also associated with the incidence of pediatric asthma (Wang et al., 2009). In contrast, the presence of elevated numbers of Th17 cells in adult airways was related to the severe type of asthma and the neutrophilic inflammation, such as an occur in steroid-resistant asthma (Zhao et al., 2010). The role of Th17 cells may thus be similar between childhood and adult asthma. Furthermore, a negative correlation between the frequency of Th17 cells and Treg cells was shown in a moderate type of child asthma and in autoimmunity (Bettelli et al., 2006; Yamamoto et al., 2010). Neutrophilic inflammation therefore seems to be ascribed to the increased activation of Th17 cells and the decline in the number and activity of Treg cells.

3. The role of Treg cells in childhood asthma

Allergy is a hyper-immunoresponse to specific antigens that also emerges as a consequence of perturbed immune tolerance. Since immune functions are initiated very early in life, the onset of allergic reactivity also appears before birth. Reflecting such a situation, the cytokine profiles such as the ratio of T helper 1 (Th1)/Th2 and elevated IgE levels in cord blood may predict those individuals who are at risk of developing allergic diseases later in life (Hinz et al., 2010). The germ-free status of the intrauterine environment favors Th2 responses, and a Th2-skewed response at birth in humans has been demonstrated (Prescott et al., 1998). A stronger maternal Th2 immune response has also been connected with childhood wheezing and atopy (Kim et al., 2008). Furthermore, reduced production of the Th2 antagonist IFN-γ during pregnancy has been associated with increasing IL-13 production in the child (Kopp et al., 2001). Maternal cells could cross the placenta and affect the regulation of immune responses after birth (Mold et al., 2008). Taken together, these results suggested that the perturbation of immune tolerance is initiated *in utero* and as a result specific responses to allergens emerge in early in life.

There are several reports implicating reductions in the numbers and functionality of $CD4^+CD25^+$ Treg cells in both human and mouse allergies; although the regulatory ability of Treg cells is still controversial in a mouse model of ovalbumin sensitized airway hypersensitivity (Suto et al., 2001; Hadeiba & Locksley, 2003; Jaffar et al., 2004). As previously noted, many studies have shown that the reduced number and the dysfunction of Treg cells were related with asthma. In adult humans, the regulatory function of Treg cells was reduced in symptomatic hay fever subjects during the pollen season but not in asymptomatic status from the same population outside of the pollen season (Ling et al., 2004). Ca^{2+} signaling is very important for lymphocyte functions and our own data have indicated that impaired Ca^{2+} regulation within $CD4^+CD25^+CD45RO^+$ Treg cells correlated with child asthma symptoms (Yamamoto et al., 2011). We showed that anergy, one of the defining human Treg cell features, is dependent on intra-cellular calcium. Importantly, intra-cellular Ca^{2+} influx in Treg cells identifies those populations that lack anergic status and may have a role in impairing their regulatory functions. Moreover, pulmonary $CD4^+CD25^{high}$ Treg cells were also functionally impaired in childhood asthma (Hartl et al., 2007). A diminished number of Treg cells were also observed in the peripheral blood of children subjects with symptomatic food allergy and atopic dermatitis implicating these cells in yet further allergic reactions (Bellinghausen et al., 2003; Karlsson et al., 2004). It has been proposed that reduced numbers of maternal Treg cells and increased production of Th2 cytokines during pregnancy might play a significant role in enhancing the allergy risk in children (Hinz et al., 2010).

Forkhead box P3 (FOXP3) is a forkhead transcription factor that has been shown to be a master regulator of Treg cell development and functions, and thereby is considered as the one of the most specific markers of Treg populations (despite its transient induction in activated human effector $CD4^+$ T cells). Human naïve and memory T cells can be distinguished by the reciprocal expression of CD45 isoforms (RA^+: naïve, RO^+: memory) (Michie et al., 1992). Human $FOXP3^+CD4^+$ Treg cells in adult peripheral blood are classified into three distinct subpopulations, namely $CD45RA^+FOXP3^{low}$, $CD45RA^-FOXP3^{high}$ and $CD45RA^-FOXP3^{low}$ T cells (Miyara et al., 2009). $CD45RA^+FOXP3^{low}$ and $CD45RA^-FOXP3^{high}$ Treg cells are resting and activated cells respectively, and both cell populations have functional suppressor activity *in vitro*. The $CD45RA^-FOXP3^{low}$ Treg cells are cytokine

secreting non-suppressive cells. $CD45RA^+$ (naïve) subset in $FOXP3^+$ Treg cells from umblical blood is far greater than $CD45RO^+$ (memory) subset, because the fetus receives little stimulation from environmental factors such as bacteria, viruses and allergens (Thornton et al., 2004). We identified two subsets of $CD4^+CD25^+$ Treg cells, $CD45RO^-FOXP3^{low}$ (nearly equal in numbers to the $CD45RA^+$ population) and $CD45RO^+FOXP3^{high}$ (nearly equal in numbers to the $CD45RA^-$ population) T cells in peripheral blood from children (Yamamoto et al., 2011). However, our data indicated that $CD45RO^+$ Treg population did not have the distinctly different FOXP3 expression levels seen in the adult subsets and Ca^{2+} unresponsiveness in the cells seemed to be similar at different FOXP3 expression levels in children. This population seems to be anergic but the suppressive function may vary with FOXP3 expression levels, like the adult Treg cells subsets, since Treg cells with low level FOXP3 remained anergic but their suppressive activities was greatly impaired (Wan & Flavell, 2007). Furthermore, intra-cellular Ca^{2+} concentration in response to TCR activation also seemed to be different between $CD45RO^-FOXP3^{low}$ and $CD45RO^+FOXP3^{high}$ Treg cells from children, suggesting that the two populations were not equally functional. Furthermore, $CD45RA^+$ Treg cells from newborns were reported to lack Treg capability (Ly et al., 2009). That is, it may be different in the functions of $CD45RO^-$ Treg cells in children unlike the adult Treg cells subsets.

What kind of factors impair the functions of Treg populations? Tumor necrosis factor-α (TNF-α) has been reported to contribute to dysfunction of Treg cells and consequent breakdown of immunological self-torelance in Rheumatoid Arthritis (RA) (Nadkarni et al., 2007). TNF-α is one of main causative factors in RA, and anti-TNF treatment (infliximab etc) led to the elevated number of Treg cells and restored the partly impaired suppressive functions. TNF-α was able to inhibit the suppressive activity of $CD45RA^-$ Treg cells, via TNF-α receptor 2 (TNFR2) on their surface, in human RA subjects (Nagar et al., 2010). In childhood asthma, escalation of TNF-α level in allergen stimulated-peripheral blood mononuclear cells (PBMC) and in asthmatic airway has also seemed to be related to the functional insufficiency of Treg cells (Lin et al., 2008). Furthermore, the frequency of $FOXP3^+$ cells in $CD4^+CD25^{high}$ Treg cells in the subjects was significantly reduced and TNF-α treatment *in vitro* compromised the function of Treg cells, which was also associated with increased TNFR2 expression. $CD45RA^+$ Treg cells can promote human Th17 differentiation, which is impaired by TNF-α (Baba et al., 2010), but $CD45RO^+CD25^{high}$ Treg cells inhibit the function of murine Th17 (Bettelli et al., 2006). In childhood asthma, $CD45RO^-FOXP3^{low}$ (nearly equal in numbers to the $CD45RA^+$ population) Treg cells may accelerate Th17 development in addition to impairing the functions of $CD45RO^+FOXP3^{high}$ (equally $CD45RO^+CD25^{high}$) Treg cells.

4. Different mechanisms of determining anergic status in Treg cells

In response to TCR activation, under some conditions, T cells can be led to an unresponsive status termed anergy. Anergic cells do not transcribe the *IL-2* gene or proliferate in response to TCR activation, even in the presence of costimulation (Fathman & Lineberry, 2007; Zheng et al., 2008; Wells, 2009). Generally, when T cells are activated via TCR and CD28 costimulation molecule, phospholipase Cγ (PLCγ), protein kinase Cθ (PKCθ) and Ras can be activated very quickly. Subsequently, the activation of the major signal transduction pathways such as MAPK, JNK, RSK and IκB kinase (IKK) and intra-cellular Ca^{2+} influx are provoked. Finally, transcription factors (NFAT, AP1 and NFκB etc), which are essential for the transcription of *IL-2*, are activated (Kane et al., 2002; Wells, 2009).

The induction of anergy is observed both *in vitro* and *in vivo*. There are several methods for the induction of *in vitro* anergy; including antigen presentation by chemically fixed antigen presenting cells (APC), ionomycin stimulation, anti-CD3 stimulation without co-stimulation, etc (Lamb et al., 1983; Jenkins & Schwartz, 1987). Likewise there are several models of *in vivo* anergy induction, systemic delivery of superantigens, administration of soluble peptide antigen into TCR transgenic mice, etc (Rammensee et al., 1992; Rellahan et al., 1990; Kawabe & Ochi, 1990). Under these conditions, the *in vitro* induced clonal anergic cells produce less IL-2, proliferate poorly, and can be long-lived and stable for weeks if they escape from apoptosis. However, Ca^{2+} influx in these T cells is normal and subsequently induces the activation of nuclear factor of activated T cells (NFAT) when the cells are re-stimulated (Fathman & Lineberry, 2007). Cyclosporin A, an inhibitor of the immediate upstream activator of NFAT, can inhibit the induction of T cell anergy (Jenkins et al., 1990). Since NFAT (the Ca^{2+}-calcineurin signal) promotes *IL-2* transcription and cooperates with Fos/Jun dimers (AP-1) (the CD28-MAPK signal). Mutation of NFAT, which prevents its binding to AP-1, also induces an anergic phenotype (Macián et al., 2002). Excessive calcium-calcineurin-NFAT signaling without AP-1 induces the negative regulatory factors for TCR/CD28 dependent signaling such as the transcription factors, early growth response (Egr) 2, Egr3 and the lipid kinase, diacylglycerol kinase-α (DGK-α) etc (Safford et al., 2005; Zheng et al., 2008). In contrast, *in vivo* adaptive tolerance models showed a defect in TCR-induced calcium influx (Chiodetti et al., 2006). *In vitro* induced anergic cells also showed the same results but a short rest period of 1-2 days after anergy induction resulted in recovery of a normal calcium flux (Gajewski et al., 1994, 1995). As mentioned above, the *in vitro* clonal anergy model showed that Ca^{2+}-calcineurin-NFAT signal functions were normal but the MAPK-AP1 signal was impaired, whereas the *in vivo* adaptive tolerance model showed Ca^{2+}-calcineurin-NFAT signal was significantly impaired. These systems thus operate at quite different mechanisms at the molecular level.

In contrast to effector T cells, $CD4^+CD25^+$ Treg cells produce less IL-2, proliferate poorly, and exhibit a low level of Ca^{2+} influx in response to TCR stimulation (Gavin et al., 2002; Yamamoto et al., 2011). The decline of *IL-2* transcription in Treg cells is mainly attributed to the master regulator FOXP3, which is able to inhibit the function of NFAT by competing with its binding to AP-1 (Wu et al., 2006). Although anergy is one of the key Treg features, the molecular mechanisms by which this phenotype are achieved are quite different from those mentioned above in the *in vitro* clonal anergy and *in vivo* adaptive tolerance models. FOXP3 acts as both a transcriptional repressor and activator, regulating the transcription of a diverse array of target genes (Marson et al., 2007; Zheng et al., 2007). Low level of Ca^{2+} influx in response to TCR activation may be either directly or indirectly regulated by FOXP3. One possible explanation for the low level of Ca^{2+} influx in Tregs is that several molecules involved in the regulation of intra-cellular Ca^{2+} concentration may be controlled by FOXP3. Intra-cellular Ca^{2+} influx, in response to TCR activation, in T cells depends on the CRAC channel, comprising the subunits ORAI1 and STIM1 etc (Zhang et al., 2005; Prakriya et al., 2006). Intra-cellular Ca^{2+} signaling events in T cells are as follows: Engagement of the TCR brings about ZAP70 phosphorylation and is followed by PLCγ1 activation. Activated PLCγ1 cleaves membrane phospholipids into two different second messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 interacts with IP_3Rs (Ca^{2+} channel) on endoplasmic reticulum (ER) and triggers this to release Ca^{2+} from the ER via IP_3Rs. Increasing intra-cellular Ca^{2+} concentration induces Ca^{2+} influx through a pore-forming unit of CRAC channels involving ORAI1 on plasma membrane (Zweifach & Lewis, 1993). Down

regulation of any component of this signaling cascade offers the possibility to reduce the Ca^{2+} influx in Treg cells. Notably, ZAP70 is one of target genes reported to be repressed by FOXP3 (Marson et al., 2007). However, the exact molecular mechanisms of low level of Ca^{2+} influx in response to TCR activation remain obscure in Treg cells.

5. Conclusion

Immunopathology of childhood asthma seems to have little difference in terms of neutrophilic inflammation when compared to the adult disease. Impaired Ca^{2+} regulation in Treg cells from asthma patients appears to compromise their regulatory functions, consequently increasing the number of Th17 cells and neutrophils. Consistent with these data, Ca^{2+} elevation in immunocytes induced by several stimuli also seems to have a key role for aggravation of airway inflammation in asthma. The intra-cellular Ca^{2+} elevation may then result in production of IL-2 via NFAT activation in asthma Treg cells. These data imply that dysregulation of Ca^{2+} unresponsiveness was concurrent with the impaired regulatory functions in Treg cells from asthma patients (Fig.1). Moreover, our Ca^{2+} analysis is a useful tool for the evaluation of Treg functions that proves particularly valuable when only small blood samples are available for study.

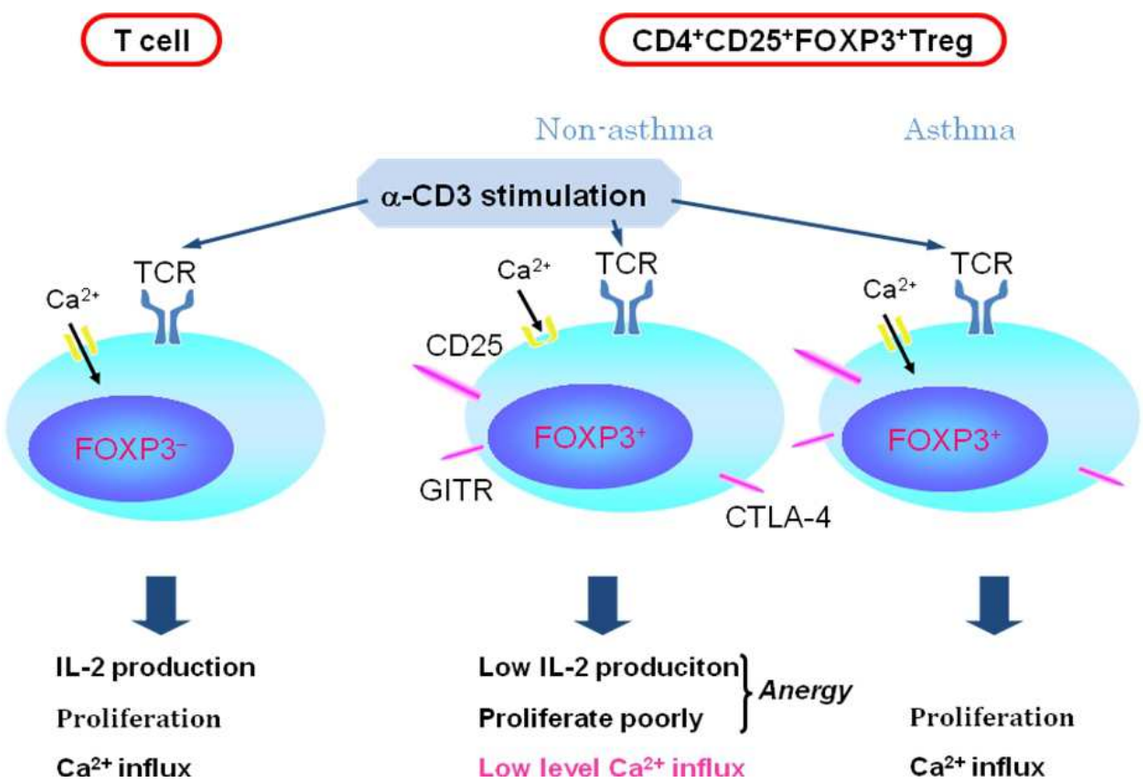

Fig. 1. Characteristics of $CD4^+CD25^+FOXP3^+$ Treg cells.

In response to TCR activation, $FOXP3^-$ T cell (effector T cell, left), $FOXP3^+$ Treg cells from non-asthma (middle) and from asthma (right) are compared in terms of anergy status (low IL-2 production and proliferation poorly) and Ca^{2+} response.

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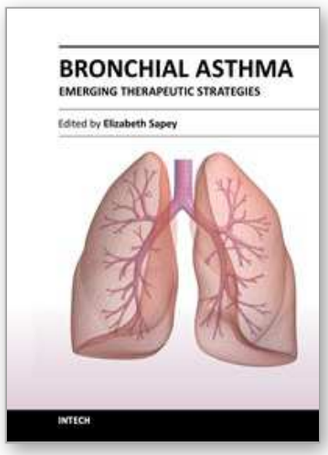

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Asthma remains a serious health concern for millions of people globally. Despite continuing research interest, there have been few advancements that impact clinically on patient care, potentially because asthma has been treated as a homogeneous entity, rather than the heterogeneous condition it is. This book introduces cutting-edge research, which targets specific phenotypes of asthma, highlighting the differences that are present within this disease, and the varying approaches that are utilized to understand it.

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