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T2-HIGH AND T2-LOW SEVERE ASTHMA: ROLE OF BASOPHIL ACTIVATION TEST (BAT)

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1. ABSTRACT

Allergic disorders are one of the most common chronic diseases worldwide. Skin prick test and the measurement of immunoglobulin (Ig)E antibody are routinely used for the diagnosis of allergic disorders. However, skin prick test and serum IgE detect only sensitization but do not prove the clinical manifestations of allergic disease. Allergic disorders (i.e., asthma, allergic rhinitis, urticaria, atopic dermatitis, anaphylaxis) include a variety of immediate IgE-mediated hypersensitivity reactions. They are characterized by the abnormal reaction to allergens, which, in sensitized subjects, induce IgE-mediated degranulation of human basophils and mast cells with the release of several proinflammatory mediators. The basophil activation test (BAT) mimics *in vitro* the allergic reaction and has manifold advantages as diagnostic test: safety profile, sensitivity/specificity, and the potential to predict the severity of an allergic reaction. There is some evidence that BAT allows the discrimination between sensitized asymptomatic subjects and truly allergic individuals and has the potential to replace expensive and risky *in vivo* allergen bronchoprovocation tests The aim of our study was to evaluate, in two groups of severe asthmatic patients (T2-high and T2-low), the ability of anti-IgE and of the *Staphylococcus aureus* enterotoxins to induce the activation of peripheral blood basophils, using the BAT. This technique employes flow cytometry to demonstrate *in vitro* the degranulation of basophils from asthmatic patients and control subjects following the activation with different stimuli. Peripheral blood basophils are identified as $CRTH2^+$ and $CD3^-$ cells.

The activation of basophils was evaluated through the expression of CD203c. Our results indicate that anti-IgE induced CD203c basophil expression is increased in T2-high and T2-low asthmatics compared to healthy controls. Staphylococcal enterotoxins can induce the activation of basophils only in a percentage of patients with severe asthma.

2. INTRODUCTION

Allergic disorders (bronchial asthma, allergic rhinitis, atopic dermatitis, urticaria, and anaphylaxis) are immune-reactions characterized by hypersensitivity reactions in IgE-sensitized subjects (**Holgate 2015**). IgE antibodies are present in low concentration in serum and are directed against usually harmless antigens, also called allergens. These substances can induce the production of IgE by plasma cells (**Hammad 2021**). Subsequent exposure to the sensitizing allergen, triggers an immune response involving circulating basophils and tissue mast cells (**Spadaro 2020**). This immunologic event can cause a local reaction or systemic manifestations (e.g., asthma and anaphylactic shock) (**Varricchi 2019a**).

Allergens are small molecular weight which can induce the synthesis of specific IgE. Among allergens, there are a variety of proteins from pollens, polysaccharides, lipids and bacterial or viral proteins (**Varricchi 2019b; Marone 2020; Cristinziano 2021**).The presence of antigenic determinants with molecular and steric conformation complementary to specific antibodies gives the molecule antigenicity. Allergens are at least bivalent and in many cases multivalent. The *in vivo* antigenicity of a molecule depends on its molecular weight, its structure, the method of introduction in the body, the quantity and the number of exposures (**Rich 2018**).

Typical feature of bronchial asthma is a variable airflow limitation associated with symptoms such as dyspnoea, cough, wheezing and chest tightness (GINA 2021). The extreme heterogenicity of the immunologic disorder is reflected in

different phenotypes that differ in etiology, pathogenic mechanisms, symptoms, and severity (**Wenzel 2021**). Based on the type of inflammation, asthma can be subdivided into type 2-high (T2-high) and -low (T2-low) (**McGregor 2019; Wenzel 2021**).

Around 20–30% of the general population are persistently colonized with *Staphylococcus aureus* (*S. aureus*) on their nasal mucosa (**Mehraj 2016**). An association of *S. aureus* colonization with several chronic inflammatory diseases of the airways such as allergic rhinitis, allergic rhinitis with nasal polyps (CRSwNPs), and allergic asthma has been described. The nasal mucosa of 44% of perennial allergic rhinitis (AR) patients is colonized with *S. aureus* (**Shiomori 2000**). In inferior turbinate biopsies of allergic rhinitis patients, *S. aureus* is significantly more abundant in IgE-high than in IgE-low patients (**Hyun 2018**). Also, 67% of CRSwNP patients with comorbid asthma are colonized with *S. aureus* (**Van Zele 2004; Tomassen 2016**). Asthma comorbidity occurs in < 20% of patients with CRSsNP, but up to 71% of CRSwNP patients with high levels of staphylococcal enterotoxin-specific IgE (SE-IgE) have asthma comorbidity (**Van Zele 2004; Tomassen 2016**).

S. aureus can synthesize and release a variety of proteins including staphylococcal enterotoxin A (SEA), SEB, SEC, and toxic shock syndrome toxin-1 (TSST-1). A link between SE-IgE formation and asthma severity was reported in approximately 60% of severe asthma patients having SE-IgE, while only 13% of healthy control subjects are SE-IgE positive (**Bachert 2012**). SE-IgE positively correlates with lower forced expiratory volume (FEV_1), higher oral glucocorticoid

use, and more hospitalization (**Bachert 2012**).

Specific IgE against SEA has been shown to be significantly associated with uncontrolled asthma compared with SEA-negative asthma patients (**Tanaka 2015**). These findings emphasize an increased prevalence of SE-IgE among both adults and children with asthma, compared to the general population (**Akinbami 2015**). Nasal *S. aureus* carriage was identified as a risk factor for asthma in adolescents (**Davis 2015**). In teenagers, SE-IgE correlates with atopy, asthma, and bronchial hyperreactivity. In elderly asthmatics, high levels of SE-IgE are associated with more severe asthma and sputum eosinophilia, than those with lower SE-IgE levels (**Song 2016**).

Several studies have identified a positive associations between staphylococcal enterotoxin-specific IgE (SE-IgE) sensitization and asthma prevalence and severity in adult populations (**Tomassen 2013; Song 2014; Bachert 2012; Song 2016**). The relevance of these associations is supported by the functionality of SEB-IgE (**Zhang 2011; Altrichter 2018**). Allergen-specific IgE sensitization and allergic response require repeated allergen exposure; these findings indicate that SA exposure may play a role in SE-related allergic diseases, including asthma. Two recent studies have reported a positive relationships between nasal *S. aureus* colonization and asthma prevalence (**Davis 2015; Sorensen 2016**).

S. aureus enterotoxin B also stimulates the production of interleukin-5 (IL-5) and induces polyclonal IgE production, which might contribute to severe inflammation *via* the activation of mast cells (**Bachert 2008; Gould 2007**). IgE

antibodies specific to the *S. aureus* superantigens are present in nasal polyp tissue, and their levels correlate with markers of eosinophil activation and recruitment (**Matsui 2007**).

Clinically, nasal *S. aureus* or serum IgE to *S. aureus* toxins are associated with wheeze, asthma, or both in children and adults (**Davis 2015; Bachert 2012; Semic-Jusufagic 2007**). Finally, the presence of *S. aureus* or IgE to *S. aureus* toxins is related to asthma severity (**Bachert 2012; Semic-Jusufagic 2007: Uong 2017**) poor asthma control (**Tanaka 2015**), and greater prevalence of aeroallergen sensitization (**Uong 2017**). IgEs specific to *S. aureus* enterotoxin A (SEA), SEB, SEC, and TSST-1 have been reported in the serum of both allergic rhinitis and asthma patients at higher incidence than in normal controls and specific IgE levels were correlated with disease severity (**Bachert 2003; Rossi 2004; Van Zele 2004; Pastacaldi 2011**). Collectively, these studies suggest that *S. aureus* and its products can be associated with a variety of allergic disorders.

2.1 TYPES OF IMMUNE REACTIONS

Immune reactions are classified into four types based on the different effector cells.

Type I reactions. These hypersensitivity reactions are caused by the cross link between a multivalent antigen and the specific IgE linked to high affinity IgE receptors (FcεRI) on the membrane of peripheral blood basophils and tissue mast

cells (**Varricchi 2018**). Figure 1 schematically illustrates the pathogenic mechanism of type I allergic reactions. Allergens engage the B cell receptor on B lymphocytes activated by IL-4 produced by Th2 cells and basophils. B cells differentiate to IgE secretory plasma cells to produce specific IgE against the allergen. IgE binds with high affinity to FcεRI on human mast cells and basophils. When the FcεRI receptors are cross linked by the antigen interacting with two IgE on the cell surface, a series of metabolic signals are generated allowing the release of preformed granules containing histamine and other inflammatory mediators (**Varricchi 2021a**). These proinflammatory mediators (histamine, tryptase, cysteinyl leukotriene C_4, prostaglandin D_2, etc.) activate specific receptors on target tissues (e.g., smooth muscle cells, blood vessels, mucous glands, sensory nerve endings) and several immune and non immune cells. Type I IgE-mediated reactions are called immediate because the manifestations appear within one hour of contact with the allergen (Fig. 2A). Bronchial asthma, allergic rhinitis, anaphylaxis, and urticaria are considered type I allergic reactions (**Spadaro 2020**).

Type II reactions (antibody-dependent cytotoxicity).

Type II reactions are cytotoxic reactions involving the binding of IgG or IgM antibodies to antigens bound to immune cells. The antigen-antibody complex activates the complement and the destruction of target cells (Fig. 2B).

Type III reactions (immune complexes).

These reactions are induced by the formation of soluble immune complexes formed by IgG-bound to specific antigens. This event occurs when there is locally or in peripheral blood an excess of antigen compared to the specific antibody. These immune complexes activate the complement system inducing the formation of anaphylatoxins (C3a, C5a). These molecules induce an inflammatory response that damages the tissue in which the reaction occurs, and compromises the physiological functions (Fig. 2C).

Type IV reactions (cell mediated).

These reactions are not mediated by antibodies but are caused by the products of antigen-specific effector cytotoxic T cells. The antigen is bound directly by sensitized T lymphocytes, with the release of several cytokines and the delayed reactions mediated by T lymphocytes (**Parham 2001; Nagao-Dias 2009**) (Fig. 2D).

3. BRONCHIAL ASTHMA

Bronchial asthma is a chronic inflammatory disorder that clinically manifests with wheezing, dyspnoea and chest tightness (**Holgate 2015**). Asthma is characterized by bronchoconstrinction mainly due to several chemical mediators (histamine, cysteinyl leukotrienes, prostaglandin D_2: PGD_2), leading to bronchial hyperreactivity and airway remodeling (**Pelaia 2021**). The latter aspect is a fundamental feature of asthma due to the involvement of several inflammatory (mast cells, eosinophils, macrophages, neutrophils) and structural cells (fibroblasts, airway smooth muscle cells) (**Ito 2019; Mostaco-Guidolin 2019; Yap 2019; Liu 2021**). Currently, it is believed that asthma is not a single nosological entity, but represents a heterogeneous complex of multiple clinical and pathobiological phenotypes also characterized by different responses to drug treatments (**Wenzel 2021**).

3.1. TYPE 2-HIGH AND TYPE 2-LOW ASTHMA

The majority of asthmatic patients achieve a good symptom control with standard treatments including inhaled glucocorticoids (ICS), $beta_2$ adrenergic bronchodilators, and leukotriene inhibitors (GINA 2021). However, despite optimal inhalation therapy, in more than 10% of patients the therapy does not control adequately the disease resulting in frequent exacerbations (**Mukherjee 2020**). Moreover, the severity of asthma in these "difficult-to-treat" patients is often furtheraggravated by the coexistence of one or more comorbidities such as chronic rhinitis, atopic dermatitis, chronic urticaria, and obesity.

Based on the type of inflammation, asthma can be subdivided into type 2-high (T2-high) and -low (T2-low) (**McGregor 2019; Fitzpatrick 2020; Wenzel 2021**). A further distinction is made between eosinophilic and non-eosinophilic asthma (Fig. 3) (**Carr 2018; Heaney 2021**)**.**

The pathophysiological process leading to the development of T2-high asthma is characterized by the release of several inflammatory and immunoregulatory mediators produced by cells of the innate and adaptive immune system. In this condition, immunologic stimuli (e.g., allergens, viral and bacterial superallergens) activate primary effector cells of allergic disorders (i.e., mast cells, basophils) through the engagement of specific IgE to release a plethora of interleukins (ILs) such as IL-3, IL-4, IL-5, IL-13, IL-33 and thymic stromal lymphopoietin (TSLP) (**Bartemes 2012; Fahy 2015**)**.** Eosinophils and their mediators contribute to the pathogenesis of both allergic and eosinophilic asthma (**Kuang 2018**).

T2-low asthma (T2-low) is a heterogeneous and incompletely characterized disorder, presumably including different phenotypes involving the activation of mast cells, macrophages, neutrophils or a mixture of these immune cells (**Fitzpatrick 2020; Wenzel 2021**). Figure 3 illustrates the different phenotypes of severe asthma. At the present time, definitions for T2-low asthma have not been established. For the purpose of this study, T2-low asthma is defined loosely as asthma in the absence of prominent T2-pathway signatures. Although the original studies of T2-high asthma relied on epithelial gene expression signatures to define immune phenotypes (**Hekking 2017; Kuo 2017**), bronchoscopy is not clinically indicated in patients with

asthma and epithelial-based definitions are therefore not practical in the real-world setting. Some emphasis has also been placed on noninvasive biomarkers for the detection of T2-high asthma (i.e., exhaled nitric oxide, blood eosinophils, or sputum eosinophils). However, the exact cutoff points for these markers are debatable (**Shah 2019**). These T2 markers may also have little concordance in the same patient (**Hastie 2013; Haughney 2016**) and may be subject to variability over time and in response to asthma treatments (**Mathur 2016**). There is also the possibility that T2-low asthma could result from suppression of T2-high asthma by glucocorticoids.

T2-low asthma is typically associated with a later age of onset, higher medication requirements, and more severe symptoms (**Haldar 2008; Hastie 2010; Holguin 2011; Modena 2014**). Patients with T2-low asthma tend to have a poorer response to short-acting bronchodilators (**Ntontsi 2017; Tliba 2019**). Obesity has also been associated with T2-low phenotypes of asthma (**Peters 2019**), which may worsen symptoms and lung function (**Dixon 2011**). Patients with T2-low asthma also have a poor response to ICS and systemic glucocorticoids (**Woodruff 2007**).

Airway and peripheral blood neutrophilia may also be a feature of some patients with T2-low asthma, but this feature has not been consistently reproduced. Cutoff points for airway neutrophils have not been established and depend on the compartment measured (i.e., biopsy tissue, bronchoalveolar lavage, or induced sputum). Indeed, various cutoff points for airway neutrophilia have been used in the literature, with a broad range (**Samitas 2017**). The consistency of airway neutrophils over time is also less than that of eosinophils (**Kupczyk 2014**), and intensive

glucocorticoid treatment may alter the number of circulating neutrophils (**Cox 1995; Zhang 2001**). Many patients with elevated airway neutrophils also have abundant airway eosinophils and other T2-high features. There is some evidence that even peripheral blood neutrophilia could represent a biomarker of T2-low asthma (**Varricchi 2021b).**

Severe asthma is defined as asthma which is not well controlled despite the administration of high-dose drug therapy. Approximately 3-10% of patients with bronchial asthma have severe asthma, which according to the latest GINA recommendations falls into the categories indicated as steps 4 and 5 (GINA 2021) (**Stirling 2001; Chung 2014; Israel 2017**). Figure 4 illustrates some of the immunologic and histologic characteristics of a healthy bronchus and a bronchus of a patients with severe asthma.

3.2. ROLE OF BASOPHILS AND MAST CELLS IN BRONCHIAL ASTHMA

There is compelling evidence that basophils and mast cells are the primary effector cells of allergic reactions because they express on their surface the high affinity receptor for the Fc portion of the IgE (FcεRI) (**Varricchi 2018**). IgE binds with high affinity to the α chain of FcεRI on the basophil/mast cell membrane. When sensitized basophils/mast cells are re-exposed to a specific allergen, there is the formation of cross-links between specific IgE and antigens (allergens), which induce cell activation and the release of specific granules containing histamine and other

inflammatory mediators (Fig. 5) (**Varricchi 2018**). Figure 6 illustrates the specific mediators that are released from human basophils and mast cells (**Varricchi 2018**). The degranulation process occurs when two neighboring IgE, bound to FcεRI receptors, bind the same allergen. This results in the immediate (≅ 1 h) release of preformed mediators stored in the cytoplasmic granules of human basophils (e.g., histamine, basogranulin) and mast cells (e.g., histamine, tryptase, chymase). At the same time, the effector cells synthesize newly formed lipid mediators (e.g., cysteinyl leukotriene C_4: LTC_4 and/or prostaglandin D_2: PGD_2), which significantly contribute to the inflammatory response (Schulman 2001).

The pivotal role of basophils in allergic disorders is also supported by the results of several recent studies indicating that these cells are a major source of Th2-like (IL-4, IL-13) cytokines (**Redrup 1998; Patella 2000; Genovese 2003; Rivellese 2014**). In particular, IgE cross-linking can induce the release of IL-4, a major cytokine controlling the production of IgE (**Genovese 2003; Patella 2000; Patella 1998; Redrup 1998**). Even more important for the pathogenesis of severe asthma is the production of IL-13, because this cytokine is involved in airway remodeling (**Marone 2019**). Several groups have demonstrated that IgE cross-linking induces the release of IL-13 from human basophils and mast cells (**Genovese 2003; Patella 2000; Redrup 1998**).

FcεRI on human basophils and mast cells is expressed as a tetramer ($\alpha\beta\gamma 2$); the presence of β chains amplifies the intracellular signal transduction. The density of FcεRI on human basophils is closely correlated to the concentrations of free IgE in

the serum (**Malveaux 1978**). The extracellular portion of α chain binds the Fc portion of IgE; α chain consists of an extracellular domain, a transmembrane domain and a small cytoplasmic domain. The β subunit, on the other hand, has four transmembrane domains and a single immunoreceptor motif that is activated at the level of a tyrosine (ITAM - *Immunoreceptor tyrosine-based activation motif*), and is associated with the Lyn kinase. Finally, the γ subunit forms a dimer through a disulfide bond, and each subunit contains an ITAM. After the aggregation of FcεRI, caused by the binding of IgE with specific antigens, Lyn phosphorylates the tyrosines of the ITAM domains of the β and γ subunits. The phosphorylation of the tyrosines of the γ subunit leads to the recruitment of the Syk kinase, whose signaling is associated with the activation of mast cells and basophils that release histamine present in the cytoplasmic granules. This complex sequence of events triggers the allergic reaction (**Stone 2010**).

Figure 6 also illustrates the constellation of mediators that are specifically released by human mast cells and basophils. It appears that there are common mediators released by human basophils and mast cells, whereas there are several mediators selectively secreted by human basophils and mast cells.

3.3. DIAGNOSIS OF ALLERGIC DISORDERS

The first step towards the diagnosis of allergic disorders must be aimed to distinguish between IgE- and non IgE-mediated diseases. Subsequently it is necessary to identify the allergen responsible for the activation of basophils and mast

cells, in order to implement effective prevention or pharmacologic or immunologic therapies. The *in vivo* identification of clinically relevant allergens is frequently problematic. In fact, there are a plethora of molecules with allergenic capacity that are not clinically relevant. All foreign substances with a high molecular weight that meets the immune system in the body can potentially trigger a hypersensitivity response. A large number of substances present in the environment or contained in food, are potentially immunogenic, but not necessarily clinically relevant.

From a diagnostic point of view, the patient's clinical history is of fundamental importance for the diagnosis of allergic reactions. Moreover, the family history, the pathological anamnesis are also important. The skin prick tests are routinely used to identify type I hypersensitivity reactions. The *skin prick test*, introduced in clinical practice several decades ago, is performed by pricking the flexor surface of the upper limbs with a sterile lancet after placing a drop of the allergen to be tested; in case of positivity, the formation of a wheal and flare is observed within 10-20 minutes. This skin reaction usually disappears within a couple of hours.

The size of the wheal depends on the local concentration of histamine and possibly other mediators released from skin mast cells and reflects the "releasability" of the skin mast cells. The releasability of primary effector cells depends on genetic factors (**Marone 1986a**), the age of the patients (**Marone 1986b**) and presumably the density of IgE on skin mast cells. In addition, the non-specific sensitivity of the skin (dermographism) can influence the size of the wheal.

It is commonly considered that a reaction with a diameter less than 2 mm indicate a negative result; each skin reaction should be compared with the skin response caused by the negative (the solution without the allergen) and the positive controls (the solution containing histamine). Skin prick tests have several practical advantages: they can be easily performed, several allergens can be tested at the same time, and are relatively inexpensive. However, there are also disadvantages because this is an *in vivo* procedure and the interpretation of results is subjective and depends on the experience of the operator. In addition, in subjects with certain skin disorders (e.g., atopic dermatitis, psoriasis, etc.) the skin tests cannot be easily performed and/or interpreted.

The *patch test* is used for the diagnosis of contact dermatitis (type IV hypersensitivity). Patch tests are performed by exposing the skin to an allergen in the form of a lipophilic preparation in order to simulate the infiltration of immune cells that are responsible for this hypersensitivity reactions. This technique evaluates a possible delayed reactions that usually last few days. The assessment is based on the presence of papules, vesicles or simple erythema (**Rich 2018**).

It is important to emphasize that these diagnostic approaches sometimes lead to results that disagree with the clinical evaluation. Therefore, *in vitro* diagnostic tests are useful to distinguish clinically irrelevant sensitization from truly allergic individuals. The absolute number of eosinophils in peripheral blood is a useful biomarker to identify asthmatic patients with hypereosinophilia (**Bagnasco 2017; Nair 2009**). However, the increase of peripheral blood eosinophils can also occur in

several non allergic conditions, such as parasitic infections (**Aceves 2020**), vasculitis, and solid and hematologic tumors (**Galdiero 2017**). Therefore, eosinophilia must be considered as a biomarker of hypereosinophilic asthma with caution (**Haldar 2009; Chupp 2017**).

The *in vitro* histamine release from peripheral blood is an excellent test used mainly in research (**Marone 1986a; Marone 1986b**). This technique is extremely accurate and essential for *in vitro* studies of allergic reactions (**Spadaro 2020**). However, this procedure has several limitations that should be pointed out. First, it requires a sample (≅ 20 ml) of fresh peripheral blood from the subject; this technique is rather complex and the measurement of histamine can be performed by expensive and/or complicated fluorometric or ELISA techniques (**Marone 2020**). Finally, this procedure is rather expensive and time consuming. It is important to remember that the release of histamine and other inflammatory mediators from human basophils are essentially modulated by a series of intrinsic metabolic pathways collectively defined basophil releasability (**Marone 1986a**). Thus, this technique is presently used only in specialized laboratories and for experimental purposes.

The measurement of total IgE in serum can be easily done in most laboratories of Clinical Pathology. Normal values are difficult to define because they depend on ethnic characteristics, environmental conditions, and age of donors. In normal subjects, there is a wide variations in the normal range of serum IgE (< 100 IU). Therefore, the presence of relatively high concentrations of serum IgE can be found in different types of subjects.

The measurement of IgE can be done with different methods: the most used is the PRIST (*Paper Radio Immuno Sorben Test*). This analytical test is performed by using a solid phase consisting of paper discs on which anti-IgE antibodies are covalently linked, with formation of the immune complex anti-IgE-IgE bound to the solid phase. After the elimination of the excess of the reagents, this complex binds to a second anti-IgE antibody, which constitutes the detection system of the reaction.

Atopic subjects generally have high concentrations of total IgE (> 100 IU). High serum concentrations of IgE tend to increase the number of FcεRI on human basophils and mast cells (**Malveaux 1978**). However, high concentrations of IgE are not necessarily correlated to clinical symptoms of allergic reactions.

IgE specific for an allergen can be measured by the RAST technique which uses a labeled antibody and a solid phase on which the allergens are chemically linked. The initial phase of the assay involves the extraction of the isotypes of all allergen-specific antibodies by the allergen adsorbent, while the non-specific ones remain in solution. After a washing phase with buffer to remove the latter and the components of the serum that have not reacted, a second incubation is carried out

with an excess of anti-IgE labeled with I^{125} or with an enzyme, with the formation of a complex in which the specific IgE are bound to "sandwich". This technique allows to measure the number of IgE molecules specifically directed against an allergen. This is a specific and sensitive technique but it is rather expensive.

3.4. BASOPHIL ACTIVATION TEST (BAT)

Diagnostic approaches of allergic disorders are also based on the functional evaluation of peripheral blood basophils, the cells clinically relevant in the pathogenesis of immediate type I IgE-mediated reactions (**Boumiza 2005; Longo 2008; Peternelj 2009**).

Flow cytometry allows to recognize the activation of basophils by evaluating the expression levels of CD203c (**Santos 2015**). This protein is present on the plasma membrane and it is overexpressed following basophil activation (i.e., IgE cross-linking) with anti-IgE or a specific allergen. The expression of CD203c reflects the percentage of activation and is correlated to the extent of the reaction (**Erdmann 2004; Gonzales-Munos 2008; Ebo 2008; Santos 2015**).

In order to evaluate the quantitative expression of CD203c, peripheral blood is incubated with three different monoclonal antibodies, anti-CHTR2, anti-CD203c and anti-CD3. CHTR2 is expressed on the surface of basophils, eosinophils, and monocytes. CD3 is a specific marker for T lymphocytes. Basophils are identified by events positive for CHTR2 but negative for CD3. In this population we examine the expression of CD203c, a marker of basophil activation (**Nagao-Dias 2009; Boumiza 2005; Peternelj 2009; Gonzales-Munos 2008; Ebo 2008; Erdmann 2004; Monneret 2010; Korosec 2009**).

This method, known in the literature as Basophil Activation Test (BAT) (**Hemmings 2018**), has been used for the *in vitro* diagnosis of food allergy to peach (**Palacin 2010**), cow's milk (**Rubio 2010**), and beef (**Joo-Hee 2010**). BAT has also

proved to be useful in the diagnosis of aspergillosis (**Gernez 2016; Mirkovic 2016**), allergic reactions to certain drugs such as quinolones (**Aranda 2010**), non-steroidal anti-inflammatory drugs (**De Weck 2009a**), and β-lactam antibiotics (**De Weck 2009b; Sanz 2002**). It should be noted that this method has several limitations because it recognizes IgE-mediated allergic reactions and not other different pathogenetic mechanisms such as complement activation or direct activation of basophils.

4. AIMS OF THE STUDY

In this study we wished to characterize immunologically T2-high and T2-low asthmatics compared to healthy controls enrolled in this study. We also evaluated the serum concentrations of IgE specific to *Staphylococcus aureus* (*S. aureus*) enterotoxin (SEs) in the three groups of subjects examined. Finally, we evaluated the ability of the SEs to induce the activation of circulating basophils obtained from healthy donors compared to severe asthmatic patients by using the BAT technique.

Several studies have indicated that several proteins synthesized and released by *S. aureus* are involved in the pathogenesis of allergic disorders (**Bachert 2008; Bachert 2012**). The BAT method evaluates the percentage of basophil activation by examining the expression of CD203c, which is normally present at low level on the basophil membrane and is overexpressed following basophil activation with a specific antigen or anti-IgE. In this study, basophils were incubated *in vitro* with the SEs which eventually reacts with IgE bound to FcεRI receptors leading to their activation. Basophil activation was measured as a percentage of basophil expression of CD203c.

A cohort of healthy donors and severe asthmatic patients (T2-high and T2-low) underwent spirometry, prick test for inhalant allergens, ENT examination with rhinofibroscopy, complete peripheral blood count, total IgE and specific IgE against staphylococcal enterotoxins type A and B. Peripheral blood basophils were examined for their response to staphylococcal enterotoxins type A (SEA), SEB, and toxic shock syndrome toxin (TSST-1).

5. MATERIALS AND METHODS

5.1. STUDY POPULATION

The study was conducted in accordance with Good Clinical Practice (GCP) guidelines and adhered to the Declaration of Helsinki II. All participants provided written informed consent to participate in the study. 51 patients with characteristics of severe asthma, according to GINA 2021 classifications, and 30 healthy controls (18 females and 12 males) were enrolled in this study. 30 patients were T2-high asthmatics (19 females and 11 males) and 21 subjects were T2-low asthmatics (12 females and 9 males). Asthmatic patients were followed in the Outpatient Clinic of the Department of Respiratory Diseases of the University of Naples L. Vanvitelli of the Azienda Ospedale dei Colli and the Outpatient Clinic of the Division of Allergy and Clinical Immunology of the University of Naples Federico II. Thirty healthy volunteers served as controls.

Patients aged between 19 and 78 years. 20% of healthy controls were ex smokers, whereas 28.6% of T2-high asthmatics and 33.3% of T2-low asthmatics were ex smokers. All patient underwent spirometry, prick test for inhalant allergens, ENT examination with rhinofibroscopy, peripheral blood count, total IgE, specific IgE for staphylococcal enterotoxins type A (SEA), SEB, SEC, and TSST-1, and BAT for the SEA, SEB, and TSST-1. The inclusion criteria included an age over 18 years and less than 79 years, a diagnosis of severe asthma according to ERS/ATS 2014

criteria (**GINA 2021**). Key exclusion criteria were acute and chronic infections, bronchiectasis, primary and secondary immunodeficiencies, autoimmune diseases, malignancies, cystic fibrosis, patient-reported smoking history or the onset of respiratory symptoms after the age of 40 years in current or previous smokers with a smoking history of at least 10 pack-years. None of the asthmatic patients has been or was treated with allergen-specific immunotherapy or monoclonal antibodies anti-IgE, anti-IL-5/IL-5Rα or anti-IL-4Rα. The majority of patients (48/60) were treated with daily low-dose of inhaled glucocorticoids (ICS) therapy [fluticasone propionate (FP), 100–200 μg or equivalent] plus two additional controllers (e.g., a long-acting β2-agonist and/or leukotriene receptor antagonist and/or long-acting muscarinic agonist. As a control, we investigated 30 healthy non-allergic subjects. Patients with T2-high asthma were characterized by increased serum IgE, blood eosinophilia, and robust improvement in FEV_1 after the initiation of inhaled glucocorticoids (**Fitzpatrick 2020**). For the purpose of this study, T2-low asthma was defined as asthma in the absence of prominent T2-pathway signatures previously described.

The following parameters were evaluated: the on-treatment forced expiratory volume in 1 second (FEV_1, in liters); the score of the Asthma Control Test (ACT; the mean score of five questions that assess asthma symptoms, use of rescue medications and the effect of asthma on daily functioning during the previous 4 weeks). Pulmonary function test (Quark PTF, COSMED, Pavona di Albano, Italy) was performed in accordance with the American Thoracic Society/European Respiratory Society (ATS/ERS) guidelines. FEV_1, Forced Vital Capacity (FVC), FEV_1/FVC

were measured and the best of three forced maneuvers was recorded. Results were expressed both as absolute values and as a percentage of the predicted values referred to ERS reference values **(Fahy 2015).** The height and weight of patients were measured using standard techniques and the Body Mass Index (BMI) was calculated as body weight (kg)/height^2 (m^2) (**Vural 2006**). Peripheral blood leukocyte counts were measured using an automated hematology analyzer (**Varricchi 2021b**). Serum samples from venous blood were stored in aliquots at −80°C until tested. The serum IgE and IgE agonist SEs were measured with ImmunoCap system (ThermoFisher, Monza, Italy).

5.2. BAT METHOD

The BAT test used in this study is the Allergenicity Kit, Cellular Analysis of Allergy (Beckman Coulter FC500). This method uses as a marker CD203c, which is normally present on the basophil membrane and overexpressed following their activation (**Bavbek 2009; Salter 2016**). In order to select the cell population in which to evaluate the expression of CD203c, other markers used were CRTH2 and CD3. CRTH2 is expressed on the membrane of eosinophils, basophils, and monocytes, whereas CD3 is selectively found on T lymphocytes. Human basophils were identified by events positive for CHTR2 and negative for CD3. Peripheral blood from patients with severe asthma and from controls was tested for a negative

control (buffer), a positive control (anti-IgE), SEA, SEB and TSST-1. A sample (25 µL) of heparinized whole blood was incubated (15 minutes at 37°C) with:

- 5 µl of MoAbs (CRTH2-FITC, CD203c-PE, CD3-PC7).
- 25 µl of activation solution.
- 10 µg/ml of *S. aureus* enterotoxins.

After incubation, a stop solution (25 µl) was added and the cells were fixed (Fix-and-Lyse mixture). Cells were maintained at 22°C for 15 minutes, centrifuged (3500 rpm, 5 minutes) and the supernatants was discarded. 200 µL of final solution (Fix-and-Lyse mixture) was added. An anti-IgE antibody was used as a positive control by binding the Fc portion of the IgE attached to the FcεRI receptor on the basophil membrane. The negative control was incubated in the presence of buffer alone.

Initial experiments were carried out to optimize the execution of the test in terms of both efficacy with increasing concentrations of anti-IgE, SEA, SEB, and TSST-1 (data not shown). In initial experiments, we used increasing concentration of the stimuli to establish the optimal concentrations of the stimuli. Similar experiments were performed to establish the optimal incubation time of basophils with different stimuli.

5.3 FLOW CYTOMETRY

Samples were analyzed by flow cytometry with a FACS COULTER FC500 (CXP Cytometry List Mode Data Acquisition & Analysis software) (Beckman Coulter Inc). The gating strategy consisted in identifying the basophils by a two-dimensional histogram in a specific area excluding lymphocytes and monocytes. The basophil population was enriched by co-expression of the CRTH2 and CD203c markers. Resting basophils were identified as $CRTH2^{+}$ $CD203c^{dim}$ $CD3^{-}$. Activated basophils were identified as $CRTH2^{+}$ $CD203c^{bright}$, $CD3^{-}$ cells. Briefly, the flow cytometric strategy was as follows: in the first dot plot SS *vs* FS all events were visualized and the “A” region was positioned in order to include all leukocytes and exclude cell debris. In the second dot plot, CD3 *vs* SS, the events of the region “A” were corrected in region “B” to include lymphocytes and monocytes and exclude $CD3^{+}$ events and granulocytes. In the third plot, CRTH2 *vs* CD203, the events of regions "A" and "B" are combined and region "C" will include only the population of $CRTH2^{+}$ basophils $CD203c^{bright}$ $CD3^{-}$ in the lower quadrant "D4" ($CD203c^{dim}$: basophils not activated). The quadrants "D1" and "D3" are aligned on the Y axis of the dot plot. The upper "D2" defines the region of activated basophils ($CD203c^{bright}$). The negative mark is placed on the negative control and must be the same for the positive control and the other enterotoxins. Within the basophil population identified, $CD203c^{+}$ cells represented activated basophils; 500 events were acquired for each analysis. The readout for each patient was considered 0 based on the negative

control. Everything outside the cloud of cells expressing CD203c indicated a non-specific activation and was subtracted from the activation rate.

5.4 STATISTICAL ANALYSIS

All data were analyzed for statistical significance by means of Prism 8 (GraphPad software, La Jolla, San Diego, CA, USA). Values from groups were compared by Student's *t*-test based on the parametric or nonparametric distribution of the continuous variables. One-way ANOVA analysis of variance was performed to compare means among multiple groups. Values were considered significant when * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; $**** $p < 0.0001$.

6. RESULTS

Table 1 summarizes the demographic, clinical, and immunological characteristics of healthy controls and patients with T2-high and T2-low severe asthma. The median age was 43.7±3.1 for T2-high asthma, 47.6±4.0 for T2-low asthma and 46.1±3.0 for healthy controls. 60% of T2-high, 63% of T2-low asthma, and 57% of healthy controls were females. FEV_1 values in both groups of asthmatics were significantly lower than in healthy controls (Fig. 7). This figure also shows that the FEV_1/FVC ratio was significantly lower in T2-high and T2-low asthmatics when compared to healthy controls, whereas no difference was found between the two groups of asthmatics. Rhinosinusitis (40% and 22%) and rhinosinusitis with polyposis (48% and 31%) were co-morbidities of the two groups of asthmatics enrolled in this study.

Table 1 and Figure 8 illustrate the immunological characteristics of healthy controls and patients with T2-high and T2-low asthma. Serum concentrations of IgE were significantly increased T2-high (398.3±61.2 IU/ml) ($p < 0.0001$) and T2-low (161.0±25.2 IU/ml) ($p < 0.0001$) compared to controls (57.4±4.9 IU/ml). Interestingly, the serum concentration of IgE in T2-high asthmatics were significantly higher than in T2-low asthmatics ($p < 0.01$) (Fig. 8). Peripheral blood eosinophils were significantly increased in T2-high (386.6±12.65 cells/mm^3) and T2-low asthmatics (214.9±12.54 cells/mm^3) ($p < 0.001$) compared to healthy controls

(119.9±9.1) (Fig. 8). FeNO was increased in T2-high (49.35±4.06 ppb) ($p < 0.01$) and T2-low asthmatics (47.43±4.32 ppb) ($p < 0.01$) compared to controls (8.06±0.59 ppb) (Fig. 9). The Asthma Control Test (ACT) was markedly increased in both asthmatic groups (14.31±0.68 *vs*. 14.00±0.77; NS) (Fig. 9).

Previous reports indicated that a percentage (≅ 60%) of patients with severe asthma had detectable levels of IgE against at least one of the *S.aureus* enterotoxins (SEA, SEB, SEC or TSST-1) (**Bachert 2012; Tanaka 2015; Song 2016**). We measured the serum concentrations of IgE against SEs in the two groups of asthmatics and in the controls. In our experiments, detectable concentrations of IgE were found in a percentage (≅ 26%) of control subjects (Fig. 10). Approximately 57% of T2-high asthmatics were positive for at least one of the SEs. This means that a large percentage (≅ 43%) of patients with T2-high asthma had undetectable concentrations of serum IgE specific to SEs. Similarly, ≅ 50% of T2-low asthmatics had detectable specific IgE to at least one of the SEs (Fig. 10).

BAT analysis was carried out after incubation of peripheral blood samples from healthy controls and asthmatic patients with anti-IgE, SEA, SEB and TSST-1. Basophils were gated as $CRTH2^{+}$, $CD3^{-}$ and their expression of CD203c was used to identify the percentage of activated basophils. For each donor a negative (buffer) and a positive control (anti-IgE) was carried out. The negative control was used for the normalization of the results, since it indicates the non-specific activation of basophils and represents the percentage with which the negative limit is established to be

subtracted from the percentage of specific activation. The positive control was assessed by stimulating basophils with an optimal concentration (0.1 μg/ml) of anti-IgE.

Figure 11 shows that CD203c was constitutively expressed on basophils from healthy controls and from patients with T2-high and T2-low asthma. Anti-IgE induced a significantly higher expression of CD203c in basophils from T2-high ($p < 0.0001$) and T2-low asthma ($p < 0.001$) compared to controls. Although the increase in CD203c basophil expression was higher in T2-high asthma compared to T2-low asthma, the difference was not significant.

It should be emphasized that the degree of specific activation caused by individual stimuli varied among different patients. Therefore, there are no absolute values of positivity, since the activation value must always be compared to its positive and negative controls. Therefore, the normalization of the results compared to the controls is fundamental and the results must be assessed by evaluating each individual subject.

In a final group of experiments, we examined the effects of activation (% CD203c expression) of human basophils stimulated with SEA, SEB, or TSST-1. Basophils from approximately 6% of control subjects were positive to at least one of the SEs examined. Basophils from 24% of T2-high and 25% of T2-low asthmatics overexpressed CD203c in response to at least one of the SEs examined (Fig. 12).

When we examined the correlations between BAT positivity for at least one SE and the serum concentrations of IgE to SEs in both groups of asthmatics no correlations were found (data not shown).

7. DISCUSSION

A first goal of this study was to characterize immunologically two groups of patients with T2-high or T2-low asthma compared to healthy controls. We found that both groups of patients had similar decrease in FEV_1 and in FEV_1/FCV ratio when compared to controls. The ACT score and FeNO were comparable between the two groups of asthmatics. However, the absolute number of peripheral blood esosinophils was more markedly increased in T2-high asthma compared to T2-low asthma. Similarly, serum concentration of IgE was more markedly augmented in T2-high asthma compared to T2-low asthma. Anti-IgE induced a similar increase in $CD203c^+$ basophils in T2-high and T2-low asthma. Collectively, these results indicate that although the two cohorts of T2-high and T2-low asthma showed similar alterations of lung function and clinical severity, they markedly differed with respect to some immunological parameters (i.e., serum IgE, eosinophils), but not in CD203c expression induced by anti-IgE.

Several studies have shown that serum IgE directed against *Staphylococcus aureus* enterotoxins are present in a subgroup of patients with atopic and non-atopic severe asthma (**Bachert 2012; Tomassen 2013; Song 2014; Song 2016**).

The diagnostic efficacy of BAT test has been validated in allergies to several allergens including those of *Aspergillus fumigates* (**Clementsen 1989; Kowalski 2011; Tanaka 2015; Gernez 2016; Mirkovic 2016; Song 2016; Hemmings 2018; Flora 2019**). Several studies have provided indirect evidence of the role of *S. aureus* toxins in the pathogenesis of severe bronchial asthma (**Tanaka 2015; Davis 2015;**

Song 2016). Compared to mild/moderate asthma patients, a higher percentage of severe asthma patients have serum IgE specific for SEs. Serum IgE levels against SEs were reported to be higher and to correlate with asthma severity parameters, such as a decrease in lung function parameters, a more frequent history of hospitalization for asthma, greater use of glucocorticoids, and poor control of asthma symptoms.

Our results indicate that detectable serum concentrations of IgE against at least one of the SEs examined (SEA, SEB, SEC, TSST-1) can be found in approximately 26% of healthy controls. 57% of T2-high and 50% of T2-low asthmatics were positive for at least one of the SEs examined. Therefore, a high percentage of T2-high (≅ 57%) and T2-low asthmatics (≅ 50%) did not possess detectable concentrations of specific IgE against at least one of the SEs examined.

Similar results were obtained when we examined the percentage of activated basophils ($CD203c^+$) in peripheral blood of the three groups examined. $CD203c^+$ basophils were 6% in controls, 24% in T2-high and 25% in T2-low asthmatics. These results suggest that *S. aureus* enterotoxins can induce degranulation of basophils only in a small percentage of patients with T2-high and T2-low asthma. In conclusion, our results indicate that the *in vitro* response of basophils to SEs and the serum concentrations of IgE against SEs cannot distinguish T2-high from T2-low asthmatics.

This study has several limitations that should be pointed out. First, the cohorts of asthmatic patients and healthy controls are rather limited, also due to the stringent

inclusion and exclusion criteria of the study. These results should be repeated with larger cohorts of patients and controls. Second, asthma is a very heterogeneous inflammatory disorders including several pheno-/endo-types (**Wenzel 2021**). In our study, we examined two groups of severe asthmatics: T2-high and T2-low asthmatics. Our cohort of T2-high asthmatics was characterized by increased serum levels of IgE, positivity of prick skin tests, and increased peripheral blood eosinophils when compared to T2-low asthmatics. T2-low asthmatics are probably heterogeneous and not yet completely characterized (**Fitzpatrick 2020**). This heterogeneous group of patients includes subjects in which lung macrophages, mast cells, and neutrophils play a prevalent pathogenic role (**Fitzpatrick 2020; Braile 2021; Varricchi 2021b**).

The preliminary results of this study do not exclude the functional involvement of *Staphylococcus aureus* enterotoxins in the pathogenesis of allergic and non-allergic severe asthma. In fact, approximately 24% of these patients had a BAT positive for at least one SE. This positivity would suggest that IgE directed against SEs are functional in a percentage of patients with severe asthma.

In conclusion, although our cohorts of T2-high and T2-low asthmatics were comparable from a clinical (ACT score) and functional point of view (FEV_1, FEV_1/FCV), they differ with respect of serum IgE and number of peripheral blood eosinophils. The BAT positivity to anti-IgE was significantly increased in the two groups of asthmatics compared to the controls, but did not differ between T2-high

and T2-low asthmatics. Finally, only a percentage of allergic asthmatics were BAT positive for at least one of the SEs of *S. aureus*. The latter results cannot exclude the possibility that *S. aureus* and its products could represent pathogenic co-factors in a percentage of T2-high and T2-low asthmatics.

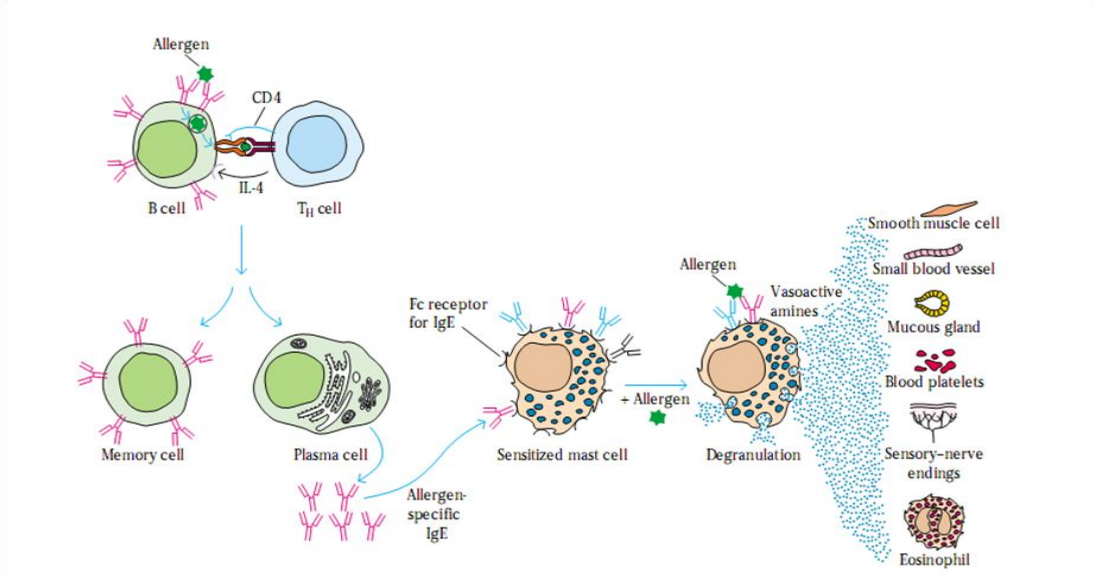

Figure 1.

Allergen engages the B cell receptor on human B lymphocytes activated by IL-4 produced by Th cells. Activated B cells can undergo two forms of differentiation. They can become B memory cells or differentiate to IgE secretory plasma cells. These cells produce specific IgE against the original allergen. IgE binds with high affinity to FcεRI expressed by human mast cells and basophils (the latter not shown in this figure). When primary effector cells of allergic reactions (mast cells and basophils) are reexposed to the same allergen, the cells are activated to release a plethora of preformed (histamine, tryptase) and *de novo* synthesized mediators (LTC_4, PGD_2) and cytokines (e.g., IL-4. IL-5, IL-13). These mediators activate specific receptors on target tissues smooth muscle cells, small blood vessels, goblet cells, immune (e.g., eosinophils, T cells, etc.) and non immune (fibroblasts, sensory-nerve endings) cells and are responsible for the clinical manifestations of allergic reactions.

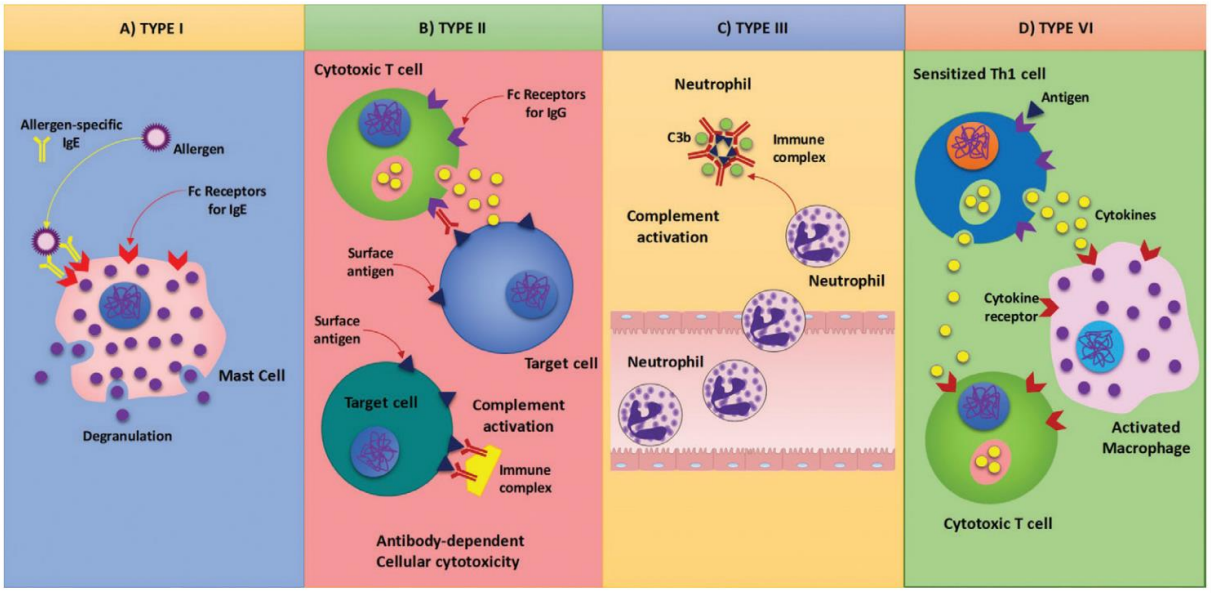

Figure 2.

(A) Type I hypersensitivity. The binding of the allergen to preformed IgE antibodies bound to the FcεRI on the surface of mast cells or basophils causes the release of inflammatory mediators such as histamine, cytokines and metabolites of arachidonic acid, which produces clinical manifestations, such as allergic rhinitis, anaphylaxis, asthma and urticaria. (B) Type II hypersensitivity. Cytotoxic reactions involve the binding of IgM or IgG antibodies to antigens bound to cells. The antigen–antibody binding results in the activation of the complement cascade and in the destruction of the cells to which the antigen is bound. (C) Type III hypersensitivity. Immune complexes are formed when the antigens bind to the antibodies. They are usually removed by the process by phagocytosis. However, the deposition of these immune complexes in the tissues or in the vascular endothelium can produce a tissue damage mediated by immune complexes. (D) Type IV hypersensitivity. These reactions are not mediated by antibodies. Delayed hypersensitivity reactions are mediated primarily by T lymphocytes (cell-mediated immunity) (modified from Munoz-Carillo *et al*., Immunoregulatory Aspects of Immunotherapy 2018).

Phenotypes of T2-high and T2-low asthma

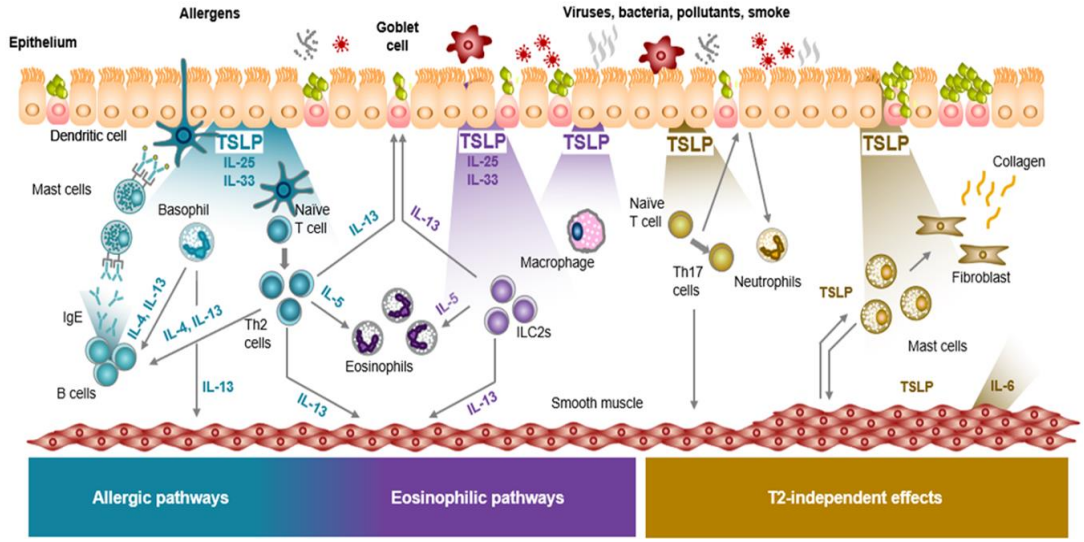

Figure 3.

Bronchial asthma is a highly heterogeneous inflammatory disorder of the airways. Several stimuli (allergens, viral, bacterial, and fungal proteins, smoke, and cytokines) can activate airway epithelial cells to release alarmins (TSLP, IL-33, IL-25) that function as upstream cytokines. Asthma can be subdivided in T2-high (allergic and eosinophilic) and T2-low asthma. In allergic asthma, TSLP and IL-33 activate dendritic cells (DCs), which polarize T cells toward a Th2 phenotype producing IL-4 and IL-13. These cytokines stimulate the production of IgE from B cells. IgE binds to FcεRI on human mast cells and basophils, which are primary effector cells of allergic reactions. In eosinophilic asthma, IL-5 produced by Th2 cells, ILC2, mast cells and eosinophil themselves induce the maturation, differentiation and activation of human eosinophils. TSLP is a potent stimulus for human eosinophil activation. T2 independent asthma is less characterized and presumably include several pathogenic mechanisms. TSLP released by epithelial cells, DCs, mast cells, and lung macrophages plays a pivotal role in T2-low asthma. Activated fibroblasts and the proliferation of airway smooth muscle cells contribute to T2-low asthma.

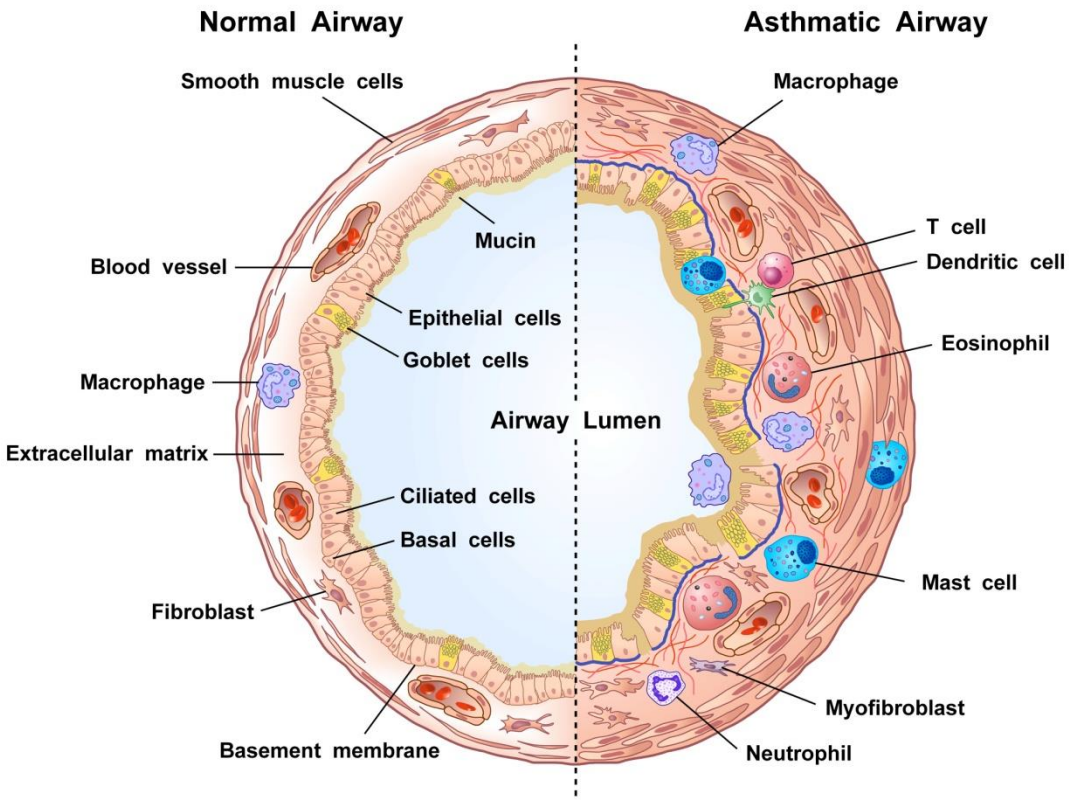

Figure 4.

Airway remodeling in patients with severe asthma characterized by several structural changes. Increased deposition of extracellular matrix (ECM) proteins in the reticular basement membrane, lamina propria, and submucosa is a characteristic of severe asthmatic airways (**Bourdin 2007**). Airway smooth muscle cells (ASM) hypertrophy and hyperplasia are features of asthmatic airway remodeling (**Howarth 2004; Araujo, 2008; Lau 2010; Yick 2012; Yap 2019**). The ASM mass increase is responsible and predictive of bronchial obstruction with consequent reduction of ventilation (**Holgate 2008**), loss of function (**Pascual 2005**), and greater susceptibility to external triggers (**Winkler 2021**). The number of blood vessels and the vascular area (angiogenesis) are increased in asthmatic patients (**Hoshino 2001; Chetta 2003**). Goblet cell hyperplasia, mucin overproduction and mucus hypersecretion are features of severe asthma (**Hauber 2006**). There is some evidence that biologics targeting IgE (omalizumab), IL-5 (mepolizumab, reslizumab), IL-5Rα

(benralizumab), IL-4Rα (dupilumab), and TSLP (tezepelumab) can modulate certain aspects of airway remodeling in severe asthma.

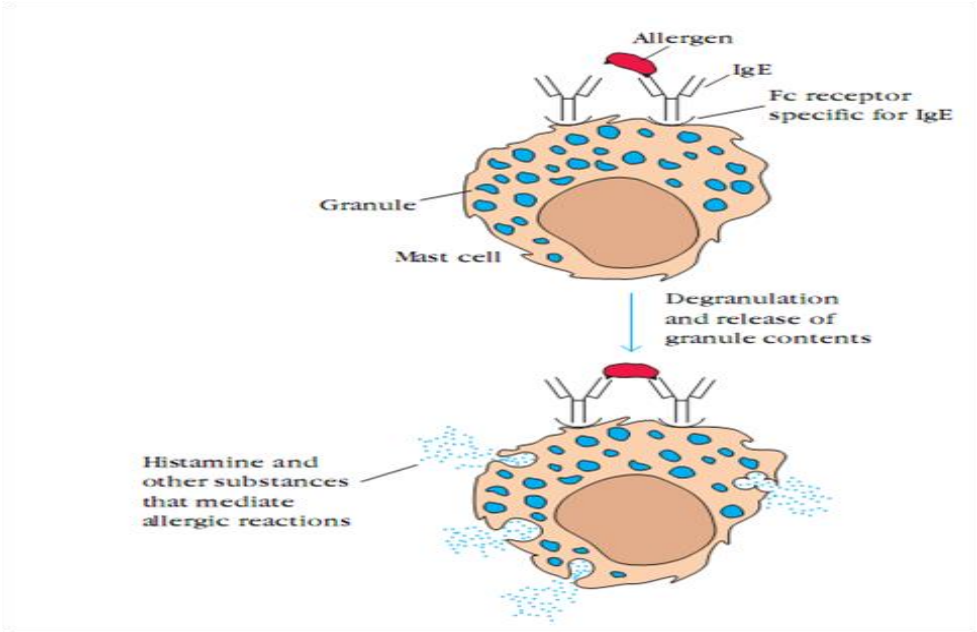

Figure 5.

FcεRI on human basophils and mast cells is expressed as a tetramer (αβγ2). The density of FcεRI on human basophils is closely correlated to the concentrations of free IgE in the serum (**Malveaux 1978**). The extracellular portion of α chain of FcεRI binds to the Fc portion of IgE; α chain consists of an extracellular domain, a transmembrane and a small cytoplasmic domain. The β subunit has four transmembrane domains and a single immunoreceptor motif that is activated at the level of a tyrosine (ITAM - Immunoreceptor tyrosine-based activation motif), and is associated with the Lyn kinase. The β subunit forms a dimer through a disulfide bond, and each subunit contains an ITAM. After the aggregation of FcεRI, caused by the binding of IgE with specific antigens, Lyn phosphorylates the tyrosines of the ITAM domains of the β and γ subunits. The phosphorylation of the tyrosines of the β subunit leads to the recruitment of the Syk kinase, whose signaling is associated with the activation of mast cells and basophils that release histamine present in the cytoplasmic granules. This complex sequence of events triggers the allergic reaction (**Stone 2010**).

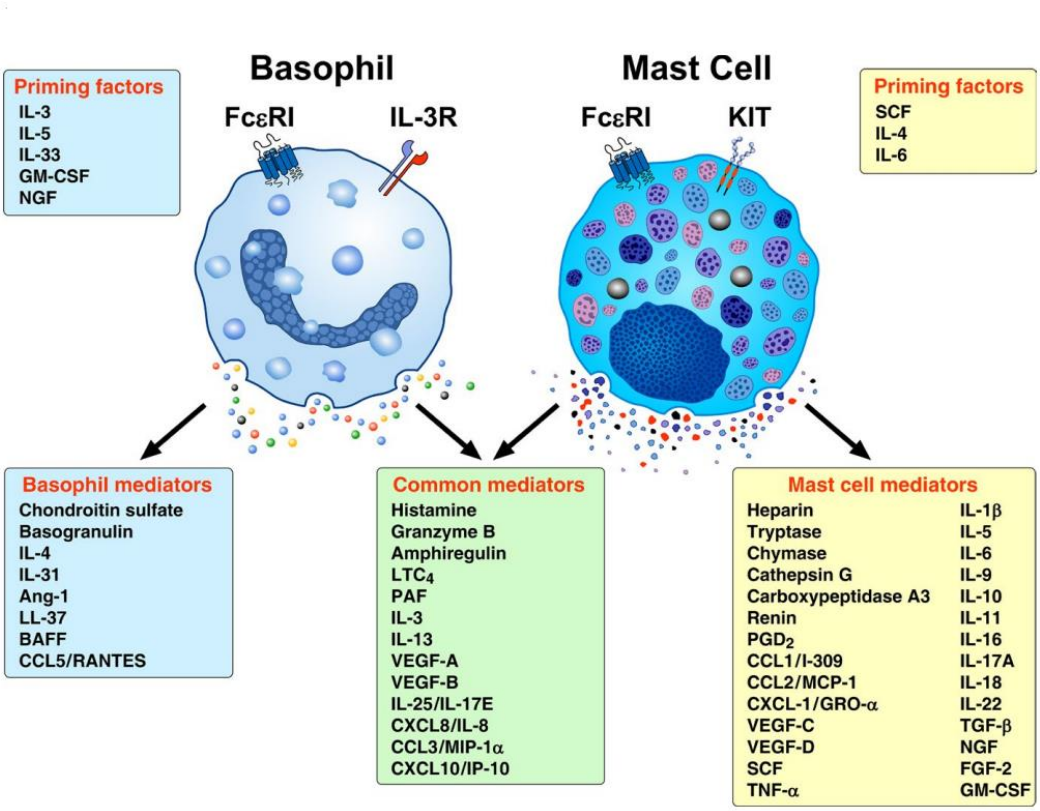

Figure 6.

Pro-inflammatory and immunomodulatory mediators of human basophils and mast cells. Basophil contain histamine in secretory granules complexed with chondroitin sulfate. Secretory granules also contain basogranulin and minute amounts of tryptase (i.e., less than 1% of mast cells). Immunologic activation of basophils leads to the selective release of histamine and basogranulin, and the production of IL-4, IL-13, IL-31, CCL5/RANTES and under specific conditions of LL-37 and BAFF. A wide spectrum of mediators are release by both basophils and mast cells (i.e., histamine, granzyme B, amphiregulin, LTC_4, PAF, IL-3, VEGF-A, VEGF-B, IL-25/IL-17E, CXCL8/IL-8, CCL3/MIP-1α, and CXCL10/IP-10). Secretory granules of human mast cells selectively contain several preformed mediators (i.e., heparin, tryptase, chymase, cathepsin G, carboxypeptidase A3, and renin). In addition, activated mast cells can produce PGD_2, chemokines (CCL1/I-309, CCL2/MCP-1, CXCL-1/GRO-α), angiogenic and lymphangiogenic factors (VEGF-C and VEGF-D), and a constellation of cytokines (SCF, TNF-α, IL-1β, IL-5, IL-6, IL-9, IL-10, IL-11, IL-13, IL-16, IL-17A, IL18, 1L-22, TGF-β, NGF, FGF-2, and GM-CSF). Several factors (i.e., IL-3, IL-5, IL-33, GM-CSF, and NGF) can prime human basophils, whereas

fewer factors (i.e., SCF, IL-4, and IL-6) prime human mast cells (Modified from Varricchi *et al.* Immunol Rev 2018).

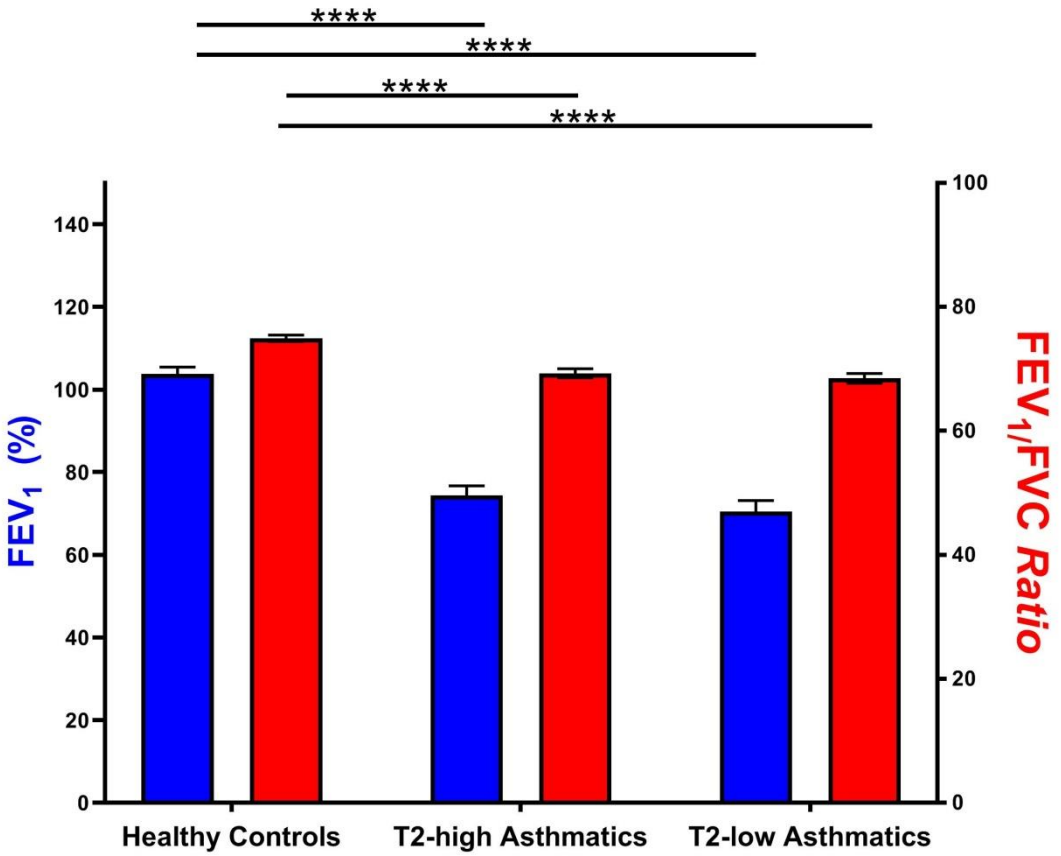

Figure 7.

FEV_1 and FEV_1/FVC ratio in healthy subjects and in patients with T2-high and T2-low asthma. **** $p < 0.0001$.

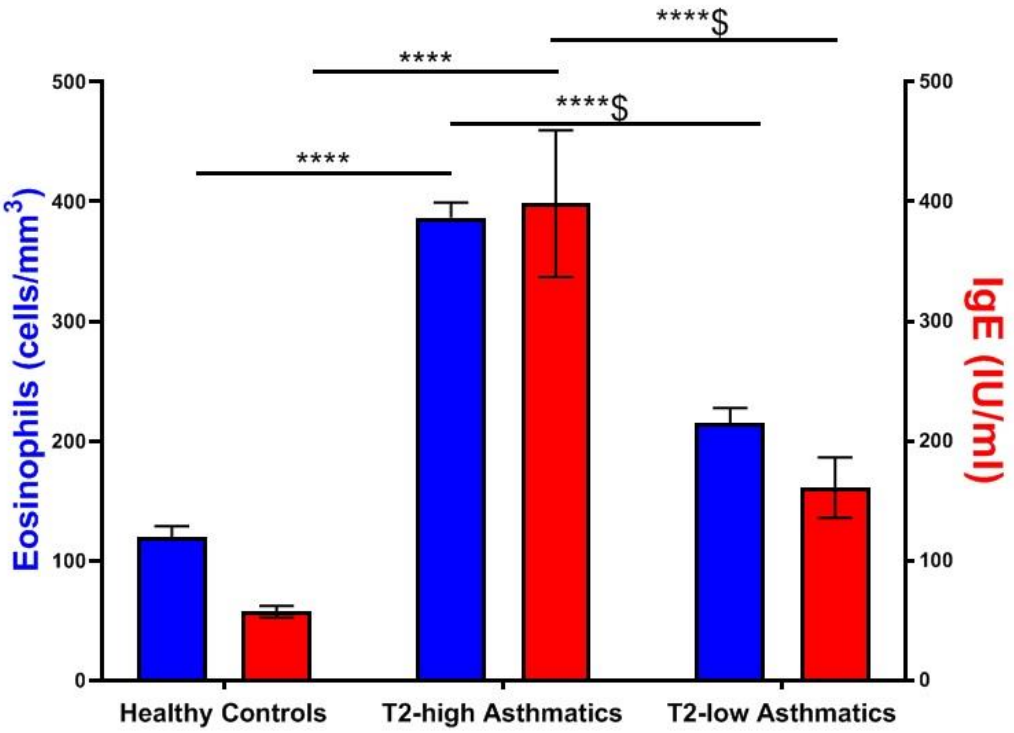

Figure 8.

Peripheral blood eosinophils and serum concentrations of IgE in healthy subjects and in patients with T2-high and T2-low asthma. **** $p < 0.0001$ when T2-high asthmatics are compared to healthy controls; $**** $p < 0.001$ when T2-high asthmatics are compared to T2-low asthmatics.

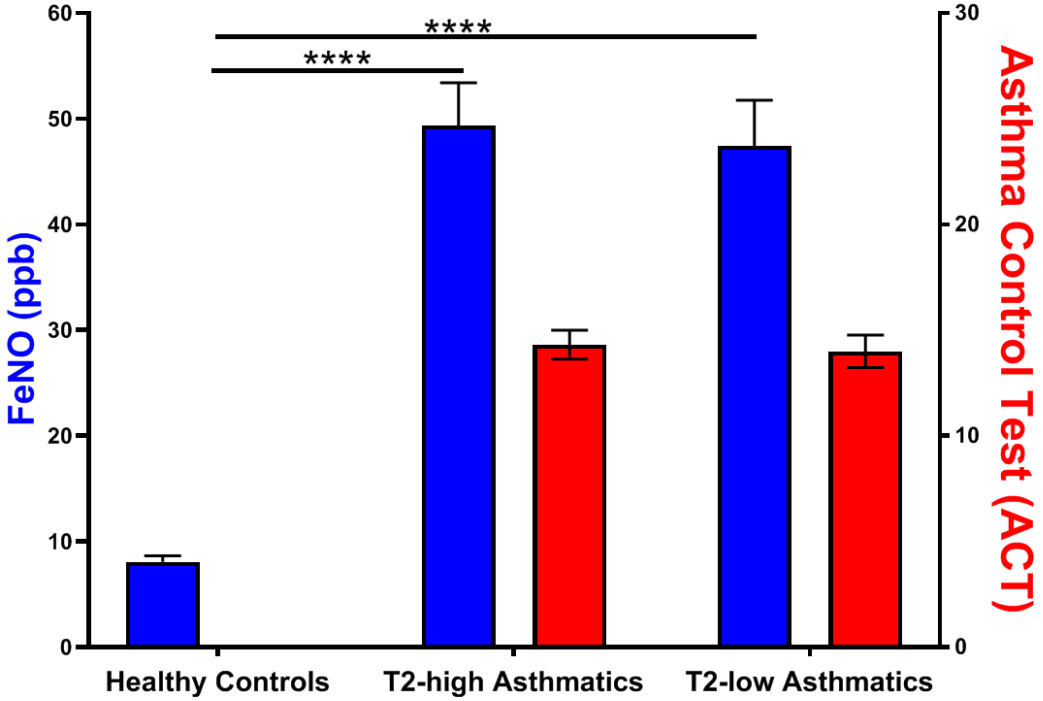

Figure 9.

FeNO and Asthma control test (ACT) score in T2-high and T2-low asthma. **** $p <$ 0.0001.

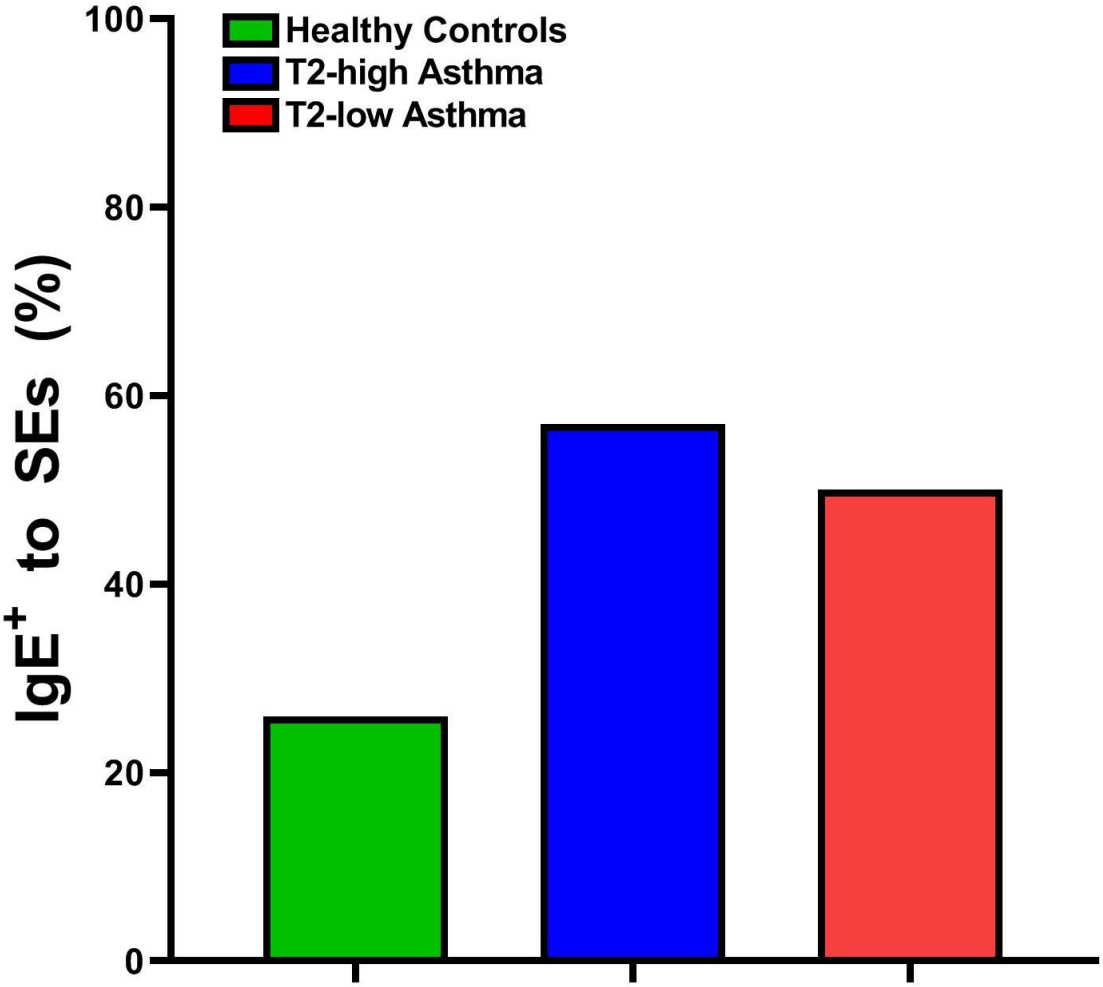

Figure 10.

Percent of healthy controls, T2-high and T2-low asthmatics who had detectable concentrations of serum concentrations of IgE against at least one of the SEs (SEA, SEB, SEC, TSST-1) examined.

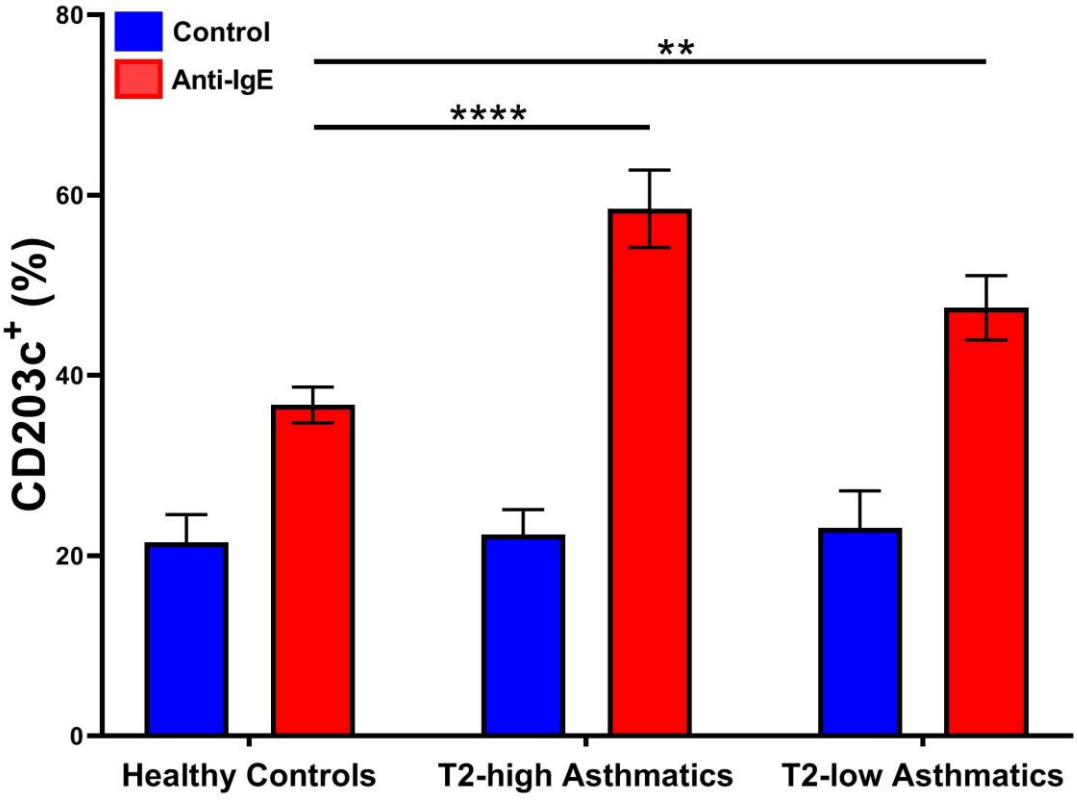

Figure 11.

Percent of unstimulated peripheral blood basophils expressing CD203c in healthy controls, T2-high and T2-low asthmatics (Blue bars). Red bars indicate the percent of $CD203c^+$ basophils after incubation with anti-IgE (0.1 μg/ml). ** $p < 0.01$; **** $p < 0.0001$.

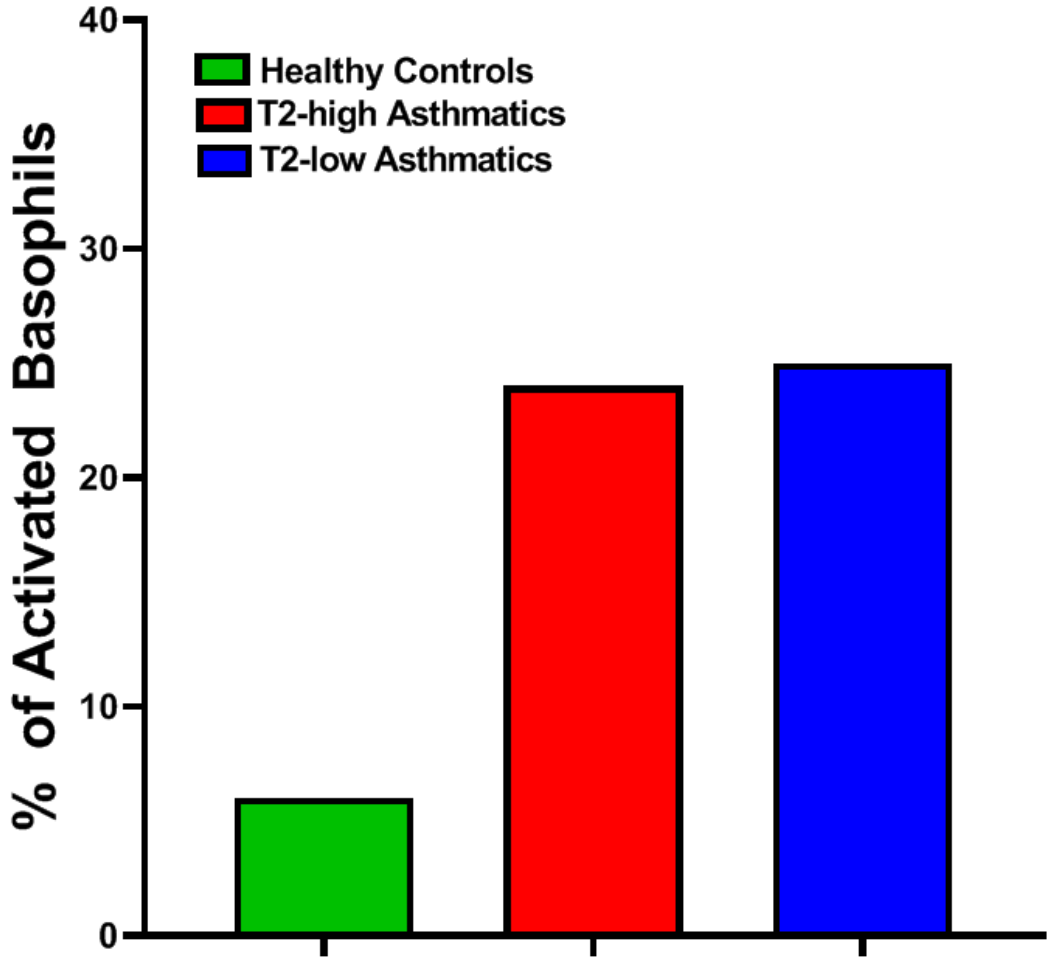

Figure 12.

Percent of activated basophils ($CD203c^{+}$) in the healthy controls and in patients with T2-high and T2-low asthma in response to at least one of the SEs (SEA, SEB,TSST-1) examined.

Table I.

Demographic, clinical and immunological characteristics of healthy controls and patients with T2-high and T2-low asthma.

CHARACTERISTICS	HEALTHY CONTROLS	T2-high ASTHMATICS	T2-low ASTHMATICS
Subjects (n.)	30	30	21
Age (years)	46.1 ± 3.0	43.7 ± 3.1	47.6 ± 4.0
Gender, female (%)	60	63	57
BMI(Kg/m^2) median	27.0	26.4	27.2
Ex Smokers (%)	20	28.6	33.3
FEV_1 (%)	103.8 ± 1.62	74.37 ± 2.30 (****)	70.43 ± 2.68 (****)
FEV_1/FVC ratio (%)	74.93 ± 0.51	69.30 ± 0.71 (****)	68.48 ± 0.77 (****)
FeNO (ppb)	8.06 ± 0.59	49.35 ± 4.06 (****)	47.43 ± 4.32 (****)
Asthma Control Test (ACT)	NA	14.31 ± 0.68	14.00 ± 0.77
Rhinosinusitis without nasal polyposis (%)	8	40	22
Rhinosinusitis with nasal polyposis (%)	7	48	31
Atopic dermatitis (%)	0	4	0
Positive skin tests (%)	0	97	15
Eosinophils ($cells/mm^3$)	119.9 ± 9.1	386.6 ± 12.65 (****)	214.9 ± 12.54 (****$)
IgE (IU/ml)	57.4 ± 4.9	398.3 ± 61.2 (****)	161.0 ± 25.2 (****$)

NA: not applicable.

****** $p < 0.0001$ when compared to healthy controls.**

$ $p < 0.001$ when compared to T2-high asthmatics.

Abbreviations:

ACT, asthma control test; AR, allergic rhinitis; ASM, airway smooth muscle cells; ATS/ERS, American thoracic society/European respiratory society; BAT, basophil activation test; BMI, body mass index; CRSwNP, allergic rhinitis with nasal polyp; DC, dendritic cell; ECM, extracellular matrix; FcεRI, high affinity IgE receptors; FEV_1, forced expiratory volume in 1 second; FP, fluticasone propionate; FVC, forced vital capacity; GCP, good clinical practice; GINA, global initiative for asthma; ICS, inhaled glucocorticoids; Ig, immunoglobulin; IL, interleukin; ITAM - Immunoreceptor tyrosine-based activation motif; LTC_4, cysteinyl leukotriene C_4; PGD_2, prostaglandin D_2; PRIST, Paper Radio Immuno Sorben Test; *S. aureus*, *Staphylococcus aureus*; SE-IgE, staphylococcal enterotoxin-specific IgE; SEA, staphylococcal enterotoxin A; T2-high asthma, type 2-high asthma; T2-low asthma, type 2-low asthma; TSLP, thymic stromal lymphopoietin;TSST-1, toxic shock syndrome toxin-1; VEGF, vascular endothelial growth factor.

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