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Investigating T_H2-mediated immune response in children with type 1 hypersensitivity

By

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Declaration

I, Elina Nepolo hereby declare that the work contained in this thesis entitled "Investigating TH2-mediated immune response in children with type 1 hypersensitivity" is my own original work and that I have not previously, in its entirety or in part, submitted it at any university or other higher education institution for the award of a degree.

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Dedication

To my beautiful, adorable family:

I love you!

Publications directly related to this thesis.
Published Manuscripts

1. Nepolo, E. P., Nkambule, B. B., Dludla, P. V., Ndevahoma, F., & Nyambuya, T. M. (2022). Association between the type of allergen and T-helper 2 mediated inflammation in allergic reactions: a systematic review and a meta-analysis. Allergologia et immunopathologia, 50(1), 37–50. https://doi.org/10.15586/aei.v50i1.470

Abstract

Background

The global prevalence of type 1 hypersensitivity is becoming more prevalent in recent years, particularly among children in developing countries. This has been attributed to rapid modernisation in low-to-middle income countries which promotes the manifestation of allergy risk factors. Type 1 hypersensitivity reactions are exaggerated immune responses to allergens that would not usually elicit immunological response. These allergic reactions are primarily characterized by T helper (T_H2)-mediated inflammation modulated by interleukin (IL)-4, a cytokine that promotes B-cell activation and class switch to secrete immunoglobin (Ig)E. Notably, the severity of T_H2-mediated inflammation in allergic reactions seems to be influenced by the type of allergen. Therefore, this study aimed to evaluate T_H2 immune responses in children with atopy.

Methods

This was an observational study involving children (< 18 years old) with allergies tested at Windhoek Central Hospital, Namibia. A total of sixty participants (n = 60) (fifty with allergy (n = 50) and ten controls (n = 10) were recruited for the purposes of this study between October 2020 and May 2021. Standard laboratory instruments and validated assays were used to measure the total and allergen-specific IgE levels as well as the levels of IL-4. All patients with allergies were confirmed using the cut-off point of 0.35 kUA/l). A p-value of < 0.05 was considered statistically significant. All statistical analysis was performed using the Graph Pad Prism 8 version 8.0.2 Software, (Graph Pad Software Inc, San Diego, CA, USA).

Results

Patients with the allergies had a mean age range of 9.50 ± 3.90 and a male to female ratio of 1:17, whilst those without had a mean age of 5.67 ± 6.09 with a male to female ratio of 0:70. The levels of total IgE were elevated in children with the allergies (298.40 ± 104.3) when compared to those without (133.70 ± 50.61), p = 0.0312. The levels of IL-4 were comparable between the patients and the control group (p = 0.7836). Further assessment of total IgE levels in children with allergies showed varying levels across the type of allergen ($F_{(5, 84)} = 7.28$, p< 0.0001). The posthoc analysis showed that the least levels of total IgE were observed in children allergic to mould spores (411.00 ± 110.10) whilst the highest were found in those allergic to epithelia (690.10 ± 190.20). In comparison to mould spores (411.00 ± 110.10), higher levels of total IgE were seen in children allergic to weed pollen (665.30 ±178.10), p = 0.0002; tree pollen (586.40 ± 144.50), p = 0.0270); epithelia (690.10 ± 190.20), p<0.0001 and house dust mite (632.20 ± 143.90), p = 0.0020. Significant differences were observed between grass pollen and epithelia (503.90 ± 134.40), p = 0.015. Further analysis of allergen specific IgE showed significant differences across the allergens ($F_{(5, 84)} = 2.62$, p = 0.0300). The Tukey's test showed that in comparison to patients allergic to weed pollen (2.88 ± 2.07), the levels of specific IgE levels were lower

in those sensitised to tree pollen (1.35 ± 1.05), p =0.0321 and epithelia (1.39 ± 1.02), p = 0.0389. Other comparable comparisons were found (p>0.05). The levels of IL-4 in children with an allergy were comparable amongst the groups ($F_{(5, 84)}$ = 1.31, p = 0.2667). The cytokine levels were negatively associated with a total IgE level of (r = -0.23, p = 0.0330). The specific IgE to tree pollen were positively associated with a total IgE of (r = 0.35, p = 0.0390) and Phadiatop levels of (r = 0.43, p = 0.0110), whilst a negative corelation was observed between specific IgE to HDM and phadiatop level (r= -0.36, p = 0.0300).

Conclusion

Although the levels of IL-4 were comparable across the allergens, total and allergen-specific IgE levels were dependent on the type of allergen in children with allergies. Moreover, these IgE levels were associated with the levels of IL-4. As a result, treatment options that inhibit IgE activity should be prioritized in Namibian children with allergies. Most significantly, while establishing treatment dosages for allergic reactions, the kind of allergen should be examined.

Key words: Allergy; allergen; immunoglobulin E; interleukin 4; type 1 hypersensitivity; T helper 2 cell

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List of Abbreviations

ANOVA	Analysis of variance
APC	Antigen Presenting Cells
BCR	B cell receptor
DC	Dendritic Cells
ELISA	Enzyme linked immunosorbent assay
ECF	Eosinophilic chemotactic factor
WHO	Health Organisation
HDM	House dust mite
HIV	Human Immunodeficiency Virus
AIDS	Acquired Immunodeficient Syndrome
Ig	Immunoglobulin
IL	Interleukin
MHC	Major Histocompatibility Complex
NUST	Namibia University of Science and Technology
RAST	Radioallergosorbent test
SADC	Southern African Development Community
SST	Serum separator tube
SLE	Systemic lupus erythematosus
TCR	T-cell receptor
T_H2	T-helper 2
WBC	White Blood Cells

Contents

Chapter 1: Introduction

Hypersensitivity or allergic reactions are exaggerated immune responses against an antigen or allergen which normally would not evoke the immune system (Buelow, 2015). These reactions are traditionally classified into 4 categories, that is type 1, 2, 3 and 4. Notably, the global prevalence of type 1 hypersensitivity in children has significantly increased in recent years (Loh & Tang, 2018a), and it is estimated to exponentially rise in the coming years (Backman et al., 2017). As a result, the World Health Organisation (WHO) has recognised allergic reactions as a growing public health burden, particularly in developing countries (Bilaver et al., 2019; Junttila, 2018; Loh & Tang, 2018b; Pawankar, 2014). Currently, the global prevalence of allergies is estimated to be between 10% and 30% (Gupta et al., 2019; Pawankar, 2014) whilst 8.6% in low-to-medium income countries (Cruz et al., 2017). The upsurge in the incidences of allergies is mainly attributed to the rapid modernisation, environmental changes and adoption of unhealthy lifestyles such as consumption of unhealthy diets (Taborda-Barata & Potter, 2012; Zhang & Zhang, 2019). Notably, these changes are prominent in developing countries and they collectively promote the manifestation of risk factors associated with type 1 hypersensitivity (Backman et al., 2017; Pawankar, 2014; D. Ryan et al., 2013; Taborda-Barata & Potter, 2012). Despite, the high levels of unreported cases and scarcity of data in these regions, the prevalence of type 1 hypersensitivity is at a staggering 13.9% in sub-Saharan Africa (Mbatchou Ngahane et al., 2016; Pefura-Yone et al., 2015) and approximately 10% in Southern African Development Community (SADC) (Atiim et al., 2018).

Type 1 allergic reactions may either be immunoglobulin (Ig)E (atopy) or non- IgE-mediated (Spergel, 2006) with atopic reactions typically occurring immediately, whilst non-IgE reactions are relatively delayed (Galli J & Tsai, 2013). Type 1 hypersensitivities are primarily characterised by T-helper 2 (T_H2)-mediated inflammation and the manifestation of allergy associated symptoms such as asthma, rhinitis, conjunctivitis, eczema and atopic dermatitis (Galli J & Tsai, 2013). Notably, atopic reactions are driven by aggravated T_H2 cytokines, particularly interleukin (IL)-4, IL-13 and IL-5 (Brandt & Sivaprasad, 2011). These cytokines mediate the inflammatory milieu in allergic reactions by activating B-cells, mast cells, eosinophils and basophils (Brandt & Sivaprasad, 2011). Briefly, the presentation of an allergen to naïve T_H cells by the Antigen Presenting Cells (APC) on the major histocompatibility complex II induces their differentiation to T_H2 cells (Kaiko et al., 2007). The binding of the allergen to the T-cell receptor followed by the transduction of co-stimulatory signalling results in the successful activation of T_H2 cells and the subsequent secretion of their cytokines (Podojil & Miller, 2009). The released IL-4 instigates the activation of B-cells and promotes their differentiation and class switch to IgE secreting plasma cells (Moens & Tangye, 2014). The allergen can also bind to the IgE-producing plasma cells and further exacerbate the release of IgE antibodies (Galli J & Tsai, 2013). Mast cells and basophils have high-

affinity IgE receptors (FcεRI) on their cell surface and as a result, they are activated by the binding of IgE antibodies on their cell membrane (Stone et al., 2010). Their activation results in cell degradation and subsequent release of histamines, trypstan, leukotrienes and other inflammatory mediators that induce allergic symptoms (Theoharides et al., 2012).

Currently, several therapeutic strategies against atopy aim to alleviate allergic symptoms and alleviate T_H2 mediated inflammation (Voskamp et al., 2020). In fact, the use of allergen-specific immunotherapy and anti-inflammatory drugs in patients with atopy is effective in modulating the activity of T_H2 cytokines and inhibiting the activation eosinophils, basophils, mast cells and IgE secreting B-cells (Benson et al., 1997; Horiguchi et al., 2008; Oda et al., 1998; Wambre et al., 2017; Wang & Shi, 2017; Wisniewski et al., 2015). Of particular interest are treatment strategies that antagonise the effects of IL-4 and IL-5 cytokines in allergic responses (Chiricozzi et al., 2020; Sun et al., 2020). Notably, the therapeutic inhibition of these cytokines is associated with the alleviation of allergy-associated symptoms, the reduction in eosinophil counts and the correction of the T_H1/T_H2 imbalance (Chiricozzi et al., 2020; Wisniewski et al., 2015). However, the levels of IL-4 and IL-5 cytokines in atopy are controversial. In fact, others have reported IL-4/IL-13 mediated immune response in allergic responses (Braddock et al., 2018; Wisniewski et al., 2015) whilst others suggested an IL-5 weighted (Roufosse, 2018; Smart et al., 2002; Voskamp et al., 2020; Wambre et al., 2017). This discrepancy seems to be influenced by the type of allergen. For example, high levels of IL-4 and 13 in serum are noted in patients allergic to house dust mite (HDM) (Rastogi et al., 2015; Wang & Shi, 2017), whereas, elevated levels of IL-5 is apparent in patients allergic to pollen (König et al., 2015; Li et al., 1998; Tang et al., 1998). These findings seem to suggest that the immune response in allergic reactions is dependent on the type of allergen. This may have a negative impact on treatment efficacy, particularly those that involve the use of cytokine-targeting biologics. Therefore, this study aimed to assess T_H2-mediated immune responses in children with atopy and the impact of allergen type on allergic reactions.

1.1 Hypothesis

The Pathogenesis of type 1 hypersensitivity is driven by T_H2 immune responses mediated by elevated levels of their cytokines in response to an allergen. As a result, the level of T_H2 cytokines and total IgE level are directly proportional to atopy severity.

1.2 Research questions

This study seeks to answer the following research questions:

1. Does T_H2-mediated inflammation in allergic reactions differ based on the type of allergen?

2. Is there any association between specific allergens and the levels of IgE (total and specific) and IL-4?

1.3 Aim

The overall aim of this study is to evaluate T_H2 immune responses in children with atopy.

1.4 Objectives

1. To determine the total and allergen specific IgE levels in children with atopy.
2. To measure the levels of IL-4, a T_H2 cytokine that induces B-cell activation and IgE secretion in children with atopy.
3. To establish whether T_H2-mediated inflammation in atopic reactions is allergen-dependent.
4. To evaluate whether there are any associations between IgE levels, inflammatory state, and specific allergens.

Chapter 2: Literature review

2.1 Introduction

The global prevalence of allergic diseases has increased over the years (Backman et al., 2017; Zhang & Zhang, 2019). This has been attributed to changes in diet and environmental settings due to t modernisation and urbanisation in most developing countries (Taborda-Barata & Potter, 2012; Zhang & Zhang, 2019). An exacerbated immune response against a foreign antigen or allergen can trigger a hypersensitivity reaction (Delves, 2017) . Whereby, reproducible signs and symptoms manifest after exposure to a specific stimulus at a dose that would be tolerated by healthy individuals with normal immune responses (Dispenza, 2019; Uzzaman & Cho, 2012). The global prevalence of allergies is estimated to be between 10% and 30% (Gupta et al., 2019; Pawankar, 2014) whilst in low-to-medium income countries it is estimated to be around 8.6% (Cruz et al., 2017). Notably, the prevalence of hypersensitivity is at a staggering 13.9% in Sub-Saharan Africa (Mbatchou Ngahane et al., 2016; Pefura-Yone et al., 2015) and approximately 10% in SADC(Atiim et al., 2018). Despite high levels of unreported cases and a scarcity of data in these regions, more incidences persist.

Hypersensitivity reactions can manifest in different organs at any age, and symptoms range from mild to life threatening (Karki, 2018). Type 1 hypersensitivity is characterised by an Immunoglobulin (Ig) E mediated adverse immune response (Berin & Sampson, 2013; S. G. O. Johansson et al., 2001; Valenta et al., 2015), type 2 hypersensitivity consists of cytotoxic reactions as a result of the binding of IgG or IgM to the surface of cells (Dispenza, 2019; Uzzaman & Cho, 2012), type 3 hypersensitivity causes tissue injury through the deposition of antibody-antigen complexes (Dispenza, 2019; Sika-Paotonu et al., 2017) and type 4 hypersensitivity is a delayed type of reaction mediated by T cells (Dispenza, 2019; Nayak, 2017). This study however focused on type 1 hypersensitivity reactions.

The prevalence of type 1 hypersensitivity has dramatically increased over the past few decades (Fernández-Benítez, 2001). Although the specific mechanisms that are involved in the pathogenesis of type 1 hypersensitivity are not fully understood, genetic predisposition and environmental aspects have been identified as risk factors (Toskala & Kennedy, 2015). The industrial and technological revolutions have led to environmental variations, including climate change, pollution, and microbial sterilisation. These revolutions have also led to an urban and sedentary lifestyle where the intensity, type and diversity of external exposures have been affected, altering the normal immune responses to different antigens/allergens (Fernández-Benítez, 2001). Despite, the rapid increase in the prevalence of type 1 hypersensitivity in Southern Africa which is mostly attributed to globalisation and urbanisation (Atiim et al., 2018), data on hypersensitivity in Namibia is very limited prompting

the researcher to contend that research on allergies in Africa is important. The Westernisation of urban lifestyle in Africa leads to an increased burden of allergic diseases (Van Ree & Yazdanbakhsh, 2007). Although allergic disorders are not commonly reported, they are mathematically more prevalent than other popular ailments like Tuberculosis or Human Immunodeficiency Virus (HIV)/Acquired Immunodeficient Syndrome (AIDS) (Mbugi & Chilongola, 2010). Type 1 hypersensitivity often presents as a chronic disorder that infrequently cause death but have a significant impact in the quality of life of the patients and their families (Longo et al., 2013). Avoidance of causal allergens can be difficult and accidental exposure may occur. Whether these accidental exposures have an impact on specific IgE, concentration is unknown (Kowalski et al., 2016). The association between exposure to allergens and the development of allergen-specific immune responses is complex (Yoo & Perzanowski, 2014). Establishing the relationship between specific allergens and the degree of the immune response that they trigger within a population, helps to determine appropriate preventative measures and treatment options (Larsen et al., 2016).

2.2 Types of hypersensitivities

2.2.1 Type I hypersensitivity

Type 1 hypersensitivity is an antibody mediated reaction that is also referred to as an mediate allergy reaction (Li et al., 1998; Pier et al., 2004). The allergic reaction is mediated by IgE antibodies (Ray, 2016). Briefly, when an atopic individual is exposed to an allergen for the first time, antigen presenting cells (APCs) process the allergen and present it to T-helper (T_H) cells (Boyton & Altmann, 2004; Kidd, 2003). The activated T_H cells, particularly T_H2 release interleukin (IL)-4 that induces the activation of B- cells and their subsequent differentiation into plasma cells (Kouro & Takatsu, 2009; E. J. Ryan et al., 2000) which in turn secretes IgE (Gernez et al., 2007; Santos et al., 2015). The IgE antibodies bind to the Fc receptors on the cell surface of mast cells and basophils, thus sensitising them (Figure 2.1) (Kips, 2001; Rouhani et al., 2005). Upon subsequent exposure to the same allergen, sensitised mast cells rapidly release inflammatory granules such as histamine that cause allergy reaction symptoms. These include, vasodilation, increased vascular permeability and oedema in the early phase of hypersensitivity (Blanca-López et al., 2015; Broide, 2007; Uzzaman & Cho, 2012). Type 1 hypersensitivity reaction is illustrated in figure 2.1.

In the late phase, mediators such as leukotrienes, prostaglandins and eosinophilic chemotactic factor (ECF) are released (Rouhani et al., 2005; Slovick et al., 2017; Valenta et al., 2015). Leukotrienes are fatty inflammatory mediators that cause difficulty in breathing by tightening the airways and induce mucus production in asthma reaction (Bosnjak et al., 2011; Carlsen, 2004). While prostaglandins are lipid pro-inflammatory mediators that are essential for their vital role in broncho-constriction and

vasodilation (S. Johansson et al., 2007; S. H. Yang et al., 2010). Moreover, prostaglandins are involved in attracting neutrophilic leukocytes to the site of inflammation (Uzzaman & Cho, 2012). The third mediator released during the late phase of immediate hypersensitivity is ECF which is a substrate released by mast cells and basophils (Kidd, 2003; König et al., 2015). These mediators are responsible for attracting eosinophils and other granulocytes to the site of inflammation (Hu et al., 2018; Valenta et al., 2015). This type of hypersensitivity include conditions such as anaphylaxis, allergic bronchial asthma, allergic rhinitis, allergic conjunctivitis, food allergy, atopic eczema and drug allergy (S. G. O. Johansson et al., 2001).

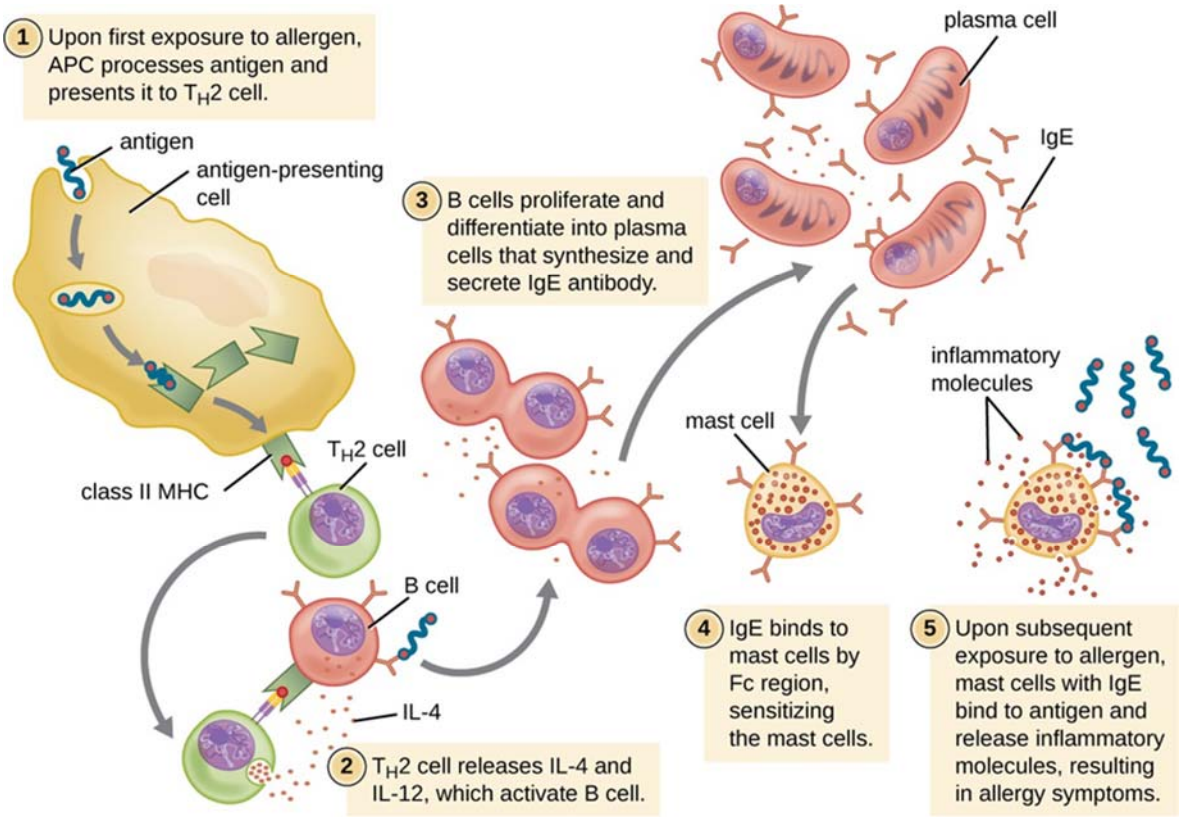

Figure 2.1: The pathogenesis of type 1 hypersensitivity. Antigen presenting cell (APC) presents the allergen to T helper (T_H) cells. T_H2 release interleukin (IL)-4 which induce the activation, proliferation, and differentiation of B cells into plasma cells that secretes immunoglobulin (Ig)E antibodies specific to the allergen. IgE binds to the Fc receptor of the mast cells. Upon subsequent exposure to the allergen, the mast cell with the IgE specific to that allergen bind to the allergen and release inflammation mediators such as histamines that promotes allergic reactions *(*adapted from (Nayak, 2017)).

2.2.2 Type II Hypersensitivity

Type 2 hypersensitivity reaction is mediated by antibodies directed against cellular or extracellular matrix antigens (Nayak, 2017). These antibody mediated responses are known to cause cellular destruction, functional loss, or damage to tissues (Pier et al., 2004). Additionally, this reaction occurs as a result of immune response to modifications of cell surface or matrix-associated antigens generating antigenic epitopes that are regarded as foreign by the immune system (Buelow, 2015). Notably, this type of reaction is mostly caused by medications such as penicillin, thiazides, cephalosporins and methyldopa (Trubiano et al., 2018). Briefly, the pathogenesis of type 2 hypersensitivity is initiated when a drug molecule (an allergen) binds to the cell surface resulting in a neoantigen or alteration of epitopes of existing self-antigen on the cell surface (Blanca-López et al., 2015). This triggers the immune system to recognise the modified antigen as an allergen and cause production of antibodies directed to self-antigens (Bolon, 2012). In this type of hypersensitivity, IgG and IgM are produced as a result of exposure to the inciting agent and it promotes a series of pathogenic outcomes (Nayak, 2017). The pathophysiology of type 2 hypersensitivity reactions can be broadly classified into three types, namely:

(i). Cell depletion or destruction without inflammation
(ii). Inflammation mediated by complement or Fc receptor
(iii). Cellular dysfunction by antibodies

Type 2 hypersensitivity reactions include conditions like, autoimmune haemolytic anaemia, systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA) (Nayak, 2017). Currently, these type of reactions are managed by avoiding the cause of reaction or by administering systemic glucocorticoids which is used as one of many important interventions to suppress antibody response and prevent tissue damage (Larsen et al., 2016; Simon, 2019). However, if left untreated, patients may develop tissue or organ damage, which depends on the clinical presentation, e.g. cytopaenia which might contribute to infections, bleeding tendencies, and severe anaemias (Dispenza, 2019; Pawankar, 2014). Acute rheumatic fever may lead to rheumatic heart disease with valvular lesions (Sika-Paotonu et al., 2017).

2.2.3 Type III Hypersensitivity

Type 3 hypersensitivity is an immune complex mediated reaction whereby (Uzzaman & Cho, 2012) antigen-antibody complexes are deposited into the tissue and activates complements (Leaker et al., 2017). In turn, complements triggers the release of lysosomal enzymes and free radicles to the site of inflammation by the monocytes and neutrophils (Pier et al., 2004). Type 3 hypersensitivity reactions

are noted in autoimmune diseases such as Rheumatoid arthritis, Systemic Lupus Erythematosus (SLE), it involves IgM and IgG antibodies (Kidd, 2003; Singh et al., 1999).

2.2.4 Type IV Hypersensitivity

Type 4 hypersensitivity is the only type that is cell mediated. It is mediated by antigen-specific activated T cells (Abbas & Lichtman, 2011). It is also known as a delayed-type hypersensitivity because of the time it takes for the symptoms to occur after antigen exposure (Dispenza, 2019). This reaction takes longer compared to all other types due to the length of time required to recruit cells to the inflammation site which is around 24 to 72 hours (Dispenza, 2019). Once the antigen enters the body, it is processed by APCs and presented together with Major Histocompatibility Complex (MHC) II to a T_H1 cell. If an individual was already exposed to that specific antigen, the T-helper cell will become activated and secrete chemokines to recruit macrophages and cytokines such as Interferon- γ (IFN-γ) to activate them (Uzzaman & Cho, 2012). Activated macrophages release pro-inflammatory factors, leading to inflammation symptoms such as local swelling, oedema, warmth, and redness (Pier et al., 2004). In addition to pro-inflammatory factors, they also secrete lysosomal elements and reactivate oxygen species, which cause local tissue damage (Pier et al., 2004). CD8+ T cells are involved in type 4 reaction where a foreign antigen is detected on a cell, such as in organ rejection which is known as cell mediated cytotoxicity, and results in recruitment and activation of macrophages (Uzzaman & Cho, 2012).

2.3 The cells of the adaptive cell immunity involved in allergic response

Allergic reactions are mediated by a variety of immune cells of both innate and adaptive immunity (Ray, 2016). In the context of the latter, the reaction begins with the activation of allergen-specific T_H2 cells by Antigen Presenting Cells (APC) (Bosnjak et al., 2011). Dendritic Cells (DC) are the essential APC that will induce T-cell differentiation (Leite-de-Moraes et al., 2012). In short, DCs stimulate naïve T cells to differentiate into distinct effector cell subsets, particularly T_H2, which secrete cytokines that mediate hypersensitivity (Chung, 2001; Mishan-Eisenberg et al., 2004; Verschoor & Von Gunten, 2019). These includes IL-4, IL-5, and IL-13 (Deo et al., 2010). These T_H2 cytokines mediate the inflammatory response by inducing the synthesis of IgE antibodies which in turn activates mast cells (Buelow, 2015). The synthesis of IgE is central to the induction and maintenance of immediate hypersensitivity (X. Yang, 1993). The antigen-specific IgE antibodies bind through its F_c portion, to high-affinity receptors located on the surface of mast cells and basophils, leaving its allergen receptor site available for future interaction with allergen, this process is called sensitisation (Deo et al., 2010; Portnoy, 2015a). When sensitized mast cells are exposed to the same allergen; they get activated and secrete mediators such as vasoactive amines and lipid mediators that are responsible for immediate hypersensitivity (Buelow, 2015; Toniato et al., 2017).In addition, they release

cytokines such as IL-5 and IL-10 which are responsible for late-phase reaction. Different allergens stimulate the production of corresponding allergen-specific IgE antibodies, which is dependent on the ratio in which the cytokines IL-4 and IL-10 after the exposure to the allergens (X. Yang, 1993) (Portnoy, 2015b).

2.3.1 T cells

T cells are one of the two primary types of lymphocytes involved in the adaptive immune response (Stone et al., 2010). They are produced in the hematopoietic stem cells in the bone marrow and move to the thymus where they mature into several distinct subtypes (Hartl et al., 2006; Junttila, 2018). T-cells have a unique protein called T-cell receptor (TCR) on their cell surface, which makes them different from other lymphocytes (Kidd, 2003; Mishan-Eisenberg et al., 2004). These cells are referred to as naïve T cells when they have not encountered an antigen yet. Nevertheless, once they encounter one, they become activated by the antigen they encounter in a two signal process (Kidd, 2003). T cells play a vital role in adaptive immunity upon activation by APC (Chu et al., 2014; Stone et al., 2010). Briefly, once an antigen is encountered, it is recognised by APC and presented to T cells via the MHC (Crocker et al., 1998; Kidd, 2003), whereby MHC I is recognised by cytotoxic T cells ($CD8^+$) whilst MHC II is recognised by T_H cells ($CD4^+$) (Ray, 2016).

The binding of the antigen MHC complex to the T-cell transduces a primary signal in T cell activation (Flynn et al., 1998; Ray, 2016). This signal is however not adequate enough to activate T-cells hence a secondary signal is required from the co-stimulation receptors such as CD28 (Kips et al., 2001; Thunberg et al., 2007). The binding of CD28 to B7 results in the successful action of T cells and induces the secretion of cytokines that modulate the effector functions of T cells and other immune cells (Chung, 2001). The cytokines mediate the differentiation of naïve T cells into different subsets. Figure 2.2 illustrates the successful activation of a T cell and its subsequent differentiation into T_H subsets.

T cells are grouped into two subtypes, namely $CD4^+$ and $CD8^+$ cells based on their immunologic functions (Kidd, 2003). $CD8^+$ T cells are responsible for primarily eliminating intracellular organisms whilst $CD4^+$ T-cells are responsible for eradicating extracellular organisms (Kips et al., 2001; Ray, 2016). This review will focus more on the T_H2 subset which mediate allergic reactions once activated, T_H2 cells secrete its signature cytokines including IL-4 which induces the activation of B cells (Cardoso et al., 2009) .

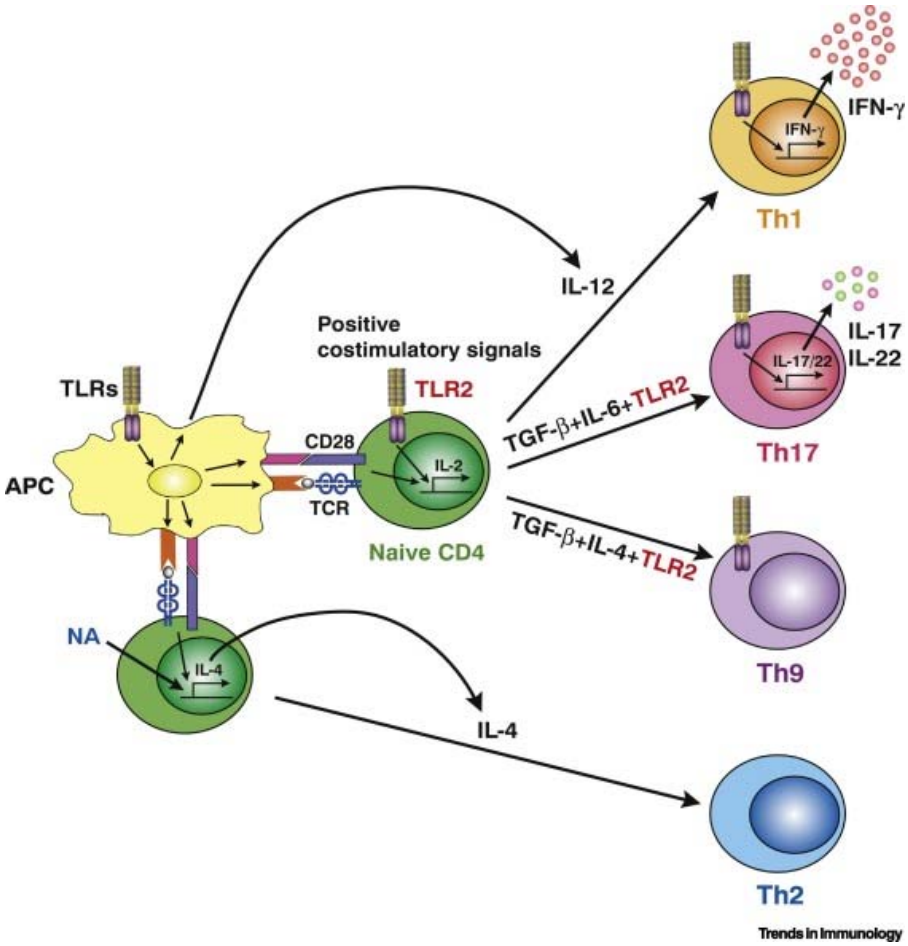

Figure 2.2: An illustration of successful activation and differentiation of T helper (T_H) cells. Once naïve T cells are activated, they secrete interleukin (IL)-4. IL-4 induces the proliferation and differentiation of naïve T cells into the T_H2 subset, while IL-12 promotes T_H1 differentiation.

(Adapted from:(Imanishi & Saito, 2020)).

2.3.2 B cells

The production of B cells starts in the early days of life, prenatally they are produced in foetal liver then move to the bone marrow (Buelow, 2015; Pier et al., 2004). Once the baby is born, the bone marrow takes over the B cell production function, then the cells move to the spleen for maturing, and differentiation (Chung, 2001; Deo et al., 2010; Moens & Tangye, 2014). They are crucial for mediating the humoral immune responses through the secretion of antibodies (Abbas & Lichtman, 2011) (Nayak, 2017). B cells have different subsets namely follicular B2 cells, marginal zone B cells and B1 cells (Lee et al., 2010). These subsets play different roles in immune response and their lifespan in blood circulation also differs (Lee et al., 2010). Naïve B cells constitutively express IgM and IgD, and undergo class switching when exposed to an antigen (Kidd, 2003). In type 1 hypersensitivity, they switch class to IgE which is specific to the exposed allergen (Alberts et al., 2002; Broide, 2007).

Like T cells, B cells also require two signals to be fully active (Moens & Tangye, 2014). The first signal is initialised by the binding of antigen to the B cell receptor (BCR) and is finalised by co-stimulation signalling (Moens & Tangye, 2014). There are two ways by which B cell can get activated namely, T cell dependent B cell activation or T cell independent B cell activation. This classification is based on the type of antigen encountered by the B cells (Moens & Tangye, 2014). In the context of the former, T dependent antigens may induce the activation of B cells through cytokine signalling with the assistance of T helper derived cytokines. Whereas T-independent antigens stimulate the activation of B cells and secretion of antibodies without any assistance from T_H cells (Deo et al., 2010; Moens & Tangye, 2014) (Figure 2.3). In T cell independent B cell activation, two signals are required for the B cell to be fully activated (Martin & Wesche, 2002). First signal is delivered from the recognition of an antigen by the B cells (Broide, 2007). The B cell bind to the antigen, clustering of the B cell receptors triggers B cell activation (Ishihara & Hirano, 2002). The Second signal is derived from other molecules that are present on the antigen (Moens & Tangye, 2014). B cell has TL receptor that can recognise various microbial surface molecules (Mesquita Júnior et al., 2010). This generates second signal for T independent B cell activation (Levy et al., 2000). Upon activation, B cell differentiate into plasma cells that mainly secrete IgM antibodies (Liu & Yang, 2015).

T cell dependent B cell activation is a 3 signals process (Imanishi & Saito, 2020). First signal is generated on antigen recognition by B cell, whereby mature naïve B cell recognise and bind the specific antigen by its B cell receptors (D. Ryan et al., 2013). B cells process the antigen and display it on their surfaces MHC II-peptide complex (Navinés-Ferrer et al., 2016; D. Ryan et al., 2013) . As a result, these B cells also start expressing co-stimulatory and cytokine receptors on their surface (Imanishi & Saito, 2020). Most important co-stimulatory receptor is CD40 (Imanishi & Saito, 2020). Meanwhile the same antigen is also recognised by mature naïve CD4+ cell (or helper T cell) (Stone et al., 2010). The dendritic cell present process the antigen in form of MHC II complex to the T cells (Navinés-Ferrer et al., 2016). T cells get activated and express T cell receptors capable of recognising the antigens (Valenta et al., 2015). The second signal is derived from the interaction of B cell with T cell (Imanishi & Saito, 2020). T helper cell recognise the antigen complex bond to the B cell and bind to it (D. Ryan et al., 2013). T helper cells start to express CD40 ligands on their surface (Shamji et al., 2019). CD40 receptor present on the B cell surface recognise and binds to CD40 ligands on the T helper cells (Shamji et al., 2019). This interaction between the B and T cells provide the second signal (Alberts et al., 2002). Finally, the third signal is provided by the release of cytokines by the T helper cells(Alberts et al., 2002). Interaction between the B cells and T cells induce the expression of new cytokine receptors on the surface of the B cells (Liu & Yang, 2015). T helper cells release cytokines such as IL-4, these cytokines binds the cytokine receptors present on the surface of B cells (Liu & Yang, 2015). As a results, B cells starts to

proliferate and differentiates into antibody secreting plasma cells and memory B cells (Gernez et al., 2007).

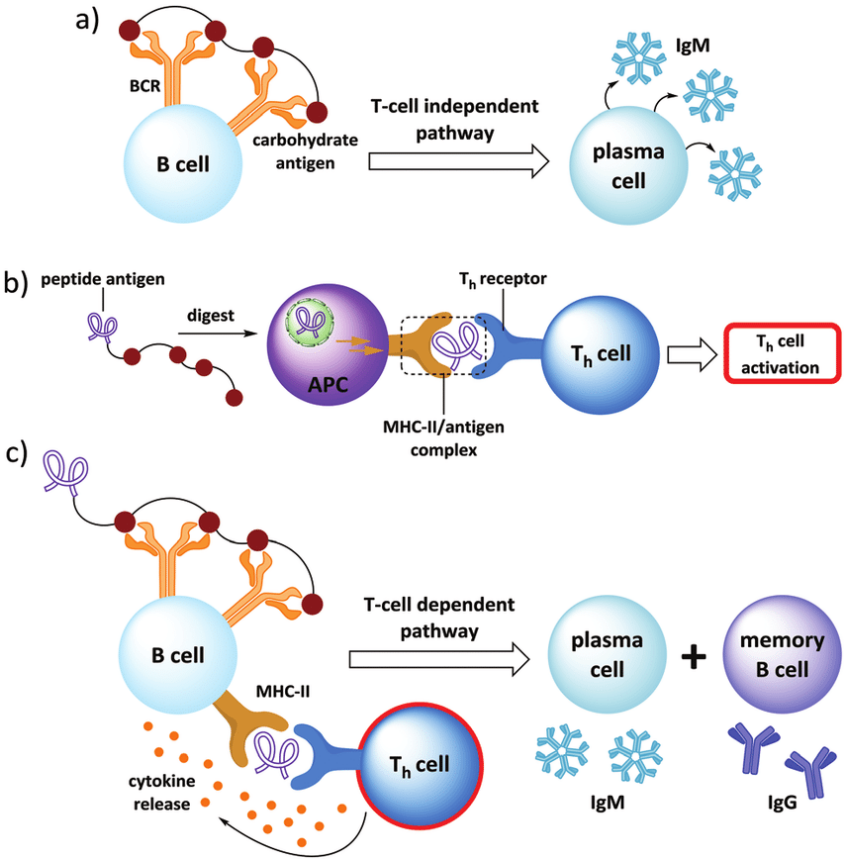

Figure 2.3: T cell dependent and independent activation of B cells, T cell dependent activation requires T cell help for B cell to be fully activated while T cell independent (Adapted from (Alberts et al., 2002)).

2.4 The pathophysiology of an allergic response

Allergic reactions are initiated with the first exposure to allergens(Platts-Mills et al., 2016). The allergen is engulfed by the APCs, processed and presented to the T_H cells in the lymph nodes (Kidd, 2003). This marks the process of sensitisation as T_H cells introduce the new allergen to the B cells (Roth-Walter et al., 2014). Activated B cells differentiate and become plasma cells that secrete immunoglobulins, predominantly IgE (Kubo, 2017; Noma et al., 2001).The IgE then binds to the F_c receptor on the mast cells and on the basophils (Navinés-Ferrer et al., 2016; Stone et al., 2010; Toniato et al., 2017) . Upon subsequent exposure to the same allergen, the IgE which is bound to the mast cell recognises the allergen and crosslinks (Asher et al., 2006; Toniato et al., 2017). This results in the

activation of mast cells to secrete IL-4 and IL-5 (Moon et al., 2014). IL-4 stimulates the immunoglobulin class switching and maturation of eosinophils and basophils (Kubo, 2017; Stone et al., 2010). Subsequently, histamine and other inflammation mediators are released causing vasodilation and increased vascular permeability (Broide, 2007; Moon et al., 2014). This leads to acute allergic reaction. In chronic allergic reaction, activated T_H2 cells release cytokines such as IL 4, IL-5 and IL-13 (Junttila, 2018; Uzzaman & Cho, 2012). These cytokines induce degranulation and release of other inflammation mediators such as leukotriene, prostaglandin, platelet activation factors and eosinophil chemotactic factor (ECF) (Chung, 2001; S. Johansson et al., 2007; S. H. Yang et al., 2010). The pathophysiology of an allergic reaction is represented in Figure 2.4 below.

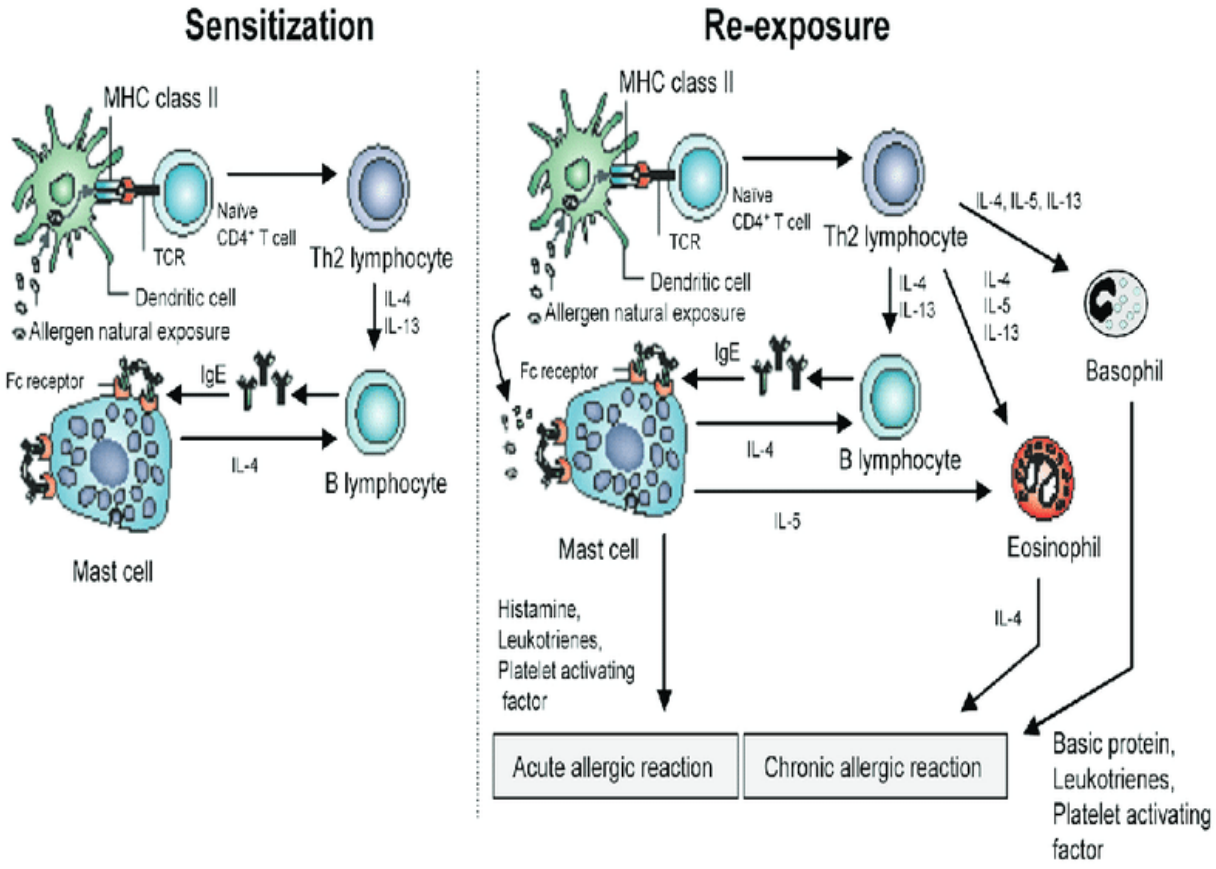

Figure 2.4: Schematic representation of the pathophysiology of allergic reactions. Individuals are sensitized upon first exposure to an allergen and only react on the subsequent exposure (Adapted from (Taher, 2007)).

2.4.1 Types of allergens

Although allergens can be categorized into two major types, namely inhalants and ingestants, this study's focused on the former. Aeroallergens are present in the air and they enter the human body through the respiratory system (Araujo et al., 2004). These includes tree pollen, grass pollen, house dust mite (HDM), mould and epithelia (Khreesha et al., 2020). Once inhaled, the allergens are captured, processed, and presented by DCs cells to T cells, and B cells activation and differentiation is induced (Kaiko et al., 2007; Voskamp et al., 2020) as described earlier in this chapter. Notably, different allergens seem to evoke different immune responses where some allergens elicit stronger reactions in comparison to others (Khasawneh et al., 2019). A recent systematic review and metanalysis conducted suggests that allergy reactions are allergen dependent (Nepolo et al., 2022).

2.4.2 Immunoglobulin E

Structurally, IgE contains two light chains (kappa, lambda) that are indistinguishable from the light chains of other immunoglobulins (Kelly & Grayson, 2016). It's two heavy chains (epsilon) contains five structural domains which makes them unique and enables it to carry it's special biological properties (Sutton et al., 2019) (Figure 2.5). Apart from mediating type 1 hypersensitivity reactions, IgE is also responsible for providing protection against parasite infections (Schroeder et al., 2010; Sutton et al., 2019). IgE is biologically active despite having a very short life span in circulation and present in low concentrations (Jenmalm & Björkstén, 1999). This is due to its high binding -affinity for the receptors on the surface of mast cells and basophils (Toniato et al., 2017). The levels of total IgE are used by clinicians to screen for IgE-mediated allergic reactions whereas specific IgE is used to diagnose the type of allergen eliciting the hypersensitivity (Naspitz et al., 2004). Notably, both total and specific IgE levels are elevated in individuals with allergies (Movérare et al., 2000; Smart et al., 2002).

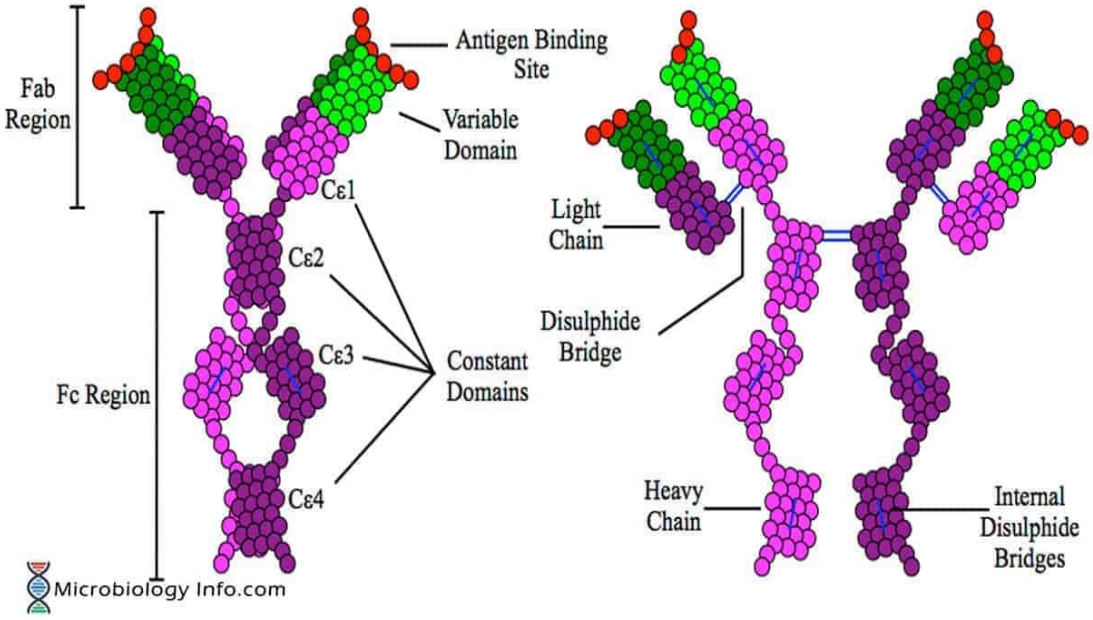

Figure 2.5: The structure of human immunoglobulin E. Human IgE has two identical allergen binding areas made up of light chain and heavy chain linked together by disulphide bonds (Adapted from (Sutton et al., 2019)).

2.5 Major cytokines that modulate IgE-mediated hypersensitivity

Cytokines are small soluble proteins that are crucial in modulating immune responses (Ishihara & Hirano, 2002). They modulate the balance between humoral and cell based immune responses (Deo et al., 2010). Cytokines secreted by T_H2 are vital in IgE mediated hypersensitivity because they are responsible for orchestrating the allergic inflammation (Barnes, 2008; Deo et al., 2010). The dysregulation of cytokine levels is implicated in mediating chronic inflammation in hypersensitivity reactions (Kips, 2001).

2.5.1 Interleukin 4

IL-4 is a T cell derived cytokine that is also secreted by mast cells, eosinophils, basophils (Gadani, Sachin P; Cronk, 2013; Wisniewski et al., 2015). IL-4 is one of the smallest cytokines with a compact globular field, stabilised by 3 disulphide bonds (Mueller et al., 2002). One half of the structure is dominated by four antiparallel juxtaposed helices (A, B, C and D) and two long loops connected by a short β-sheet packed against helices B and D (Chow et al., 2002) (Figure 2.6). It shares common receptors and biological functions with IL-13 and is involved in inducing B-cell activation and differentiation (Chapoval et al., 2011; Junttila, 2018). IL-4 has a broad range of biological effector functions which are exerted through it binding to its complementary receptors that are expressed on cell surfaces of different cells (Kips, 2001). The IL-4 receptor is a heterodimer, consisting of the alpha

chain (IL- 4Rα) and gamma chain or alpha chain of the IL-13 (IL-13Rα) (Mueller et al., 2002). The biological roles of IL-4 includes the initiating of antibody production, haematopoiesis and development of effector T cell responses (Kips et al., 2001). The exacerbated production of IL-4 is associated with IgE mediated hypersensitivity reaction, hence it is considered to be a significant cytokine in the pathogenesis of allergic reactions (Rastogi et al., 2012; Wisniewski et al., 2015).

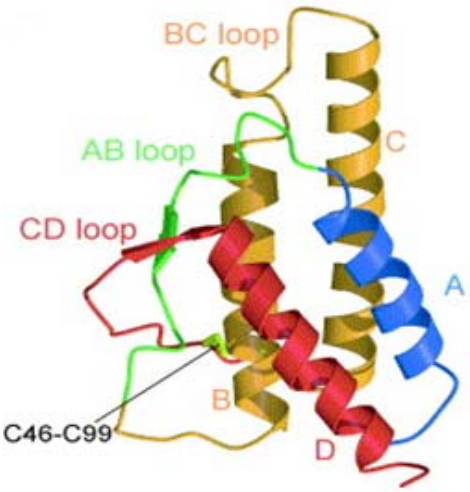

Figure. 2.6. The structure of human interleukin 4. IL-4 is made up of a compact globular field stabilised by 3 disulphide bonds. Half of the structure is dominated by four antiparallel juxtaposed helices (A, B, C and D) and two long loops connected by a short β-sheet packed against helices B and D (Adapted from (Mueller et al., 2002)).

In allergic reactions, IL-4 induces mucus production, bronchial constriction and airway eosinophilia by activating eosinophils and inhibiting their apoptosis (Bagnasco et al., 2016; Borish & Steinke, 2001; Lee et al., 2010; Tsai et al., 2005). Moreover, it skews the T_H1/T_H2 balance by inducing T_H2 differentiation and proliferation, whilst inhibiting that of T_H1 (Kips, 2001; Kips et al., 2001; Scott-Taylor et al., 2010). The release of histamines by mast cells and basophils is also believed to be induced by IL-4 in the early phase of type 1 hypersensitivity reaction (Broide, 2007; Haselden et al., 2001).

2.5.1.1 The effector functions of IL-4 and its role in allergic reactions

IL-4 is regarded as a key regulator in humoral and adaptive immunity through its ability to induce the differentiation of naïve T cells to T_H2 cells (Borish & Steinke, 2001). Briefly, the naïve T cells interact with APCs and gets activated into effector cells (Borish & Steinke, 2001). Activated $CD4^+$ cells differentiates into different T_H cells such as T_H1, T_H2, T_H9, T_H17, and Treg depending on the cytokine milieu (Bao & Reinhardt, 2015; Woodfolk, 2007). Of interest is T_H2, which plays a significant role in in allergic reactions (Cooper et al., 2008; Toniato et al., 2017). The T_H2 signature cytokines includes IL-4,

IL-5, IL-6, IL-10, IL-13, and IL-31. Of these, IL-4 is strongly implicated in mediating allergic reactions (Borish & Steinke, 2001). Upon activation by IL-4, T_H2 subsequently secrete IL-4 in a positive feedback loop by regulating T_H1/ T_H2 balance and decreasing the differentiation of T_H1 and the release of interferon gamma (IFN-γ) and IL-12 (Deo et al., 2010). In addition, IL-4 stimulates the activation and differentiation of B cells into IgE, producing plasma cells in response to allergens (Mesquita Júnior et al., 2010). The effector functions of IL-4 and IL-13 in type 1 hypersensitivity reaction are illustrated in Figure 2.7.

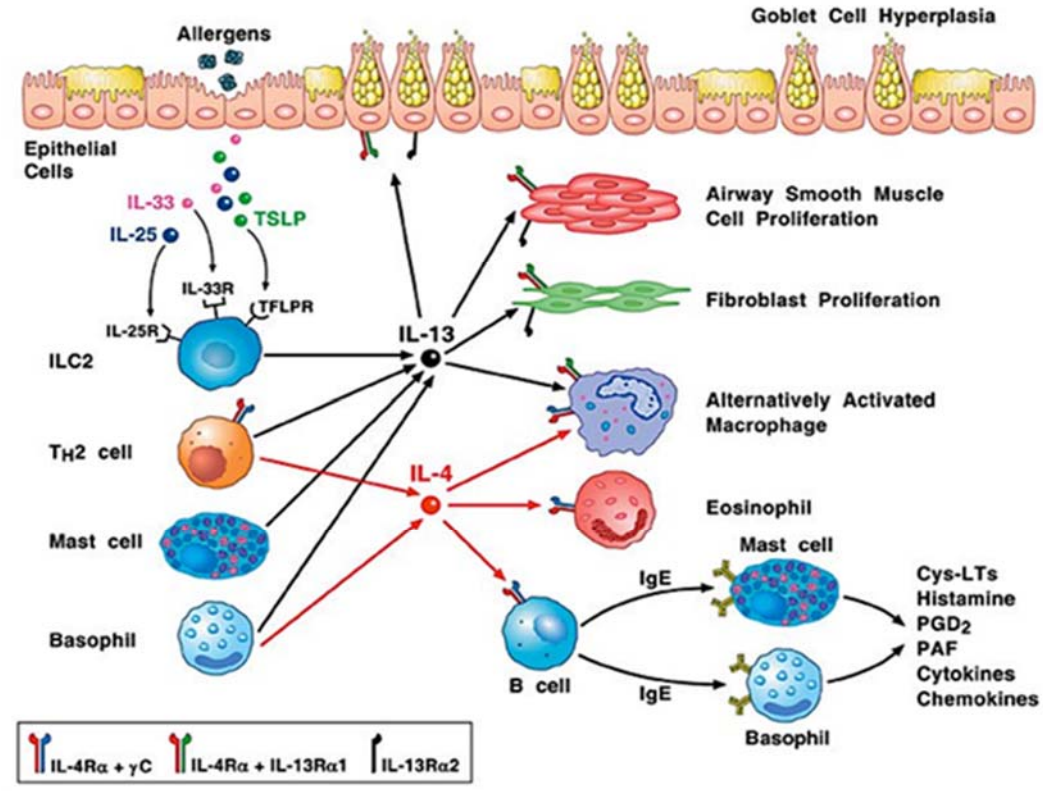

Figure 2.7: The potential cellular effects of IL-4 and IL-13 on inflammatory and structural cells in type 1 hypersensitivity reaction**.** IL-4 is involved in production of IgE, secretin of histamine chemokines and cytokine. IL-3 induces fibroblast proliferation and airway smooth muscle cell proliferation (Adapted from (Bagnasco et al., 2016)).

Previous studies showed elevated IL-4 and IgE levels in patients with allergies (Movérare et al., 2000; Smart et al., 2002; Wisniewski et al., 2015). Notably, the levels of IL-4 in these patients are associated with high levels of IgE and obesity (Benson et al., 1997; Rastogi et al., 2012). The latter has a huge impact in changing the pathophysiology of allergic responses as it skews T_H1/T_H2 towards the T_H1 subset (Rastogi et al., 2012). This is a concern as it has a negative impact on treatment efficacy (Bagnasco et al., 2016; Uzzaman & Cho, 2012; Vaillant, 2020).

2.5.2 Interleukin 13

IL-13 is one of the major cytokines involved in type 1 hypersensitivity and it is closely related to IL-4 (Bao & Reinhardt, 2015). Notably, it shares a lot of immunoregulatory and biological functions with IL-4 in allergic reactions (Bagnasco et al., 2016; Chu et al., 2014). These cytokines regulate the function of B cells (Kips, 2001; Kips et al., 2001; Scott-Taylor et al., 2010). In addition, they are also involved in promoting the growth of basophils and eosinophils (Borish & Steinke, 2001; Lee et al., 2010; Tsai et al., 2005). However, unlike IL-4, IL-13 is unable to regulate the differentiation of T cells due to the lack of IL-13R on the T lymphocytes (Junttila, 2018). IL-13 is primarily responsible for inducing the production of mucus in the airways, smooth muscle contraction and eosinophilia in allergies (Junttila, 2018).

2.5.3 Interleukin 5

IL-5 is another important cytokine secreted by T_H2 cells and mast cells, it promotes the maturation, activation and proliferation of eosinophils (Ansotegui et al., 2020; Kips, 2001). Notably, the levels of IL-5 are elevated in individuals with allergies and are associated with eosinophilia (Kanari et al., 2010; König et al., 2015; Kouro & Takatsu, 2009). IL-5 stimulates eosinophil production and maturation in the bone marrow (Kips, 2001). Matured eosinophils are then induced by IL-5 to move into blood circulation (Roufosse, 2018). Thereafter activated eosinophils migrate to the site of inflammation where they release their granular contents and inflammatory mediators that induce allergy symptoms (Bagnasco et al., 2016; Kips et al., 2001) (Figure 2.8). Mast cells are activated through cross linking of FceRI by allergen specific IgE which results in the release of inflammatory mediators (Broide, 2007). To maintain eosinophilia in allergic inflammation, IL-5 is responsible for prolonged eosinophil survival and decreased apoptosis (Nechama et al., 2018).

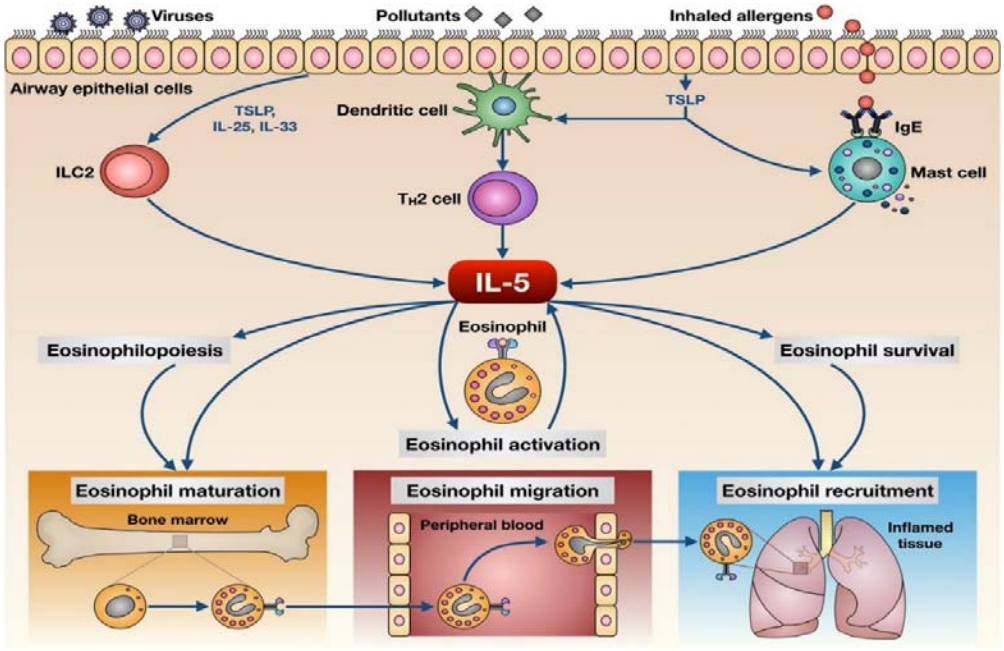

Figure 2.8: Effector functions of IL-5 on eosinophils. The secretion of IL-5 by T helper (T_H)2 is triggered once allergens presented to it by dendritic cells. Thereafter, IL-5 induce eosinophilopoesis and the maturation of eosinophils. Mast cells are activated through the cross-linking of FceRI by allergen specific IgE which results in the release of inflammatory mediators. (Adapted from (Pelaia et al., 2019).

2.6 Diagnosis of type 1 hypersensitivity reaction

Currently, the diagnosis of type 1 hypersensitivity is based on medical history, physical examination, skin prick test, oral food challenge and serum IgE levels (Boyce, 2010). Medical history is essential when allergy is suspected, it is presented to the physician by the patient whereby the patient is asked to answer certain questions including clinical and dietary history (Portnoy, 2015a). The physician examines the patient physically to assess the signs and symptoms of allergy (Boyce, 2010). Finally, laboratory tests such as serum total IgE test and Radioallergosorbent test (RAST) are ordered (Naspitz et al., 2004). The algorithm for the diagnosis of type 1 hypersensitivity is descried in Figure 2.9 below.

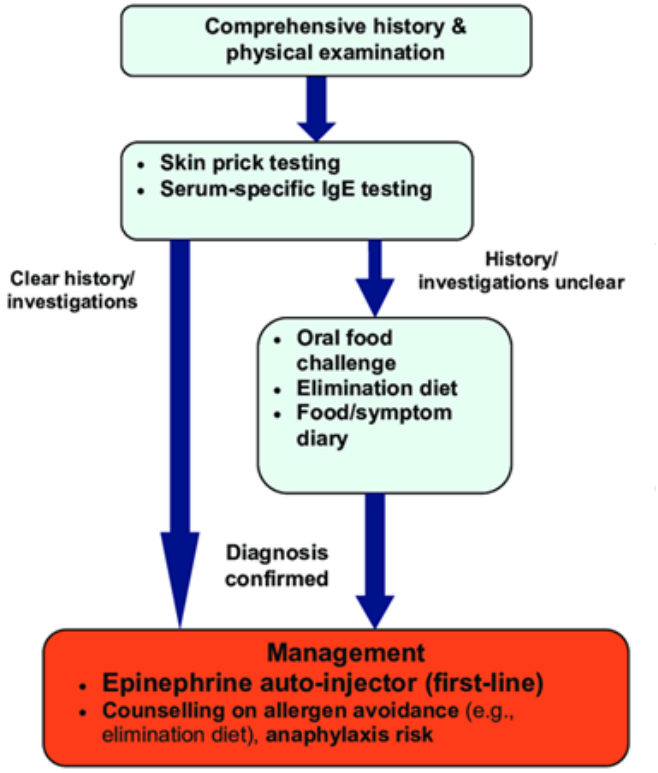

Figure 2.9: Algorithm for allergy examination and management**.** This figure outlines the steps involved in allergy management where physical examination is contacted and the positive results are confirmed by the laboratory tests (Adapted from (Waserman & Watson, 2011)**.**

2.7 The management and treatment strategies of allergic reactions

2.7.1 Cytokine-biologics

The hallmark of several therapeutic strategies against allergic reactions aim to alleviate the associated symptoms modulated by T_H2 mediated inflammation (Voskamp et al., 2020). In fact, the use of allergen-specific immunotherapy and anti-inflammatory drugs in patients with allergies is effective in modulating the activity of T_H2 cytokines and inhibiting the activation of eosinophils, basophils, mast cells and IgE secreting B-cells (Benson et al., 1997; Horiguchi et al., 2008; Oda et al., 1998; Wambre et al., 2017; Wang & Shi, 2017; Wisniewski et al., 2015). Of particular interest are treatment strategies that antagonises the effects of IL-4 and IgE in allergic responses (Bedoret et al., 2012).

The therapeutic inhibition of these biologics is associated with the alleviation of allergy-associated symptoms, the reduction in eosinophil counts and the correction of the T_H1/T_H2 imbalance (Chiricozzi et al., 2020; Wisniewski et al., 2015). However, the levels of IL-4 and IL-5 cytokines in atopy are controversial. In fact, others have reported IL-4/IL-13 mediated immune response in allergic responses

(Braddock et al., 2018; Wisniewski et al., 2015) whilst others suggested an IL-5 weighted (Roufosse, 2018; Smart et al., 2002; Voskamp et al., 2020; Wambre et al., 2017). This discrepancy seems to be dependent on the type of allergen. For example, high levels of IL-4 and 13 in serum are noted in patients allergic to house dust mite (HDM) (Rastogi et al., 2015; Wang & Shi, 2017), whereas, elevated levels of IL-5 is apparent in patients allergic to pollen (König et al., 2015; Li et al., 1998; Tang et al., 1998). There is limited literature available on associations between IgE, IL-4 levels, inflammatory state, and specific allergens in patients with allergies (Deo et al., 2010). A deep understanding of this will pave a way in improving therapeutic strategies that aim to alleviate allergic symptoms.

2.7.2 Antihistamines

Anti-histamines are designed to alleviate allergy symptoms by blocking the binding of histamines to their receptors (Blanca-López et al., 2015; Kirmaz et al., 2011; Noma et al., 2001). Histamines are released during the degranulation of mast cell and basophils in defence of an allergen (Buelow, 2015).Notably, this mediator is only released upon the second and subsequent exposure to the same allergen (Haselden et al., 2001; Moon et al., 2014). In type 1 hypersensitivity, histamines are responsible for triggering smooth muscle contraction, vasodilation, increased vascular permeability and mucus production (Bagnasco et al., 2016; Blanca-López et al., 2015; Kuperman & Schleimer, 2008). Their use is known to effectively treat rhinorrhoea, sneezing, nasal itching and eye symptoms, albeit less effective in relieving nasal congestion (Boyce, 2010; Navinés-Ferrer et al., 2016; D. Ryan et al., 2013; Van De Pol et al., 2012). Oral anti-histamines are considered safe and effective in children, however Terfenadine and astemizole are not approved in many countries due to their adverse side effects (D. Ryan et al., 2013). Anti-histamines may also be administered via intranasal in allergic reactions (Hu et al., 2018).

2.1 Conclusion

It is apparent that IL-4 and IgE play a pivotal role in the pathogenesis of allergic reactions. However, it remains to be elucidated whether their levels and activity in type 1 hypersensitivity is dependent on the allergen. Furthermore, a deep understanding of the relationship between inflammatory state, allergen type, IgE and IL-4 level may pave a way for a better therapeutic strategy for type 1 hypersensitivity. This study therefore aimed to evaluate T_H2 immune responses in children with atopy.

Chapter 3: Materials and methods

3.1 Study design and population

This was an observational study involving children (< 18 years old) with allergies that were tested at Windhoek Central Hospital, Namibia between October 2020 and May 2021. The study recruited a total of sixty participants (n = 60) of which fifty (n = 50) had allergy and ten (n = 10) were controls. The Namibia Institute of Pathology (NIP) laboratory at the Central Hospital is the only national laboratory that test for allergies; thus, the samples were a true representation of the Namibian population. All of the patients with allergies were confirmed using the cut-off point of 0.35 kUA/l (Nilsson et al., 2004). The ethical clearance for this study was obtained from the Namibia University of Science and Technology (NUST) Research Ethics committee, reference number (FHAS /2020) (see Appendix 1), the Ministry of Health and Social Services (EPN 2020) (see Appendix 2) and from NIP ethics committee (see Appendix 3). The study was conducted based on the Code of Ethics for the World Medical Association (Declaration of Helsinki 2013). Patients' confidentiality was maintained by using laboratory barcode numbers instead of their names for identification.

3.1.1 Inclusion Criteria

The study included children that were younger than 18 years old with or without allergy. In order to minimise bias, the samples were collected from the participants during the same season. Participants were recruited into the allergy or control group if their phadiatop values were >0.35 kUA/L or <0.35 kUA/L, respectively.

3.1.2 Exclusion criteria

All adult patients (> 18 years old) were excluded from this study since the immune response is influenced by age (De Martinis et al., 2017; Ventura et al., 2017).

3.1.3 Sample size

The sample size was determined using the prevalence of allergy in Kenya (Odhiambo et al., 1998), which has the same geographic and socioeconomic status as Namibia, using the formula adopted from (Alemseged et al., 2015). The sample size was calculated using the formula:

$$n = (\frac{(z)^2 * p(1-p)}{d^2})$$

$$n = (\frac{(z)^2 * p(1-p)}{d^2})$$

$$n = \left(\frac{(1.96)^2 * 0.0342(1-0.0342)}{0.05^2}\right)$$

$$n = \left(\frac{3.8416 * 0.03303}{0.0025}\right)$$

$$n = \left(\frac{0.126888}{0.0025}\right)$$

$$\underline{\boldsymbol{n = 50.72}}$$

Therefore: n = 51 patients

Where: n= Number of participants to be tested

Z=value of 95% confidence interval (1.96)

P= Proportion of children with allergy (3.42%) = 0.0342

D= Absolute sampling error (Margin of error) that can be tolerated (5%) =0.05.

3.1.4 Sample and data collection

Blood for testing was collected into 5 ml serum separator tubes (SST) by qualified registered/ enrolled nurses. As per the standard operating procedure, the samples were transported to the laboratory within the time frame of 2-3 hrs at a temperature of 2-8 °C. Upon receipt, patient's details were recorded on the laboratory information system (MEDITECH) and the SST tubes were spun at 3000rpm for 10 minutes before analysis to separate serum from the red blood cells. The samples were stored at a temperature of 2-8 °C if the test was delayed and were allowed to reach the room temperature prior testing. The samples were tested for total immunoglobulin (Ig)E and specific IgE for phadiatop, pollen, mould spores, epithelia, and house dust mite, as well as interleukin (IL)-4. The patients' characteristics (age and gender) were retrieved from MEDITECH and recorded into the data extraction template that was developed for this study. Patients' confidentiality was maintained by using computer-generated laboratory barcodes instead of their names for identification.

3.2 Laboratory measurements

3.2.1 Immunoglobin E measurements

The total IgE and specific IgE levels were measured in an ISO 15189:2012 accredited NIP laboratory by the researcher using the ImmunoCap Phadia 250 analyser (Phadia, Uppsala, Sweden). The analyser used the enzyme linked immunosorbent assay (ELISA) principle. All calibrations for the instrument were done and internal quality controls were run prior to samples being analysed, as per the laboratory's standard operating procedure. For the total IgE levels, low, medium, and high controls were used, whilst for specific IgE levels, cat dander, betula verrucosa and house dust mite were used as a low, medium, and high-level controls, respectively.

3.2.2 The determination of IL-4 levels

The levels of IL-4 were determined using the Invitrogen human IL-4 uncoated ELISA kit (Thermo Fisher Scientific, Waltham, MA USA). The kit is based on the sandwich ELISA concept where two antibodies, a capture antibody and a detection antibody are used. Briefly, IL-4 ELISA kit is designed to measure the amount of the target bound between a matched antibody pair. The testing wells on the microtiter plate are coated with the target-specific antibody and incubated overnight, the unbound antibodies were washed off using the Biotek ELx50 automatic plate washer (Biotek, Winooski, Vermont, United States). The unbound protein binding sites on the surface are blocked to reduce background and nonspecific binding. 100µL of samples, standards, and controls were then added into these wells and bound to the immobilized (capture) antibody. Meanwhile, 100µL of ELISA diluent was added to blank wells to rule out diluent contamination. The plate was sealed and incubated for 2 hours at room temperature to allow antigen to bind to the capture antibody. Unbound antibodies were then washed off before the 100µL of detection antibody was added. The plate was sealed again and left to incubate for 1 hour at room temperature. Thereafter, the plate was washed and the 100µL enzyme (streptavidin-HRP) was added to the reaction wells and then sealed to incubate for 30 minutes at room temperature. Finally, the substrate was added, and then incubated for 15 minutes before the stop solution was added to stop the reaction. After the addition of the stop solution, the microtiter plate was loaded unto the Biotek ELx800 microtiter plate reader (Biotek, Winooski, Vermont, United States) and absorbances were read at 450nm, See detail in manufacturer's Instructions (appendix 4). The measured parameters and the methods of testing used in this study are indicated in Table 3.1.

Table 3.1. The principles used in the laboratory measurements

Parameter	**Testing method used**	**Instrument used**
Total Immunoglobulin E	Enzyme linked immunosorbent assay	Phadia 250- (Phadia, Uppsala, Sweden)
Specific Immunoglobulin E	enzyme linked immunosorbent assay	Phadia 250 (Phadia, Uppsala, Sweden)
Interleukin-4	Enzyme linked immunosorbent assay	Biotek plate washer and Biotek plate reader (Biotek, Winooski, Vermont, United States)

3.3 Statistical Analysis

Normality testing for data distribution was performed using D'Agostino & Pearson test and all data were expressed as mean ± SD. Parametric data was analysed using the two-tailed independent student's t-test. The One-way analysis of variance (ANOVA) test was used to assess the comparison between different allergens. A posthoc Tukey's multiple comparisons test was performed if the F-value reached statistical significance ($p<0.05$). The spearman coefficient test was used to evaluate the associations between two non-parametric variables (correlation). A p-value of < 0.05 was considered statistically significant. All statistical analysis were performed using Graph Pad Prism 8 version 8.0.2 Software, (Graph Pad Software Inc, San Diego, CA, USA).

Chapter 4: Results

4.1 Characteristics of included participants

A total of 60 participants were recruited in this study, of which 50 were patients with allergy with a mean age range of 9.50 ± 3.90 years and a male to female ratio of 1.17 were included. A total of 10 participants were used as controls and their mean age was 5.67 ± 6.09 with a male to female ratio of 0.70 (Table 4.1). The total Immunoglobulin (Ig) E for included patients was 298.40 ± 133.70 while for control was 104.30 ± 50.61. The Interleukin (IL)-4 level for patients was 2.55 ± 0.25, while for control was 2.53 ± 0.15. The characteristics of participants are well summarised in Table 4.1.

Table 4.1: Characteristics of included participants (n=60)

	Control group (n=10)	**Patients with allergy (n=50)**	**P value**
Male % (n)	70% (7)	54% (27)	0.004
Age (years)	5.67 ± 6.09	9.50 ± 3.90	<0.0001
Total immunoglobulin E (kU/l)	133.70 ± 50.61	298.40 ± 104.30	0.0312
Interleukin-4 (pg/ml)	2.53 ± 0.15	2.55 ± 0.25	0.7836

As expected, the levels of total IgE were elevated in children with allergies (298.40 ± 104.3 kU/l) when compared to those without (133.70 ± 50.61 kU/l), p = 0.0312 (Table 4.1, Figure 4.1A). To assess T-helper 2 (T_H2) mediated inflammation in allergic reactions, the levels of IL-4 were measured. Notably, the levels were comparable between the two groups (p = 0.7836) (Table 4.1, Figure 4.1B).

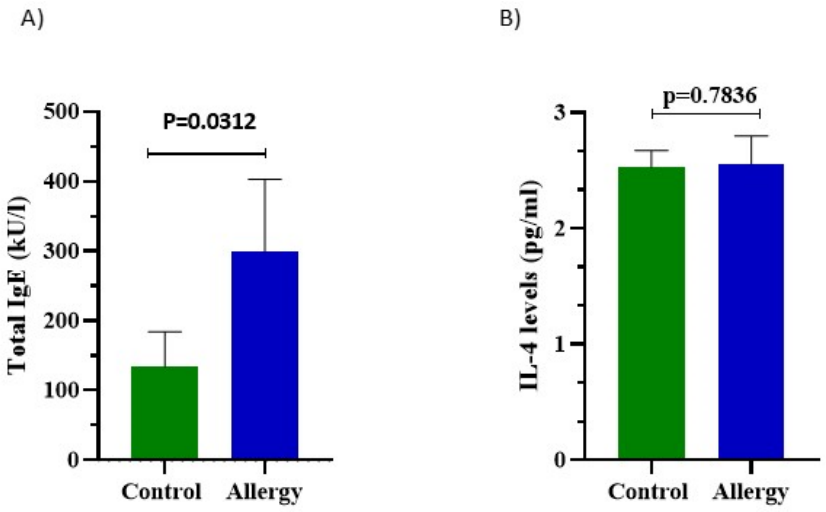

Figure 4.1: A comparison of total immunoglobulin E (IgE) (A) and interleukin 4 (IL-4) levels (B) in children with allergy versus those without. Results are reported as mean ± standard deviation.

4.2 Basic characteristics of included patients

A total of 50 patients were included in this study, and over two thirds of these individuals were polysensitised. Forty-one (84%) of included patients were allergic to grass pollen and had a mean age of 9.43 ± 4.07 years and a male to female ratio of 1.10. Thirty-four patients (68%) were allergic to weed pollen and had a mean age and a male to female ratio of 9.35 ± 4.05 and 1.27, respectively. Thirty patients (60%) were allergic to tree pollen whilst 30% (n =15) were allergic to mould and 38% (n = 19 and 66% (n = 33) were allergic to epithelia and house dust mite (HDM), respectively (Table 4.2).

Table 4.2: Characteristics of included patients (n=50)

	Grass pollen	Weed pollen	Tree pollen	Mould spores	Epithelia	House dust mite
Patients (n)	42	34	30	15	19	33
Percentage (%)	84%	68%	60%	30%	38%	66%
Age (Years)	9.35 ± 4.07	9.35 ± 4.05	9.17 ± 4.15	8.89 ± 5.09	9.32 ± 4.35	9.52 ± 4.09
Male % (n)	52% (22)	56% (19)	57% (17)	73% (11)	58% (11)	0.58% (19)

4.3 The levels of total and specific IgE are dependent on the type of allergen

Since mould spores had the least number of patients (n = 15), a total of 15 patients per group were included in this analysis. The levels of total IgE varied across the type of allergen in children with allergies ($F_{(5, 84)} = 7.28$, $p < 0.0001$). The posthoc analysis showed that the least levels of total IgE were observed in children allergic to mould spores (411.00 ± 110.10 kU/l) whilst the highest were found in those allergic to epithelia (690.10 ± 190.20 kU/l). In comparison to mould spores (411.00 ± 110.10 kU/l), higher levels of total IgE were seen in children allergic to weed pollen (665.30 ±178.10 kU/l), p = 0.0002; tree pollen (586.40 ± 144.50 kU/l), p = 0.027); epithelia (690.10 ± 190.20 kU/l), p<0.0001 and house dust mite (632.20 ± 143.90 kU/l), p = 0.002 (Figure 4.2A). Significant differences were also observed between grass pollen and epithelia (503.90 ± 134.40 kU/l), p = 0.015. Further assessment of specific IgE showed significant differences across the allergens ($F_{(5, 84)} = 2.62$, p = 0.0300). The Tukey's test showed that in comparison to patients allergic to weed pollen (2.88 ± 2.07 kU/l), the levels of specific IgE levels were lower in those sensitised to tree pollen (1.35 ± 1.05 kU/l), p =0.0321 and epithelia (1.39 ± 1.02 kU/l), p = 0.0389. Other comparisons were comparable (p>0.05) (Figure 4.2B). T_H2-mediated inflammation is modulated by IL-4, a cytokine that promotes B-cell activation and class switch to IgE. The assessment of IL-4 levels in children with allergy showed comparable quantities amongst the groups ($F_{(5, 84)} = 1.31$, p = 0.2667) (Figure 4.2).

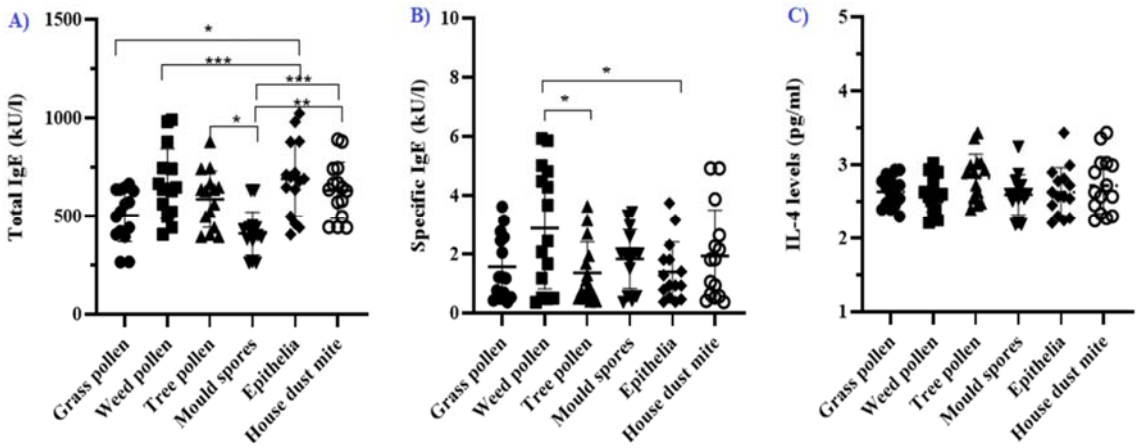

Figure 4.2: The levels of total immunoglobulin (Ig)E and interleukin (IL-4) based on the type of allergen**.** Figure A shows significant differences in the levels of IgE based on the type of allergen whilst figure B shows comparable levels of IL-4 across all allergens. Results are reported as mean +- standard deviation. ***p=0.0002, **p=0.0020, *p<0.05.

4.4 Corelation analysis between total IgE, IL-4 and different types of allergens

A multiple bivariate analysis was performed to evaluate whether there are any significant associations between IL-4, total IgE and the different types of allergens. The analysis showed that IL-4 levels are

negatively associated with total IgE level ($r = -0.23$, $p = 0.0330$). The specific IgE to tree pollen are positively associated with total IgE ($r = 0.35$, $p = 0.0390$) and phadiatop levels ($r = 0.43$, $p = 0.0110$). Lastly, a negative correlation was observed between specific IgE to HDM and phadiatop level ($r= -0.36$, $p = 0.0300$).

Chapter 5: Discussion

T helper 2 (T_H2) inflammation in allergic reactions is characterised by elevated immunoglobulin E (IgE) and interleukin 4 (IL-4) levels which mediate the manifestation of its associated symptoms (Fernández-Benítez, 2001; Kanari et al., 2010; Smart et al., 2002). Therefore, this study aimed to evaluate T_H2 immune responses in children with type 1 hypersensitivity reactions. The study showed that although the levels of total IgE were elevated in children with allergies when compared to those without, the levels of IL-4 were comparable between the groups. Furthermore, total and specific IgE levels were dependent on the type of allergen and were associated with the levels of IL-4. Therefore, it is important to consider the type of allergen in determining an effective dosage when using IgE neutralisation therapy to treat allergic reactions.

The binding of IgE to F_C receptor on the mast cells induce their activation and release of various inflammatory mediators, including histamines (Abdelnoor et al., 2002; Karki, 2018; Sutton et al., 2019). Histamines modulate allergic responses by enhancing the secretion of T_H2 cytokine whilst inhibiting T_H1 cytokine production. Thus, promoting T_H2-mediated inflammation, a hallmark of allergic reactions (Broide, 2007; Thunberg et al., 2007). These changes induce bronchoconstriction, the main symptom of allergic reactions (Bonini et al., 2015). The levels of IgE are very low in healthy individuals but are significantly increased in response to allergen exposure in patients with allergies (Fernández-Benítez, 2001; Khajuria, 2015). As a result, the levels of total IgE are used as one of the reliable biomarker to screen type 1 hypersensitivity reaction (Portnoy, 2015b). This study showed elevated total IgE levels beyond the normal limit (1.5 - 144 kU/L) in children with allergies (Carosso et al., 2007; Kelly & Grayson, 2016). These findings are in agreement with previous studies that also demonstrated higher levels of total IgE in children with IgE-mediated allergy when compared to children without allergies (Kim et al., 2013). However, some previous studies of this type conducted in Africa have indicated that total IgE level can be influenced by different factors such as genetics or obesity status (Portelli et al., 2015). Total IgE was much high in black African and mixed race children as compared to Caucasians children (Levin et al., 2008). The prevalence of obesity in Namibia was 0.9 in 2013 (Mtambo & Debusho, 2021), this could have influenced our results because obesity is associated with the exacerbated release of pro-inflammatory cytokines that modulate chronic inflammation (Nyambuya et al., 2020; Wellen & Hotamisligil, 2005).

The levels of allergen specific IgE levels in children with allergies varied across the types of allergens. This revelation is important when it comes to treatment that antagonises IgE activities and the determination of dosage thereof. To increase treatment efficacy, patients that are allergic to weed pollen should be

given high doses of drugs that inhibit IgE activity, whilst those allergic to mould spore and epithelia should be given low dosages, whereby total IgE is higher in polysensitized patients as compared to monosensitised patients (Novikova et al., 2020). However, allergies are highly influenced by one's socioeconomic status, parents' education level, geographical area and parents' income which were never considered in this study (Kim et al., 2013; Taborda-Barata & Potter, 2012). This current study also showed that the levels of IgE specific to tree pollen was directly proportional to total IgE in patients allergic to tree pollen. Although there is an association between total IgE levels and specific IgE to different allergens, it was noted that different allergens evoke different allergy reactions (Khasawneh et al., 2019). A recent systematic review and meta-analysis also proved that allergic reactions are significantly influenced by the type of allergen (Nepolo et al., 2022).

One of the major T_H2 major cytokine, IL-4, plays an important role in the pathogenesis of type 1 hypersensitivity reaction (Cardoso et al., 2009; Kips, 2001). The cytokine is responsible for B-cell activation, promoting and initiating class switching of IgM to IgE and is significantly elevated in type 1 hypersensitivity (Junttila, 2018; Kubo, 2017). Several current therapeutic strategies also aim to primarily ameliorate the effects of IL-4, thus alleviating allergic symptoms and T_H2-mediated inflammation (Bagnasco et al., 2016; Bosnjak et al., 2011; Chiricozzi et al., 2020). Although this current study showed that the levels of IL-4 were comparable between patients with allergies and control group, previous studies have cited exacerbated levels in the former group (Attia et al., 2010; Qiao et al., 2005; Rastogi et al., 2015; Wisniewski et al., 2015). In a recent systematic review and meta-analysis conducted (see Addendum 1) by (Nepolo et al., 2022) it showed elevated IL-4 levels in patients with allergy. This discrepancy may be attributed to the fact that most of the included patients in this study were polysensitised and the lifespan of IL-4 in blood circulation after allergen exposure (Reinhart & Kaufmann, 2018). Notably, IL-4 has a short life span as compared to IgE which was used as diagnosis marker for type 1 hypersensitivity reaction (Reinhart & Kaufmann, 2018). Therefore, the levels of IL-4 might have declined by the time the samples were collected and analysed. Future studies need to consider the sensitisation status of the patients before recruiting them into the study.

5.1 Strength and Limitations

The main strength of this study is that it was the first study to evaluate T_H2-mediated inflammation in children with allergies in Namibia. Moreover, it revealed the association between allergens and the levels of IgE and IL-4. These findings are important as they will aid in improving treatment efficacy of therapeutic strategies that target IgE and IL-4 strategies. Notable, are some limitations distinguished from this study.

Firstly, the study included patients that were polysensitised. This could have attributed to comparable IL-4 between the two groups. It remains to be elucidated whether T_H2-mediated inflammation in Namibia is also dependent on the type of allergen. Secondly, there were no clinical outcomes reported that could have been linked to the measured laboratory parameters. This could have provided crucial information on any associations between disease severity and laboratory parameters, and the impact of treatment and obesity on the immune responses. All samples were collected during the same time, and this did not carter for allergens seasonal variations. Therefore, the reported variations in immune responses may have been influenced by the allergen concentrations during the testing period, particularly seasonal aeroallergens as pollen. Finally, the treatment status of included patients was not known, and this could have influenced the immune responses observed.

5.2 Recommendations

Since data and inferences on allergic reactions is limited in Namibia, there is a need to conduct research in this field. This is important in providing crucial information on the prevalence of allergies and immune responses based on different factors such as geographical location, socioeconomic status, industrialisation, and educational level. Future researchers should consider assessing clinical outcomes of patients, and the sensitisation status of included participants. These will be useful to make a concrete conclusion on how the immune system responds in children with allergies. Also, the type of treatment should be evaluated to assess the effects of treatment and their mode of action in alleviating allergic reactions and symptoms.

Chapter 6: Conclusion

The levels of total IgE in children with allergy are elevated when compared to those without. Notably, these levels together with allergen specific IgE levels are allergen-dependent and are associated with the levels of IL-4, a major cytokine that modulates T_H2-mediated inflammation. Therefore, therapeutic strategies that antagonises IgE activity should be prioritised in children with allergies in Namibia. Most importantly, the type of allergen should be considered when determining effective dosages in therapies that neutralise IgE effector functions.

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Appendices

Appendix 1: Ethics clearance: Namibia University of Sciences and Technology

NAMIBIA UNIVERSITY OF SCIENCE AND TECHNOLOGY
13 Storch Street
Private Bag 13388
Windhoek
NAMIBIA
T: +264 61 207 9111
F: +264 61 207 2444
W: www.nust.na

FACULTY OF HEALTH AND APPLIED SCIENCES RESEARCH ETHICS COMMITTEE (FHAS-REC)

DECISION/FEEDBACK ON RESEARCH PROPOSAL ETHICAL CLEARANCE

Dear: Prof/Dr/Mr/Ms/Other — Ms. Elina Nepolo
NUST Student Number: 200849697

Research Topic:	Investigating T_H2-mediated immune response in children with type 1 hypersensitivity
Supervisor (if applicable):	Dr Maurice Nyambuya
Co-supervisor(s): if applicable	Prof Bongani Nkambule
Qualification registered for (if applicable):	Masters of Health Science.

Re: Ethical Screening Application No: FHAS /2020

The Faculty of Health and Applied Sciences Research Ethics Screening Committee has reviewed your application for the above-mentioned research project. Based on the recommendation of the expert reviewer, the research as set out in the application is hereby:

(Indicate with an X)

Approved: i.e. may proceed with the project, subject to Ministry of Health and Social Service Clearance.	X
Approved provisionally: i.e. may proceed but subject to compliance with recommendation(s) listed below	
Not approved: Not to proceed with the project until compliance with recommendation listed below and resubmit ethics application for consideration	

It is important to note that as a researcher, you are expected to maintain ethical integrity of your research. You are encouraged to strictly adhere to the research ethics policy of NUST. You should remain within the scope of your research proposal and support evidence as submitted to the FHAS-REC. Should any aspect of your research change from the information as presented, which could have an impact or effect on any research participants/subjects/environment, you are to report this immediately to your supervisor and to the FHAS-REC as applicable in writing. Failure to do so may result in withdrawal of approval.

Kindly consult the committee if you need further clarification in this regard. We wish you success in your research endeavour and are of the belief that it will have positive impact on your career as well as the development of NUST and the society in general.

Ethical issues that require compliance/ must be addressed : None

No.	Ethical issues	Comment/recommendation

NB: May attach additional page as required

Sincerely Yours,

Name: Prof Sylvester R Moyo Signature: Date: 4th March 2020
Chairperson: FHAS Ethics Screening Committee.

Appendix 2. Ethics clearance from the Ministry of Health and Social Services

REPUBLIC OF NAMIBIA

Ministry of Health and Social Services

Private Bag 13198
Windhoek
Namibia

Ministerial Building
Harvey Street
Windhoek

Tel: 061 – 203 2507
Fax: 061 – 222558
E-mail: itashipu87@gmail.com

OFFICE OF THE EXECUTIVE DIRECTOR

Ref: ESN 2020
Enquiries: Mr. A. Shipanga

Date: 08 December 2020

Mr. /Ms Elina P. Nepolo
P.O. Box 30885
Pioneerspark

Dear Mr. /Ms Nepolo

Re: Academic Research Proposals Approval –NUST – Masters of Health Sciences.

Title: Investigating T-helper 2 mediated immune responses in children with type 1 hypersensitivity.

1. Reference is made to your application to conduct the above-mentioned study.
2. The proposal has been evaluated and found to have merit.
3. **Kindly be informed that permission to conduct the study has been granted under the following conditions:**

3.1 The data to be collected must only be used for completion of your Masters of Health Sciences;
3.2 No other data should be collected other than the data stated in the proposal;
3.3 No any specimen should be collected from Human Subjects;

3.4 Stipulated ethical considerations in the protocol related to the protection of Human Subjects' information should be observed and adhered to; any violation thereof will lead to termination of the study at any stage;

3.5 A quarterly report to be submitted to the Ministry's Research Unit;

3.6 Preliminary findings to be submitted upon completion of the study;

3.7 Final report to be submitted upon completion of the study;

3.8 Separate permission should be sought from the Ministry for the publication of the findings.

4. All the cost implications that will result from this study will be the responsibility of the applicant and **not** of the MoHSS.

Yours sincerely,

BEN NANGOMBE
EXECUTIVE DIRECTOR

"Health for All"

Appendix 3. Ethics clearance Namibia Institute of Pathology

NAMIBIA INSTITUTE OF PATHOLOGY LIMITED

Reg. No. 2000/431

Tel: +264-61-295 4200 • Fax: +264-61-255 566 • P.O. Box 277 • Windhoek • Namibia • info@nip.com.na, www.nip.com.na

OFFICE OF THE CHIEF OPERATIONS OFFICER

Enquiries: Gerhard van Rooyen; Tel.: 061-295 4228

Ms. Elina Nepolo
Windhoek
Namibia

Date: 26 May 2021

Dear Ms Nepolo

Re: Investigating Th2-mediated immune responses in children with type 1 hypersensitivity

1. The above-mentioned research proposal was referred to the Research\Ethics Committee of the Namibia Institute of Pathology Limited.
2. After review, it is a pleasure to inform you that approval was granted for you to proceed with the research on condition that the following be complied with:
3. Inclusion of data on allergy prevalence in SADC / Namibia if available, as well as information as per the review comments on the attached protocol.
4. Consultation with the office of the Senior Medical Scientist and Senior Manager Specialised Services at Windhoek Central Reference Laboratory upon starting with your research.
5. Observe and adhere to all ethical considerations and confidentiality to protect your clientele information.
6. Report to the Senior Manager: Specialised Services and Senior Medical Scientist upon starting your research.
7. Final report to be shared with the Namibia Institute of Pathology Limited.

Yours Sincerely

.....

Gerhard van Rooyen
Acting Chief Operations Officer

NAMIBIA INSTITUTE OF PATHOLOGY PTY LTD
TECHNICAL OPERATIONS
2021 -05- 26
PO Box 277, Windhoek
Tel: +264-61-2954200
Fax: +264-61-256066
REPUBLIC OF NAMIBIA

Appendix 4. Product information sheet for IL-4 ELISA

invitrogen PRODUCT INFORMATION SHEET

Human IL-4 Uncoated ELISA

Enzyme-linked immunosorbent assay for quantitative detection of human IL-4

Catalog Number 88-7046

Pub. No. MAN0017361 Rev. C.0 (32)

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from thermofisher.com/support.

Product information

Symbol	Contents	Human IL-4 Uncoated ELISA
REF	Catalog number	88-7046
—	Sensitivity	2 pg/mL
—	Standard curve range	2-200 pg/mL
	Temperature limitation	Store at 2-8°C
LOT	Batch code	Refer to vial
	Use by	Refer to box label
	Caution	Contains preservatives

Description

This Human IL-4 Uncoated ELISA contains the necessary reagents, standards, buffers and diluents for performing quantitative enzyme-linked immunosorbent assays (ELISA). This ELISA set is specifically engineered for accurate and precise measurement of human IL-4 protein levels from samples including serum, plasma, and supernatants from cell cultures.

Components of 2-plate format (2x96 tests)

- **Capture Antibody:** Pre-titrated, purified anti human IL-4 antibody
  1 vial (100 µL) Capture Antibody Concentrate (250X)
- **Detection Antibody:** Pre-titrated, biotin-conjugated anti human IL-4 antibody
  1 vial (100 µL) Detection Antibody Concentrate (250X)
- **Standard:** Recombinant human IL-4 for generating standard curve and calibrating samples
  2 vials human IL-4 Standard (lyophilized): 200 pg/mL upon reconstitution
- **Coating Buffer**
  1 vial (2.5 ml) Phosphate Buffered Saline Concentrate (PBS, 10X)
- **5X ELISA/ELISPOT Diluent**
  1 bottle (30 ml) Diluent Concentrate (5X)
- **Enzyme**
  1 vial (250 µL) pre-titrated Streptavidin-HRP Concentrate (100X)
- **Substrate Solution:** Tetramethylbenzidine (TMB) Substrate Solution
  1 bottle (20 ml)
- **96-well plates:** Corning Costar 9018
  2 plates

Components of 10-plates format (10x96 tests)

- **Capture Antibody:** Pre-titrated, purified anti human IL-4 antibody
  1 vial (500 µL) Capture Antibody Concentrate (250X)
- **Detection Antibody:** Pre-titrated, biotin-conjugated anti human IL-4 antibody
  1 vial (500 µL) Detection Antibody Concentrate (250X)
- **Standard:** Recombinant human IL-4 for generating standard curve and calibrating samples
  10 vials human IL-4 Standard (lyophilized): 200 pg/mL upon reconstitution
- **Coating Buffer**
  1 vial (12 ml) Phosphate Buffered Saline Concentrate (PBS, 10X)
- **5X ELISA/ELISPOT Diluent**
  1 bottle (150 ml) Diluent Concentrate (5X)
- **Enzyme**
  1 vial (1.25 mL) pre-titrated Streptavidin-HRP Concentrate (100X)
- **Substrate Solution:** Tetramethylbenzidine (TMB) Substrate Solution
  1 bottle (100 ml)
- **96-well plates:** Corning Costar 9018
  10 plates (only included with product catalog numbers ending in suffixes -86)
  20 plates (only included with product catalog numbers ending in suffixes -76)
  Note: Product catalog numbers ending in suffixes -77 and -88 do not contain any 96-well plates.

Other materials needed

- Buffers
  - Wash Buffer: 1X PBS, 0.05% Tween™-20 or eBioscience™ Wash Buffer (20X) Cat. Nos. BMS408.0500 or 00-0400-46
  - Stop Solution: 1 M H_3PO_4 or 2 N H_2SO_4 or eBioscience™ Stop Solution Cat. Nos. BMS409.0100, SS03, SS03100, or SS04
- Pipettes and pipettors
- Refrigerator
- 96-well plate (Corning™ Costar™ 9018)
  Note: The use of ELISA plates that are not high-affinity protein-binding plates will result in suboptimal performance, e.g., low signal or inconsistent data. Do not use tissue culture plates or low protein absorption plates. Use only the Corning™ Costar™ 9018 or Nunc™ MaxiSorp™ 96-well plates provided or suggested.
- 96-well ELISA plate reader (microplate spectrophotometer)
- ELISA plate washer
- *(Optional)* Microplate shaker

Note: To ensure optimal results from using this kit, use only the components included in the set. Exchanging of components is not recommended because a change in performance may occur.

For Research Use Only. Not for use in diagnostic procedures.

ThermoFisher SCIENTIFIC

Stability

This kit is guaranteed to perform as defined if stored and handled as instructed according to this datasheet and the Certificate of Analysis, which is included with the reagents. Expiration date is indicated on the box label.

Storage instructions for kit reagents

Store at 2-8°C.

Reagent preparation

Note: If crystals form in the buffer concentrate, warm them gently until they completely dissolve.

- **Coating Buffer (1X)**

  Make a 1:10 dilution of PBS (10X) in deionized water.
- **Capture Antibody**

  Dilute capture antibody (250X) 1:250 in Coating Buffer (1X).
- **5X ELISA/ELISPOT Diluent**

  Dilute Diluent Concentrate (5X) 1:5 in deionized water.
- **Standard**

  Reconstitute human IL-4 standard by addition of distilled water. Reconstitution volume is stated on the label of the standard vial. Allow the standard to reconstitute for 10–30 minutes. Swirl or mix gently to ensure complete and homogeneous solubilization (concentration of reconstituted standard = 200 pg/mL).

  Mix well prior to making dilutions. The standard has to be used immediately after reconstitution and cannot be stored.
- **Detection Antibody**

  Dilute detection antibody (250X) 1:250 in ELISA/ELISPOT Diluent (1X).
- **Enzyme**

  Dilute HRP Concentrate (100X) 1:100 in ELISA/ELISPOT Diluent (1X).

Experimental procedure

Note: Shaking is recommended for all incubation steps.

Note: In case of incubation without shaking, the obtained O.D. values may be decreased. Nevertheless the results are still valid.

Note: Be certain that no sodium azide is present in the solutions used in this assay, as this inhibits HRP enzyme activity.

1. Coat Corning™ Costar™ 9018 ELISA plate with 100 µL/well of capture antibody in Coating Buffer (dilute as noted in point 1 of Reagent preparation). Seal the plate and incubate overnight at 4°C.
2. Aspirate wells and wash 3 times with >250 µL/well Wash Buffer.

   Allowing time for soaking (~1 minute) during each wash step increases the effectiveness of the washes. Blot plate on absorbent paper to remove any residual buffer.
3. Block wells with 200 µL of ELISA/ELISPOT Diluent (1X). Incubate at room temperature for 1 hour.
4. Prepare Standard (see Reagent preparation).
5. Aspirate and wash at least once with Wash Buffer.
6. Perform 2-fold serial dilutions of the top standards to make the standard curve for a total of 8 points.

   For that add 100 µL of ELISA/ELISPOT Diluent (1X) to the wells leaving the first wells empty. Add 200 µL/well of top standard concentration to the first empty wells A1/A2. Transfer 100 µL of top standard from wells A1/A2 to wells B1/B2. Mix the contents of the wells B1 and B2 by repeated aspiration and ejection and transfer 100 µL to wells C1/C2. Take care not to scratch surface of the microwells. Continue this procedure 5 times.
7. Add 100 µL/well of samples to the appropriate wells.
8. Add 100 µL of ELISA/ELISPOT Diluent (1X) to the blank well.
9. Seal the plate and incubate at room temperature for 2 hours (or overnight at 4°C for maximum sensitivity).
10. Prepare the Detection Antibody (See Reagent preparation).
11. Aspirate and wash as in Step 2. Repeat for a total of 3-5 washes.

    Allowing time for soaking (~1 minute) during each wash step increases the effectiveness of the washes. Blot plate on absorbent paper to remove any residual buffer.
12. Add 100 µL/well diluted Detection Antibody to all wells.
13. Seal the plate and incubate at room temperature for 1 hour.
14. Prepare the Streptavidin-HRP (see Reagent preparation).
15. Aspirate and wash as in Step 2. Repeat for a total of 3-5 washes.

    Allowing time for soaking (~1 minute) during each wash step increases the effectiveness of the washes. Blot plate on absorbent paper to remove any residual buffer.
16. Add 100 µL/well of diluted Streptavidin-HRP.
17. Seal the plate and incubate at room temperature for 30 minutes.
18. Aspirate and wash as in Step 2, making sure to allow time for soaking for 1 to 2 minutes prior to aspiration. Repeat for a total of 5-7 washes.
19. Add 100 µL/well of 1X TMB Solution.
20. Incubate at room temperature for 15 minutes.
21. Add 100 µL/well of Stop Solution.
22. Read plate at 450 nm. If wavelength substraction is available, substract the values of 570 nm from those of 450 nm and analyze data.

ELISA troubleshooting guide

Problem	Possibility	Solution
High background	Improper and inefficient washing.	Improve efficiency of washing. Fill plates completely, soak for 1 minute per wash, as directed.
	Cross contamination from other specimens or positive controls.	Repeat ELISA, be careful when washing and pipetting.
	Contaminated substrate.	Substrate should be colorless.
	Incorrect dilutions, e.g., conjugate concentration was too high.	Repeat test using correct dilutions; check with manufacturer.
No signal	Improper, low protein binding capacity plates were used.	Repeat ELISA, using recommended high binding capacity plates.
	Wrong substrate was used.	Repeat ELISA, use the correct substrate.
	Enzyme inhibitor present in buffers; e.g., sodium azide in the washing buffer and Assay Diluent inhibits peroxidase activity.	Repeat ELISA, make sure your system contains no enzyme inhibitor.
Very weak signal	Improper and inefficient washing.	Make sure washing procedure is done correctly.
	Incorrect dilutions of standard.	Follow recommendations of standard handling exactly as written in Reagent preparation and Experimental procedures.
	Insufficient incubation time.	Repeat ELISA, follow the protocol carefully for each step's incubation time.
	Incorrect storage of reagents.	Store reagents in the correct temperature, avoid freeze and thaw, avoid using the frost free freezer.
	Wrong filter in ELISA reader was used.	Use correct wavelength setting.
	Wrong plate used.	Use the recommended Corning™ Costar™ 9018 or Nunc™ MaxiSorp™ flat bottom 96-well plates.
Variation among replicates	Improper and inefficient washing.	Make sure washing procedure is done correctly.
	Poor mixing of samples.	Mix samples and reagents gently and equilibrate to proper temperature.
	Plates not clean.	Plates should be wiped on bottom before measuring absorbance.
	Improper, low binding capacity plates were used.	Use recommended high binding capacity plates.
	Reagents have expired.	Do not use if past expiration date.
Variation of kit performance	Different buffers, plates. Handling can strongly affect kit performance.	Use recommended buffers, plates, and kit components.

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  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

  Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at www.thermofisher.com/us/en/home/global/terms-and-conditions.html. If you have any questions, please contact Life Technologies at www.thermofisher.com/support.

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www.all-imm.com

ORIGINAL ARTICLE

Association between the type of allergen and T-helper 2 mediated inflammation in allergic reactions: a systematic review and a meta-analysis

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Received 22 June 2021; Accepted 19 October 2021
Available online 1 January 2022

KEYWORDS
Allergen; interleukin-4; interleukin-5; inflammation; T-helper 2 cells; type 1 hypersensitivity

Abstract

Objectives: To determine whether the levels of T-helper (T_H) 2 cytokines (interleukin (IL)-4 and IL-5) in allergic reactions are allergen dependent and evaluate the impact of various treatment strategies on the levels of these cytokines.

Methods: The PubMed search engine was used from inception until January 2021. The random-effects residual maximum likelihood model was performed, and effect sizes were estimated using the Hedge's *g* statistic. All data analysis was performed using STATA 16.0 (StataCorp LP, TX, USA).

Results: Fourteen studies reporting on 794 participants were included in this study. House dust mite was associated with eliciting a stronger immune response mediated by both IL-4 and IL-5 when compared to pollen. Whereas a mixture of house dust mite and pollen was associated with IL-4-weighted inflammation. Comparisons of IL-4 and IL-5 levels amongst the allergens showed significant differences. The treatment with anti-corticosteroids or allergen-specific immunotherapy was effective in normalising the T_H2 responses and alleviating allergy symptoms.

Conclusion: T_H2-mediated inflammation in allergic reactions is allergen-dependent. Therefore, the type of allergen should be considered when using cytokine-targeting biologics in allergic reactions.

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Introduction

A balance between T-helper (T_H)1 and T_H2 immune responses is important in modulating inflammation and maintaining immune homeostasis.[1] An alteration in the T_H1/ T_H2 ratio is a hallmark of several immune-mediated diseases, including immediate hypersensitivity disorders.[2,3] In fact, allergic reactions are characterised by an exacerbated T_H2-skewed immune response and the manifestation of allergic symptoms such as atopic dermatitis, allergic rhinitis and asthma.[4,5] Notably, the resulting inflammatory milieu is modulated by interleukin (IL)-4 and IL-5, the two major cytokines that mediate T_H2-mediated inflammation.[6] The aggravated elevation of these cytokines is associated with increased activation of B-cells, eosinophils, basophils and the secretion of immunoglobulin (Ig)G and IgE in allergic reactions.[7-9] As a result, several current therapeutic strategies aim to alleviate these symptoms and ameliorate T_H2-mediated inflammation.[10-13]

Although allergic responses are characterized by a predominant T_H2 response, there are some discrepancies with regards to the levels of IL-4 and IL-5 in patients with allergies. For instance, the allergic responses in patients hypersensitive to house dust mite (HDM) are associated with elevated serum IL-4 and IL-13- levels.[14,15] Whereas increased IL-5 levels have been reported in patients allergic to pollen.[16] A comparison between patients allergic to HDM and pollen showed that the levels of IL-5 are in fact elevated in the former group.[17] In patients allergic to peanuts, the immune response is skewed towards IL-4,[10] albeit others suggested it to be IL-5-weighted.[18] Therefore, these inconsistencies suggest that the immune responses in allergic reactions may be dependent on the type of allergen.

The use of allergen-specific immune therapy, antihistamines, and corticosteroids in allergic reactions has been to a greater extent successful in alleviating T_H2-mediated inflammation and the associated symptoms, albeit poor efficacy due to various factors such as age and obesity has been noted in some patients.[19,20] Consequently, cytokine-targeting biologics are currently being explored as an alternative treatment approach in allergic reactions.[11,21,22] In this systematic review and meta-analysis, we aimed at assessing available literature reporting on T_H2-mediated inflammation in allergic reactions. The primary objective of this study was to investigate whether the levels of IL-4 and IL-5, the key cytokines that mediate inflammation in an allergic response, are allergen-dependant. The secondary objective was to assess the impact of treatment on the levels of these cytokines.

Methods

This systematic review and meta-analysis was prepared following the preferred reporting items for systematic reviews and meta-analysis (PRISMA) guidelines.[23] A protocol was designed and agreed upon by all authors before conducting the qualitative and quantitative synthesis (Appendix 1). The protocol was however not registered. We conducted a comprehensive and systematic search of available literature to answer the following research question: are T_H2 immune responses in allergic reactions influenced by the type of allergens?

Sources of evidence and search strategy

A comprehensive search was designed and independently conducted by two reviewers (EPN and TMN) using the PubMed search engine from inception until January 2021.

The following search terms were used to retrieve all relevant studies; "allergy", "allergens", "IL-4, "IL-5", "T_H2" and "type 1 hypersensitivity". Moreover, grey literature and the reference lists of included studies were scanned to identify any additional studies. No language restrictions were applied on the search strategies, and a third reviewer (BBN) was consulted for arbitration in cases of disagreements. A detailed MEDLINE search strategy on PubMed search engine is shown in Supplementary Table S1.

Eligibility criteria and study selection

Two independent investigators (EPN and FN), with the help of third reviewer (TMN), screened all titles and abstracts of the retrieved studies. Studies were included if they reported on the levels of both IL-4 and IL-5 in patients of all ages with allergies.

Inclusion and exclusion criteria

The following PICO was used in the inclusion criteria:

Participants: patients of all ages with allergies
Intervention: none
Comparisons: healthy individuals without allergies (controls).
Outcome: T_H2-mediated inflammation

We excluded reviews, case studies, letters to the editor and animal studies from this systematic review and meta-analysis. In addition, studies that were not relevant to the topic of interest or with no suitable comparator were also excluded.

Data extraction and management

Two independent reviewers (EPN and FN) extracted detailed study information and characteristics using a predefined data extraction form adapted from the Cochrane Consumers and Communication Review Group data extraction for included studies template.[24] The extracted data consisted of names of authors, publication year, study design, study size, age and gender, types of allergens, IL-4 and IL-5 levels and the main findings of each study. The extracted data items were verified by FN, and disagreements were resolved through discussions or by consulting the third reviewer (TMN).

Assessment of risk of bias

The risk of bias in all included studies was independently assessed by two reviewers (EPN and FN) using the modified Downs and Black checklist.[25] The checklist consists of four domains, reporting bias, external validity, internal validity and selection of bias. The studies were rated as follows: excellent (a score between 24–27), good (score of 19–23), fair (score of 13–18) or poor (score of <12). A third reviewer (PVD) was consulted for arbitration in cases of disagreements. The Cohen's kappa scores were used to measure inter-rater reliability, and a score of 0.00 was considered poor, slight (0.01–0.20), fair (0.21–0.40), moderate (0.41– 0.60), substantial (0.61–0.80) and perfect (0.81–1.00).[26]

Statistical analysis

The mean and standard deviation for each effect measure was either extracted or estimated using GetData Graph Digitizer software or calculated from the median range using Hozo et al.'s method.[27] The continuous outcome was reported as standardised mean differences and 95% confidence interval (CI), and effect sizes were estimated using the Hedge's *g* statistic to correct for small study bias. A random-effects residual maximum likelihood (REML) model was performed to minimise the bias of variance estimator. A sensitivity analysis was performed based on study designs to evaluate the robustness of the reported overall effect estimates. A P value of < 0.05 was considered significant. All data analysis was performed using STATA 16.0 (StataCorp LP, TX, USA).

Results

Selected studies

The search identified a total of 71 studies which were assessed for eligibility. A total of 50 studies were excluded at the abstract screening stage as these were not describing findings related to the outcomes of the present study. The remaining 21 studies were assessed for eligibility using full texts and a total of seven citations were excluded because five were not relevant to the topic of interest, while two studies had no suitable comparators. As a result, only 14 studies met the inclusion criteria and were included in this systematic review and meta-analysis (overall agreement, 97.62%; kappa=0.95), and only eight were included in the quantitative synthesis (Figure 1).

Characteristics of included studies

The included studies consisted of two randomised controlled trials (RCTs), three non-RCTs and nine observational studies published in peer-reviewed journals between 1997 and 2017. Of these studies, four were from Asia,[12,15,28,29] three from Europe,[13,30,31] five from North America[10,14,16,18,32] and two from Oceania.[17,33] A total of 794 participants with a male/female ratio of 0.53 were included in this systematic review and meta-analysis. Of which, 565 participants had allergies, and 229 were healthy controls. The cohort consisted of 64% of the participants being children (Table 1) and 36% adults (Table 2) with an average age of 11.10 ± 4.44 years and 44.03 ± 17.45 years, respectively. A total of 26% of patients from the allergic group were allergic to pollen, 29% to HDM, 4% to

peanut and 13% consisted of a mixture of patients allergic to pollen and HDM, whilst 28% were not specified.

Study quality and risk bias

The median score range of included studies was 15 (14-24) out of a possible score of 27 (Supplementary Table S2).[14-24] All studies were rated as fair[10,12, 13-18, 28,31-33] except for one study which was scored as good.[29] Thus, the included studies had a moderate risk of bias. Assessments based on the four domains showed that the included studies had a low risk of reporting bias with a median score of 7.5 (6–10) out of the possible score of 11 (overall agreement, 92.85%; kappa=0.86) and internal validity bias, a median of 4 (3–6) out of the possible score of 7 (overall agreement, 92.85%; kappa = 0.86). However, the studies had high risk of external validity bias with a median of 1 (0–2) out of the possible score of 3 (overall agreement, 88.10%; kappa = 0.76) and selection bias with a median of 2.5 (2–6) out of the possible score of 6 (overall agreement, 97.62%; kappa = 0.95).

The levels of IL-4 in patients with allergies are influenced by the type of allergen

Most of the included studies (36%) involved patients allergic to pollen, followed by those allergic to HDM (29%) and peanuts (14%). Interleukin-4 shares common receptor and functional properties with IL-13,[34] and 57% of the included studies reported elevated levels of both cytokines. Allergic responses irrespective of the allergens were reported to be IL-4-weighted in 36% of the included studies,[10,14,15,29,32] whereas a few reported undetectable low levels.[17,18] Notably, the levels of IL-4 and IL-13 were associated with those of allergen-specific IgE[10,31] and disease severity.[12] We performed a subgroup analysis based on the type of allergen and participants' characteristics. The test for subgroup differences was only significant in the allergen subgroup (P = 0.01), which showed that the type of allergen in these patients influenced the levels of IL-4 and had an impact on the reported effect size. Studies that included patients allergic to HDM (Hedges' *g*: 0.68 [95% confidence interval [CI]: 0.12, 1.24], P = 0.018) and those that involved a mixture of patients allergic to pollen and HDM (Hedges' *g*: 1.02 [95% CI: 0.39, 1.65], P = 0.002) had a large increase in IL-4 levels than healthy controls. However, pollen (P = 0.579) and unspecified allergens (P = 0.065) did not affect the reported effect measure of T_H2 immune responses (Figure 2). We further compared the standardised means of IL-4 levels in each antigen. Notable differences were observed in pollen versus HDM (P = 0.0430) versus pollen and HDM (P = 0.0009) and versus unspecified antigens (P = 0.0002). In addition, HDM vs. unspecified antigens (P = 0.0024) (Supplementary Table S3). We performed a sensitivity analysis based on the study design. Notably, Hedges's *g* did not change direction nor the magnitude of effect size (Supplementary Table S4).

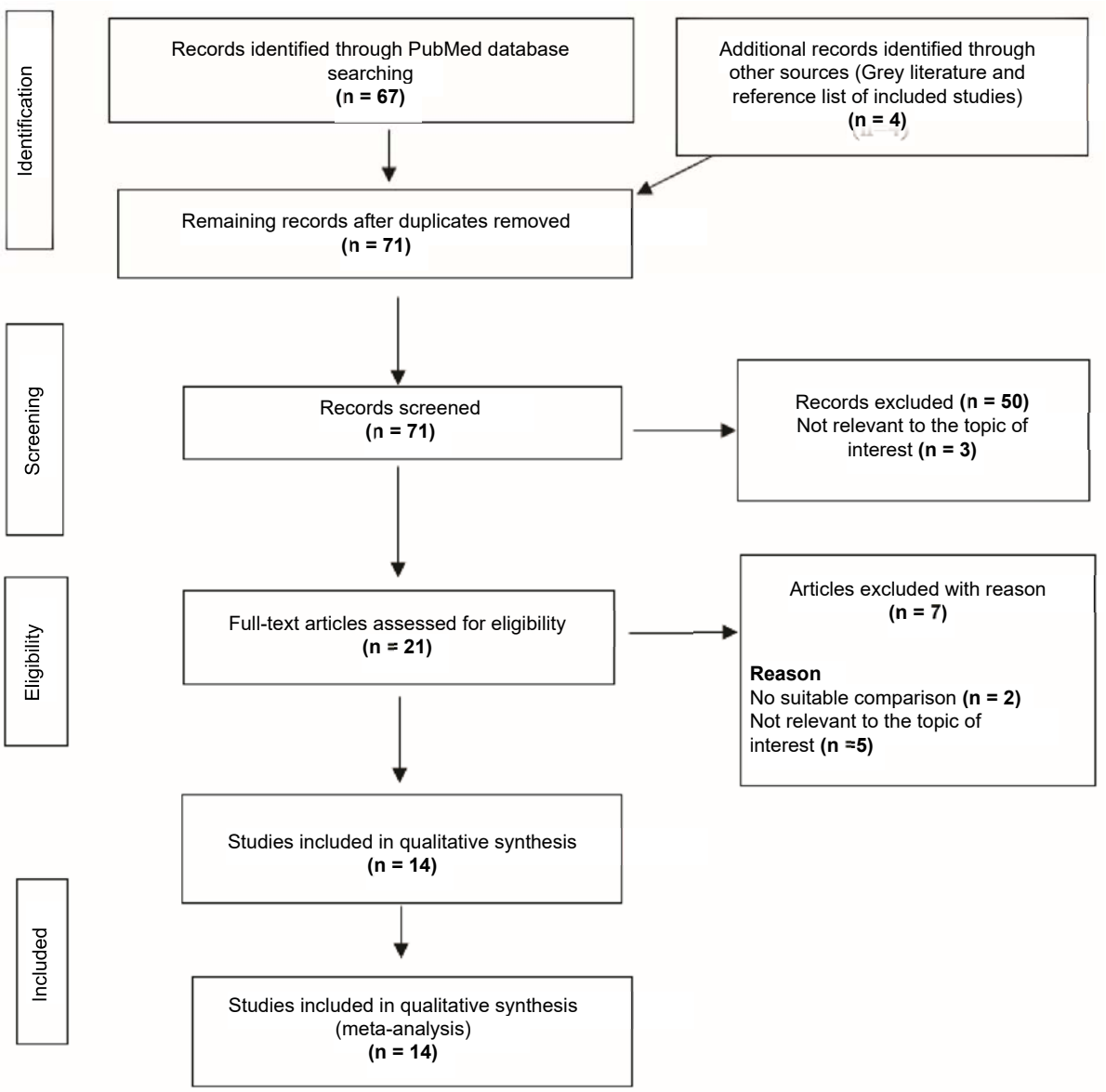

Figure 1 PRISMA flow diagram showing the study selection process.

Different types of allergen influence IL-5 levels in patients with allergies

A total of 43% of the included studies[12,16-18,28,33] reported IL-5-weighted immune responses in patients with allergy, whereas 14% reported on comparable or undetectably low levels.[14,15] The elevated levels of IL-5 in patients with allergy were also associated with increased levels of IgE,[17] eosinophils[12,13] and disease severity.[28,33] A comparison between patients allergic to HDM and pollen showed a significant increase in IL-5 levels in the former group.[17] We, therefore, performed a meta-analysis to assess the levels of IL-5 in patients with allergies. Similarly to IL-4, the quantitative synthesis of included studies revealed that IL-5 levels are dependent on the type of allergen and not participants' characteristics. The test for subgroup differences in the allergen subgroup was significant (P = 0.01), suggesting that allergens modified the reported effect size of T_H2 immune responses. Studies involving participants allergic to HDM (Hedges' *g*: 0.80 [95% CI: 0.02, 1.59], P = 0.044) and unspecified allergens (Hedges' *g*: 2.04 [95% CI: 1.10, 2.98], P < 0.01) had a large increase in the levels of IL-5 versus healthy controls (Figure 3). However, studies including patients allergic to pollen (P = 0.857) or a mixture of patients allergic to pollen and HDM (P = 0.082) did not modify the effect estimate. Comparisons of standardised means of IL-5 levels amongst the allergens showed significant differences in unspecified antigens versus pollen (P <
0.0001) versus HDM (P = 0.0086) versus pollen and HDM (P = 0.0001) (Supplementary Table S3). The sensitivity analysis showed that only the cohort study changed the direction of the effect size (Supplementary Table S4).

Table 1 Characteristic features of included studies reporting on interleukin (IL)-4 and IL-5 in children with allergies (n=7).

Study	Country	Study design	Age (years)	Male *n* (%)	Allergen/s	Main findings	Risk of bias
Benson et al.[13]	Sweden	Cohort study involving patients with allergic rhinitis and 19 healthy controls.	Allergic (8 ± 2.5) Control (13 ± 19)	Allergic (52%) 20 Control (58%) 11	Grass and birch pollen	The levels of both IL-4 and IL-5 were exacerbated during pollen season when compared to pre-pollen season. The increment of both cytokines during pollen season was associated with an elevation in eosinophil counts. Notably, treatment with budesonide, a corticosteroid, induced a significant expression of soluble IL-4 receptor and reduced the levels of eosinophils.	Medium
Smart et al.[17]	Australia	Cross-sectional study involving children patients with atopy and 23 healthy controls.	Allergic (6.9 ± 3.6) Control (7.3 ± 4.2)	Not reported	House dust mite and rye grass pollen	*Staphylococcal enterotoxin* B (SEB)-induced plasma levels of IL-4 and IL-5 were significantly increased in the symptomatic group when compared to the non-atopic group, which were comparable with the asymptomatic group. Activation of T-helper (T_H) cells with house dust mite and rye grass pollen, induced a significant release of IL-5 and IL-13 in both groups when compared to the controls. The levels of IL-5 were higher in children allergic to house dust mites when compared to RYE grass pollen. The levels of IL-4 were however, below the lower limit of detection in both groups. The elevated levels of IL-5 were concomitant with elevated immunoglobulin (Ig) E levels.	Medium
Rastogi et al.[32]	USA	Cross-sectional study involving patients with atopy and 30 healthy controls.	Allergic (9.2 ± 1.2) Control (9.3 ± 1.5)	Not indicated	House dust mite	Serum levels of IL-4 and IL-13 were higher in the lean asthmatic group than the obese group. Activation of T_H cells from children with atopy using the house dust mite-induced allergic rhinitis model released significantly increased levels of IL-4 in the lean asthmatic group vs. their lean counterparts.	Medium
Wisniewski et al.[10]	USA	Non-randomised controlled trial RCT involving 21 patients allergic to peanuts and 8 control patients.	Allergic (9.59 ± 5.0) Control (9.75 ± 4.9)	Allergic (43%) 9 Control (25%) 2	Ara h1 and Ara h2 peanuts	Ara h2 is the most common predictor of peanut allergy. More than 85% of the children were sensitised to both Ara h1 and Ara h2. The levels of IL-4, IL-5 and IL-13 were elevated in children with atopy vs. controls. Notably, Ara h2 specific IgE levels were higher than that of Ara h1 specific antibodies. In addition, high levels of peanut specific IgE were associated with IL-4 skewed T_H cell responses. Interestingly, both allergens induced similar degree of T-cell activation between children who received oral immunotherapy and those that did not. Nevertheless, oral immunotherapy reversed the IL-4-biased T-cell response.	Medium

continues

Table 1 Continued

Study	Country	Study design	Age (years)	Male *n* (%)	Allergen/s	Main findings	Risk of bias
Rastogi et al.[14]	USA	Cross-sectional study involving patients with asthma and healthy controls.	Allergic (15.70 ± 1.8) Control (16.3 ± 1.7)	Allergic (54%) Control (36%)	House dust mite	Patients with asthma had poor pulmonary function when compared to the non-asthmatic group. House mite activated T_H cells in the lean group expressed increased levels of IL-4 in comparison to the obese group. Notably the IL-4 levels in the obese group were significantly higher than that of the healthy controls. However, the levels of IL-5 were *below detectable levels.*	Medium
Wambre et al.[18]	USA	RCT involving 80 patients with atopy and 34 healthy controls.	4 to 26	Not reported	Peanuts	Allergy-associated T_H2 (T_H2A) cells are nearly non-existent in individuals without atopy and unlike conventional T $H2$ they co-express CD161 and CR45RB or CD27 on their cell surface. Notably, activated T_H2A expressed elevated levels of IL-5 in comparison to conventional T_H2 cells. However, the levels of IL-4 were comparable between the two T_H2 subsets. A subgroup analysis of patients with peanuts allergy showed that *oral desensitisation immunotherapy* reduced the levels of T_H2A when compared to pre-treatment.	Medium
Wang et al.[15]	Taiwan	Non-RCT involving patients with asthma and healthy controls.	Allergic (10.49 ± 1.7) Control (11.1 ± 3.5)	Allergic (67%) Control (67%)	House dust mite	Activation of peripheral blood mononuclear cells with house dust mite was associated with increased levels of IL-4 and IL-13 in the asthmatic allergic group when compared to the healthy controls. However, the levels of IL-5 were comparable between the groups. Notably, the levels of IL-4 and IL-5 were significantly lower in the allergen-specific immunotherapy group in comparison to the asthmatic allergic group.	Medium

Table 2 Characteristic features of included studies reporting on interleukin (IL)-4 and IL-5 in adult patients with allergies (n=7)

Study	Country	Study design	Age (years)	Male n (%)	Allergen/s	Main findings	Risk of bias
Li et al.[16]	Canada	Cross-sectional study involving 20 patients with allergic rhinitis and 20 healthy controls.	18 to 35 (All participants)	Not reported	Grass pollen	Activation of peripheral blood mononuclear cells with grass pollen antigens elevated the levels of IL-4, IL-5 and IL-13 in patients with allergy when compared to healthy controls. Notably, CD4 T-cell responses mediated by IL-13 was positively associated with IL-5 and not IL-4 levels.	Medium
Oda et al.[12]	Japan	Non-RCT involving seven patients with house dust mite sensitive asthma and three healthy controls.	18 to 26 (All participants)	Not reported	House dust mite	The activation of T-cells by house dust mite specific allergen skewed the immune response towards IL-4 and IL-5 weighted. The elevation of IL-5 and IL-4 in these patients was associated with poor pulmonary function and disease severity. Notably, increased levels of IL-5 were directly associated with an elevation of eosinophils. Interestingly, there was a significant reduction in the secretion of both cytokines by the T-cells following a modified allergen-specific immunotherapeutic procedure (rush immunotherapy). This reduction was associated with an increase in interferon (IFN)-γ levels.	Medium
Tang et al.[33]	Australia	Cross-sectional study involving 28 patients with atopy and healthy controls.	Allergy (40.36 ± 13.1) Control (29.3±7.8)	Allergy (75%) 21 Control (70%) 7	Rye grass pollen and house dust mite	Unstimulated T-cells had a slight increase in IL-4 and significant release of IL-5 when compared to healthy controls. Notably, there was no significant changes in the levels of both cytokines released by T-cells post specific allergen stimulation. However, subgroup analysis showed that patients with atopy and non-asthmatic had significantly elevated levels of IL-5 vs. the control group. Increased levels of IL-5 were associated with increased airways obstruction and decreased pulmonary function.	Medium
Movérare et al.[31]	United Kingdom	Cross-sectional study involving seven patients allergic to birch pollen and six healthy controls.	Allergy 24 to 38 Control 26 to 47	Allergy (29%) 2 Control (17%) 1	Birch pollen	Activation of T-cells with birch-pollen extract skewed the immune response towards IL-4, IL-5 and IL-13 and reduced the levels of IFN-γ. Elevated levels of IL-4 and IL-13 directly correlated with increased levels of birch-pollen-specific IgE levels.	Medium

continues

Table 2 Continued

Study	Country	Study design	Age (years)	Male n (%)	Allergen/s	Main findings	Risk of bias
Lee et al.[28] 2001	South Korea	Cross-sectional study involving 80 patients with asthma and 10 healthy controls	Allergy (53.83 ± 16.7) Control (48.6 ± 10.2)	Allergy (60%) 48 Control (60%) 6	Not indicated	The serum levels of IL-4, IL-5, and IL-13 were elevated, whereas IFN-γ levels were reduced in patients with asthma than heathy controls. Subgroup analysis showed that patients with acute asthma had exacerbated levels of both IL-4, IL-5 and IL-13 in comparison to asymptomatic group. Notably, the increased levels of IL-5 were directly associated with severe airway obstruction in patients with acute asthma.	Medium
Bullens et al.[30]	Belgium	Cross-sectional study involving 15 adults allergic to pollen and healthy controls.	Not reported for all participants	Allergy (47%) 7 Control (40 %) 4	Birch pollen	Activated T-cells with birch pollen antigen significantly released elevated levels of IL-4, IL-5, and IL-13 in comparison to unstimulated levels. Notably, activation of T-cells with IL-4 augmented IL-5 and IL-13 levels in comparison to without IL-4.	Medium
Horiguchi et al.[29]	Japan 10	RCT involving 67 patients allergic to cedar pollen.	Treated (26.8 ± 5.4) Placebo (26.4 ± 5.9)	Treated (51%) 22 Placebo 11 (46.8)	Cedar pollen	In comparison to before pollen season, the levels of IL-4 were significantly increased post pollen season only in the untreated group. The level of IL-5 remained comparable between both seasons. Immunotherapy reduced the increase fold of IL-4 and IL-5 during the pollen season which was concomitant with mild clinical symptoms and elevated serum IgG4 activity during the pollen season.	Low

The effect of treatment on the levels of IL-4 and IL-5 in patients with allergies

As expected in allergic inflammation, a comparison of IL-4 levels between symptomatic patients and healthy controls showed a large effect size (Hedges' *g*: 1.30 [95% CI: -0.10, 2.70], P = 0.068) (Figure 2) whilst revealing a small effect size in IL-5 levels (Hedges' *g*: 0.38 [95% CI: -0.14, 0.90], P = 0.150) (Figure 3). Hence, treatment strategies are designed to antagonise the effect of these cytokines in allergic responses. A total of 43% of included studies reported on the effect of treatment on these T_H2-type cytokines. Treatment with corticosteroids,[13] oral immunotherapy[12,15,19] and rush immunotherapy
reduced IL-4 and IL-5 levels.[10,18] This decrease was associated with reduced eosinophil counts and increased levels of T_H1-type cytokines. Notably, treatment lowered IL-4 levels to almost like that of healthy controls (Hedges' *g*: 0.11 [95% CI: -0.34, 0.56], P = 0.638) (Figure 2). Only one study reported on the levels of IL-5 in the treated group, hence we could not perform a meta-analysis. Nonetheless, there was a large effect size in the levels of IL-5 between untreated and treated individuals with allergies (Hedges' *g*: 0.82 [95% CI: -0.89, 2.53], P = 0.346) (Figure 3).

Discussion

This systematic review and meta-analysis aimed to comprehensively assess available literature reporting on $T_H2$mediated inflammation in allergic reactions. The primary objective of this study was to determine whether the levels of IL-4 and IL-5, the key cytokines that mediate inflammation in allergic reactions, are influenced by the type of allergen. Moreover, the secondary objective was to evaluate the effect of various treatment strategies on the levels of these cytokines. The pooled estimates showed that the levels of IL-4 and IL-5 are allergen-dependent. Notably, HDM elicited greater IL-4 and IL-5 mediated immune responses when compared to pollen. Moreover, treatment with the anti-inflammatory corticosteroid drugs or allergen-specific immunotherapy (rush immunotherapy) was effective in normalising the levels of T_H2-cytokines and ameliorating the allergy-associated symptoms.[10,13,15,18,30]the total nasal symptom scores (TNSS).

The secretion of IL-4 and IL-13 by activated T_H2 cells drive the immune responses triggered by invading allergens in immediate reactions. The subsequent binding of IL-4 and IL-13 to their respective receptors, IL-4Rα and IL-13Rα, activates Janus kinase/signal transducers and activator of transcription-6 (JAK/STAT6) signalling transduction pathway.[35] This results in the activation of various promoter genes such as transforming growth factor-beta 1 (TGF-ß1) and signalling pathways which are essential for the activation and differentiation of naïve T-cells into T_H2 and B-cells to produce IgE antibodies.[36-38] Notably, the IL-4 and IL-13-induced STAT6 signalling is enhanced in patients with atopic asthma compared to healthy individuals.[39] The activation of STAT6 signalling in allergic responses is associated with increasing levels of IL-4 and IgE, and a decrease in T_H1 cytokines.[39,40] Interestingly, in an animal study of asthma, $STAT6^{null}$ mice had reduced airway inflammation congruent with impaired T_H2 differentiation and reduced T_H2 immune responses.[41,42] Although the studies included in our meta-analysis did not assess STAT signalling, our data synthesis showed that the levels of IL-4 and IL-13 were increased in patients with allergy and are associated with increased allergen-specific IgE,and disease severity,[10,12,31] and reduced IFN-γ levels.[28,31] This highlights the shift towards T_H2 in the T_H1/T_H2 paradigm in allergic responses, possibly induced by enhanced IL-4/IL13-mediated JAK/STAT6 signalling.

The increased activation of eosinophils is also associated and implicated in T_H2 inflammation in allergic responses.[43] Even though various cytokines such as IL-4 and IL-3 may induce the differentiation and activation of eosinophils, IL-5 also facilitates these processes.[44] Notably, the binding of IL-5 to its receptor results in the downstream activation of the PI3K-AKT and MAPK signalling pathways, various STAT transcription factors and tyrosine kinases, which collectively promotes cell differentiation, activation and proliferation

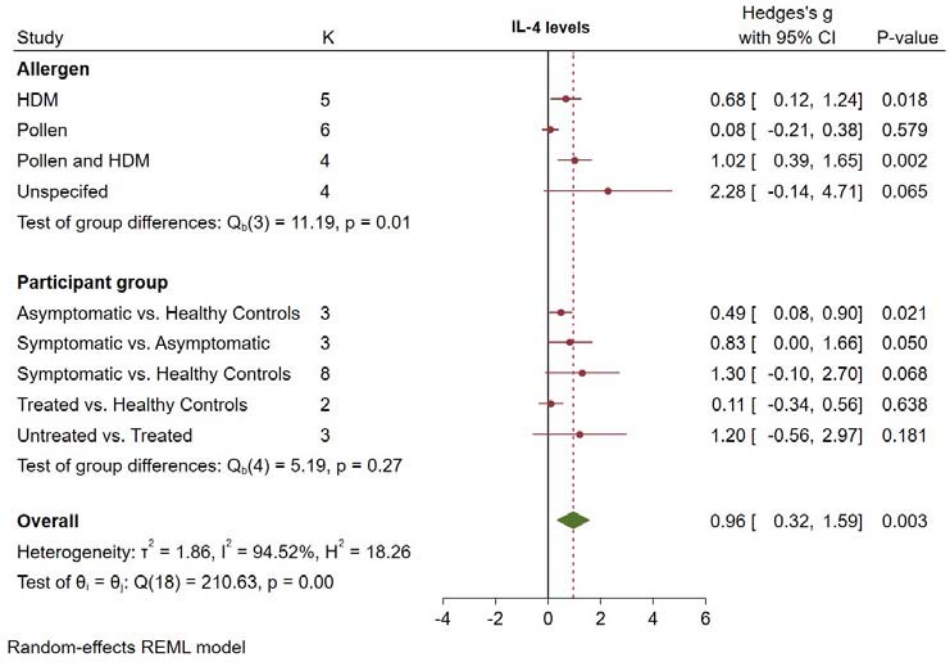

Figure 2 The levels of IL-4 in patients with allergies as an effect measure of T_H2 immune response.

and inhibit apoptosis.[45] As expected, we report on increased levels of IL-5 in allergic responses, which were concomitant with elevated eosinophil counts and increased airways obstruction.[12,13,28,33]

The use of allergen-specific immunotherapy and anti-inflammatory drugs in patients with allergies is effective in lowering the levels of allergy-associated T_H2 cells, IL-4, IL-13 and IL-5.[10,12,13,15,18,29] However, because of the heterogeneous nature of allergic responses and confounding factors such as obesity that alter immune responses,[3] there is an increased interest in cytokine-targeting biologics as an alternative treatment strategy for allergic responses.[11] In that context, while both IL-4 and IL-5 play pivotal roles in mediating type 2 inflammation in allergic patients, our meta-analysis showed that IL-4/IL-5 immune responses are weighted towards IL-4 when compared to IL-5 as indicated by a larger effect size in the levels of the former cytokine. Thus, cytokine-targeting biologics that directly antagonises IL-4/IL-13 may be effective in ameliorating allergic responses. Most importantly, for optimal therapeutic benefit, the type of antigen should be considered when determining dosage. However, it should be noted that utilisation of IL-4/IL-13 or IL-5 biologic antagonists should be done with caution as it requires a delicate balance in order to correct and maintain the T_H1/T_H2 paradigm, as reported elsewhere.[35,37]

The main strength of our study is its uniqueness. To our knowledge, this is the first systematic review and meta-analysis to assess the effect of allergen type on the levels of IL-4 and IL-5 in patients with allergies. In addition, the comprehensive literature search and the subsequent data extraction were independently carried out by two reviewers, thus minimising the risk of errors. Moreover, the inter-rater reliability scores were high in the study selection and the risk of bias assessment. Even though the included studies had a medium risk of bias overall, the studies scored poor in the external validity domain. As a result, the findings must be adopted with caution in a population outside this study's populace. Nonetheless, the findings are a true representation of T_H2 responses in allergies as denoted by a high score in internal validity. The main limitation of our study is that some of the included studies did not report on the exact levels of IL-4 and IL-5, so we estimated some levels from the reported graph values using the GetData Graph Digitizer software.

Conclusion

T_H2-mediated inflammation in patients with allergies is allergen dependent and treatment with anti-inflammatory corticosteroid drugs or allergen-specific immunotherapy is effective in normalising the levels of T_H2-cytokines and ameliorating the allergy-associated symptoms. Therefore, to further improve the efficacy of treatment in allergic reactions, the type of allergen should be considered when planning therapeutic strategies, particularly those involving the usage of cytokine-targeting biologics that antagonise the activities of T_H2 signature cytokines.

Abbreviations

IL: interleukin; cardiovascular diseases; JAK/STAT: Janus kinase/signal transducers and activator of transcription; TH2: T helper 2

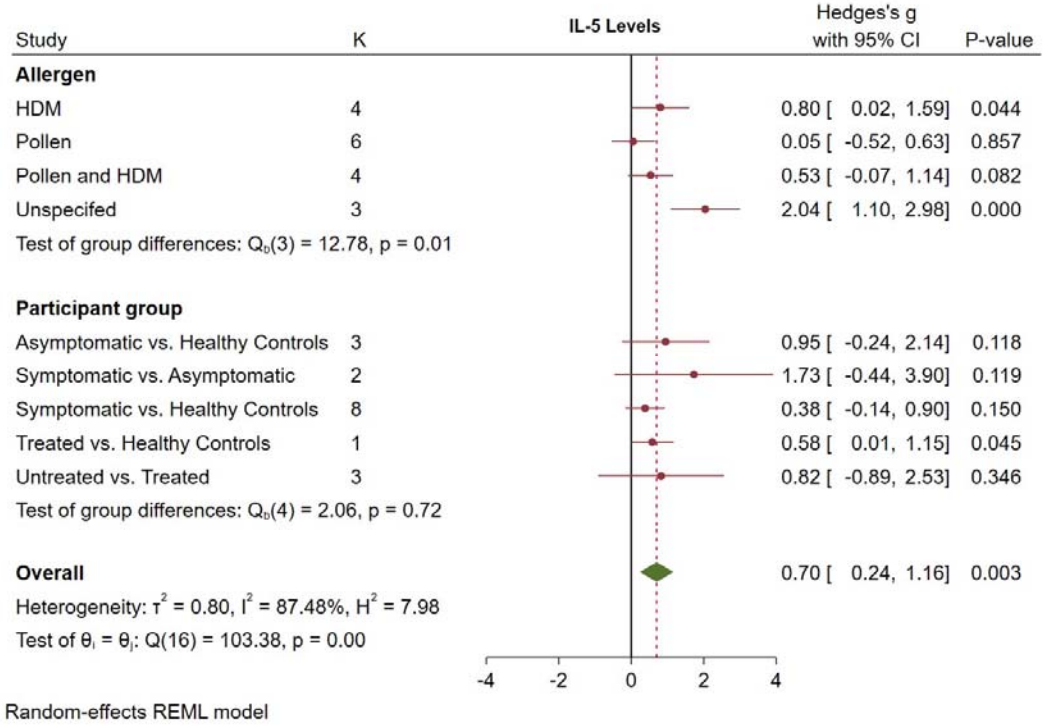

Figure 3 Effect estimates of T_H2 immune response in patients with allergies measured by the levels of IL-5.

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable

Availability of data and materials: The authors confirm that the data supporting the findings of this study are available within the article and its supplementary files.

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Supplementary

Table S1 Search strategies

Terms	Search terms
#1	(T helper cells [MeSH Terms]) =45,274 hits
#2	(IL-5[MeSH Terms]) =5,938 hits
#3	(IL-4[MeSH Terms])= 22,802 hits
#4	(allergen [MeSH Terms])= 41,348 hits
Combined #1 and #2 or #3 and #4	(((T helper cells[MeSH Terms]) AND (IL-5[MeSH Terms])) OR (IL-4[MeSH Terms])) AND (allergen[MeSH Terms]) **=46 hits** (Age: 2-12 years)
Combined #1 and #2 and #3 and #4	(((T helper cells[MeSH Terms]) AND (IL-5[MeSH Terms])) AND (IL-4[MeSH Terms])) AND (allergen[MeSH Terms]) **=67 hits**

Table S2 Modified Downs and Black check list scores for included studies (n = 14)

Author		Domain	Benson	Smart	Rastogi	Wisneiwski	Rastogi	Wambre	Wang	Li	Oda	Tang	Moverare	Lee	Bullens	Horiguchi
		1997	2002	2012	2015	2015	2017	2018	1998	1998	1998		2000	2001	2004	2008
Reporting bias	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	3	1	1	1	1	0	0	0	1	1	1	1	1	0	1	
	4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	6	1	1	1	1	1	1	1	1	0	1	1	1	1	1	
	7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	8	0	0	0	1	1	0	0	0	0	0	0	0	0	1	
	9	0	0	0	0	0	0	1	0	0	0	0	0	0	1	
	10	0	1	1	0	1	1	1	0	0	0	0	1	1	1	
	Score	**7**	**8**	**8**	**8**	**8**	**7**	**8**	**7**	**6**	**7**	**7**	**8**	**7**	**10**	
External validity	11	1	1	1	1	0	0	1	0	1	1	1	0	1	1	
	12	0	0	0	0	0	0	0	0	0	0	1	1	0	1	
	13	1	0	0	1	0	0	0	1	0	0	0	0	0	0	
	Score	**2**	**1**	**1**	**2**	**0**	**0**	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**2**	
Internal validity	14	0	0	1	0	0	1	0	0	0	0	0	0	0	1	
	15	0	0	0	0	0	1	0	0	0	0	0	0	0	1	
	16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	17	0	0	0	0	0	1	1	1	1	1	1	0	1	1	
	18	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	19	0	1	1	1	0	0	0	0	0	0	0	1	0	0	
	20	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	Score	**3**	**4**	**5**	**4**	**3**	**6**	**4**	**4**	**4**	**4**	**4**	**4**	**4**	**6**	

Selection 21 bias	1	1	1	1	1	1	1	1	1	1	1	1	1	1
22	1	1	1	1	1	0	0	1	1	0	0	0	1	1
23	0	0	0	0	0	1	0	0	0	0	0	0	0	1
24	0	0	0	0	0	1	0	0	0	0	0	0	0	1
25	0	1	1	0	1	1	1	1	1	1	1	1	0	1
26	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Score	**2**	**3**	**3**	**2**	**3**	**4**	**2**	**3**	**3**	**2**	**2**	**2**	**2**	**6**
Overall score	**14**	**16**	**17**	**16**	**14**	**17**	**15**	**15**	**14**	**14**	**15**	**15**	**14**	**24**

Table S3 Comparisons of standardised means of interleukin 4 and 5 levels based on the type of allergen

Allergens	Standardised means differences	95%CI	p-value
Interleukin 4 Levels			
HDM *vs* Pollen	0.60	0.02 to 1.18	**0.0430**
Pollen and HDM *vs* Pollen	0.94	-0.02 to 1.90	**0.0009**
Unspecified *vs* Pollen	2.20	1.07 to 3.33	**0.0002**
HDM *vs* Unspecified	1.60	0.57 to 2.63	**0.0024**
Pollen and HDM *vs* Unspecified	0.94	-0.02 to 1.90	0.0544
HDM *vs* Pollen and HDM	0.34	-0.50 to 1.18	0.4232
Interleukin 5 levels			
HDM *vs* Pollen	0.75	-0.11 to 1.61	0.0878
Pollen and HDM *vs* Pollen	0.48	-0.21 to 1.17	0.1730
Unspecified *vs* Pollen	1.99	1.40 to 2.57	**<0.0001**
HDM *vs* Unspecified	1.24	0.32 to 2.16	**0.0086**
Pollen and HDM *vs* Unspecified	1.51	0.75 to 2.28	**0.0001**
HDM *vs* Pollen and HDM	-0.27	-1.26 to 0.72	0.5923

Table S4 Sensitivity analysis of interleukin 4 and 5 levels based on study design

Study design	Number of studies	Omitted studies	Hedges's g [95% CI]	p-value
Interleukin 4				
All	**8**	**0**	**0.96 [0.32, 1.59]**	**0.003**
Cross-sectional	6	2	1.54 [-0.31, 3.40]	0.103
Cohort	1	7	0.18 [-0.34, 0.90]	0.619
Non-RCT	1	7	1.00 [0.19, 1.80]	**0.016**
Interleukin 5				
All	**8**	**0**	**0.70 [0.24, 1.16]**	**0.003**
Cross-sectional	6	2	0.56 [0.04, 1.07]	**0.034**
Cohort	1	7	-0.96 [-1.70, -0.24]	**0.012**
Non-RCT	1	7	0.57 [-0.21, 1.34]	0.151