# Effects of Toll-like Receptor 2 Ligands on T-cell Responses to Mite Allergen in Humans.

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# ABSTRACT

The last few decades have witnessed an increase in the prevalence, morbidity and economic burden associated with asthma and allergic disease. This rising incidence cannot be completely explained by changes in genetic factors or by improvements in diagnostic procedures. Environmental factors, particularly those associated with a westernised lifestyle, are considered to be involved in this increase. In the late 1980's Strachan was the first to link environmental factors with allergic disease, this theory became to be known as the 'hygiene hypothesis'. This hypothesis links the "cleaner" more "healthy" environment we now live in, with an increased risk of developing allergic disease. This effect is highlighted by studies linking farm and animal exposure (rich in microbial compounds) during early life with a decrease in allergic disease. Since then numerous studies have been undertaken to ascertain the factors present in the microbe rich environment, which elicit this protective effect. Many studies have revolved around endotoxin, however microbial components (mainly from Gram-positive bacteria) which signal through Toll-like receptor 2 (TLR2), have also shown that they can alter the allergic immune response. In mice models TLR2 has been shown to both exacerbate and inhibit allergic disease. The above research highlights the need for further studies into the effect of TLR2 ligands, and to define the mechanisms by which they exert their effects in human allergic disease. These mechanisms will be relevant to understanding the pathogenesis of allergy, but also might provide novel ways to treat allergy.

The aims of the study outlined in this thesis were to determine whether *in vitro* exposure to TLR2 ligands could modify the established immune response to house dust mite allergen (HDM), and to examine the mechanisms by which this occurs.

Blood mononuclear cells were obtained from both HDM sensitive (n=23) and non-allergic (n=22) adults, and stimulated with HDM in the presence or absence of TLR2 ligands. The TLR2 ligands chosen for this study were heat-killed *Staphylococcus aureus* (Pansorbin, PS), staphylococcal lipoteichoic acid (LTA) and the synthetic lipoprotein Pam3CSK4. These compounds have been well documented as ligands for TLR2.

In HDM-allergic subjects, IL-5 and IL-13 responses by mononuclear cells stimulated with HDM were inhibited by heat-killed *Staphylococcus aureus*, staphylococcal lipoteichoic acid (LTA) and the synthetic lipoprotein Pam3CSK4 ($p<0.005$; all stimuli). While the whole staphylococcal bacteria increased IFNγ responses, the purified TLR2 ligands LTA and Pam3CSK4 inhibited HDM-specific IFNγ synthesis. In contrast, TLR2 ligands had minimal effects on responses to HDM in non-allergic subjects. TLR2 ligands induced upregulation of HLA-DR expression, but did not inhibit antigen uptake or processing by antigen presenting cells. The addition of glucocorticoids to LTA enhanced the ability of this TLR2 ligand to inhibit IL-5 and IL-13 production by HDM-activated blood mononuclear cells.

In conclusion, this study shows that TLR2 ligands have the ability to inhibit the Th2 response to mite allergen in previously sensitized individuals by an as yet unknown mechanism. However the findings described herein do provide an impetus for future studies designed to uncover novel mechanisms by which allergic responses can be ameliorated, and may open new treatment modalities.

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# ABBREVIATIONS

APC – Antigen Presenting Cell

DC – Dendritic Cell

HDM – House Dust Mite allergen

LTA – Lipoteichoic Acid

MFI – Mean Fluorescence Intensity

Mono48 – 48hr Monocyte derived DC

Pam3CSK4 – Synthetic lipopeptide

PBMC – Peripheral Blood Mononuclear Cells

PmB – Polymyxin B

SPT – Skin Prick Test

TBds – T-cell activation Beads

Th1/2 – T helper cell type 1/2

TLR – Toll-like receptor

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# STATEMENT OF CANDIDATE CONTRIBUTION

The work detailed within this thesis was carried out by the candidate (except where stated otherwise).

Most of the work described in Chapter 2 and 3, were published,

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The experimental work covered within this publication was carried out solely by the candidate. Permission for the work to be used within this thesis has been granted by the co-authors.

The results outlined within this thesis have also been presented at local, national and international conferences and meetings. (Appendix: Abstracts and Publication)

# 1 LITERATURE REVIEW

## 1.1 Worldwide burden of allergic disease

There has been a sharp increase in the global prevalence, morbidity and economic burden associated with asthma and allergic disease over the last 40 years[1], with allergic diseases/disorders affecting millions of individuals worldwide. In Australia it is estimated that approximately 1 in 5 people are effected by an allergic disorder such as hayfever, asthma, eczema, food allergy or hives (urticaria). It is not surprising that large financial costs to both the individual and community are associated with asthma and allergies[2-8]. Furthermore, there are high personal costs associated with these diseases, resulting in school and work absenteeism, effects on social life and personal relationships[9, 10].

The high impact of asthma and allergic diseases on society highlights the importance of understanding why the rates have increased so dramatically over the last few decades. It is also important to determine the aetiology of allergic diseases associated with the development of allergies and to identify what preventable measures can be undertaken to try and stem the worldwide burden of allergic disease.

## 1.2 Allergy - what is it?

Allergic diseases produce a very broad range of symptoms as summarised in Table 1.1, that includes relatively minor symptoms causing distress and discomfort, to life threatening anaphylaxis in response to environmental allergens.

Atopy is usually defined as the genetic propensity to develop IgE antibodies in response to exposure to allergens as assessed by skin prick test responses to common allergens. Although it is generally agreed that atopy is an important risk factor for allergic diseases

such as asthma, rhinitis, and eczema, the extent to which atopy accounts for these diseases is controversial[11]. Allergy is defined as a hypersensitivity reaction to foreign substances, and is mediated by cell- and antibody-mediated (humoral) mechanisms. In allergic disease, there is a polarization of T-lymphocyte responses towards a Th2 response, and enhanced secretion of cytokines involved in the regulation of IgE, mast cells, basophils and eosinophils, ultimately leading to inflammation and disease onset[9], as discussed in more detail later.

| Disease | Symptoms | Triggers | At risk |
|---|---|---|---|
| Asthma | Wheeze<br>Cough<br>Shortness of breath<br>Tightness in chest | Colds and flu<br>Exercise<br>Inhaled allergens (eg pollens, moulds, animal hair and dust mite)<br>Cigarette smoke<br>Changes in temperature and weather<br>Certain drugs (eg aspirin)<br>Chemicals and strong smells<br>Some foods and food preservatives, flavourings and colourings | All ages<br>More boys than girls, however is more common in adult women than men |
| Eczema<br>- Dermatitis | Itch<br>Rash - It is patchy and starts out as flaky or scaly dry skin on top of reddened, inflamed skin. | Soaps<br>Detergents<br>Weather (hot, cold, humid, or dry)<br>Environmental allergens<br>Jewelry<br>Creams<br>Food handling<br>Clothing<br>Sweating<br>Gloves<br>Rubbing<br>Bacteria<br>Emotional or mental stress | All ages<br>20% children have<br>1-2% adults have |
| Allergic Rhinitis<br>- Hay fever<br>- Allergic conjunctivitis | Nasal congestion<br>Clear runny nose<br>Sneezing<br>Nose and eye itching<br>Tearing eyes | Airborne allergens such as pollen | All ages |
| Hives (Urticaria) | Raised, often itchy red welts on the surface of the skin. | Medications<br>Foods (berries, shellfish, fish, nuts, eggs, milk, and others)<br>Pollen<br>Animal dander (especially cats)<br>Insect bites<br>Infection or illness<br>Emotional stress<br>Extreme cold or sun exposure<br>Excessive perspiration | All ages<br>Very common, especially in people who have experienced other allergic reactions |

**Table 1.1. Common allergic diseases and their symptoms.**

Individuals with a family history of atopy have an increased risk of developing IgE sensitisation, and the atopic constitution is also a major risk factor for the development of allergic diseases such as allergic asthma, rhinitis or atopic dermatitis/atopic eczema.

Interestingly, it appears that asthma in the mother confers greater risk for asthma in the child, relative to paternal asthma[12], suggesting that *in utero* events may influence the later development of asthma.

Allergic disease may manifest at different stages of childhood as eczema, allergic rhinitis, wheeze, or asthma and the prevalence of each varies with age. Eczema and wheeze are the most commonly diagnosed atopic conditions in children under the age of two years. Asthma is not prevalent in early life, but becomes more persistent and common during the school years[13, 14]. Furthermore the presence of an allergic disorder such as eczema in childhood is associated with greater risk of developing atopic diseases such as asthma later in life.

Different environmental allergens are generally associated with different allergic diseases. In the case of respiratory allergy, it is exposure to aeroallergens that is most important, with asthma tending to be associated with indoor allergens derived from dust mites, pets and fungi, whereas seasonal rhinitis tends to be associated with grass and tree pollens.

## 1.3 Adaptive immune response in allergy

Allergy is considered to be regulated by the adaptive immune system, which includes both cellular-mediated and humoral components. The immune response in allergy can be classified into two phases, the first involves primary sensitisation when the immune system is first exposed to an allergen followed by a memory response when the immune system is re-exposed to the allergen leading to an allergic response[11, 17].

The cellular mediated immune response is induced by the recognition of allergens by T-cells[14, 15]. For this to occur soluble antigens are first taken up by antigen presenting cells (APC) such as dendritic cells (DC), which then process the antigen and express antigenic peptides on their surface along with major histocompatibility complex (MHC) class II molecules ready for presentation to naive T-cells. The activation of naive Th cells is the result of interaction between Th cells and antigen loaded DC, and three signals are required. Signal one or *stimulation,* is the recognition by the T-cell receptor (TCR) of antigenic peptides presented by MHC class II molecules. Signal two or *co-stimulation,* is mainly provided by triggering CD28 on the T-cell by CD80, CD86 and related molecules on the DC. Signal three or *polarisation,* directs T-cell differentiation into various effector phenotypes such as Th1 and Th2[15]. Factors that select for Th2 responses, as seen in allergy, include low-affinity binding of the allergen peptide to the groove of MHC class II molecules, exposure of APC to soluble factors such as thymic stromal lymphopoietin (TSLP), selective use of co-stimulatory molecules (eg engagement of T-cell CD28 by CD86 in preference to CD80) and reduced dendritic cell secretion of IL-12[16]. Th2 cells produce the pro-inflammatory cytokines IL-4, IL-5, IL-9 and IL-13 that are characteristic of the allergic immune response[15, 16]. However Th2 cells are not the only source of proallergic cytokines, which can also be produced by mast cells, basophils, eosinophils and CD8+ T-cells[18].

B-cells are involved in immune surveillance and make up the basis of the humoral immune response[14]. Once a B-cell encounters its cognate antigen and becomes activated, it can further differentiate into one of two types of B-cell, plasma B-cells and memory B-cells. The main function of plasma B-cells is to secrete antibodies (eg IgG or IgE), which assist in destruction of microbes/antigens by binding to them and making them targets for phagocytes. Memory B-cells are formed from activated B-cells that are

specific to the antigen encountered during the primary immune response[20]. These cells are able to live for a long time, and can respond quickly following a second exposure to the same antigen.

B-cell recognition of antigen is not the only element necessary for B-cell activation. B-cells can be activated in a T-cell independent or dependent manner. Some antigens are T-cell independent, meaning they can deliver both of the signals required to activate the B-cell without the need of T-cells. There are two types of T-cell independent activation: Type 1 T-cell independent (polyclonal) activation, and Type 2 T-cell independent activation in which macrophages present several of the same antigen in a way that causes cross-linking of antibodies on the surface of B-cells. However most antigens are T-cell dependent, meaning T-cell help is required for maximal antibody production[21, 22].

With a T-cell dependent antigen, the first signal comes from antigen cross-linking the B-cell receptor (BCR) and the second signal from the T-cell[15]. The first signal occurs when a B-cell identifies its target antigen in its natural state. The antigen is engulfed and processed by the B-cell, and antigen fragments are then bound to MHC class II molecules. This complex is then moved to the outside of the cell membrane, where it can be recognised by corresponding T-cells usually Th2 cells[27]. The second signal is then formed when the B- and T-cells interact[15]. The T-cells activate the B-cells through the release of an array of cytokines that directly or indirectly 'program' the leukocytes that are responsible ultimately for acute and chronic allergic inflammation in the airways[24]. The principal Th2 cytokines implicated in this process include IL-4 which is required to drive production of allergen-specific IgE by the plasma B-cells, IL-13 which controls the mast-cell and basophil development, and IL-5 in conjunction with GM-CSF which regulate the eosinophil component of allergy[18]. Once formed and released into

circulation IgE binds through its Fc portion to high affinity receptors on mast cells. This allows the immune system to rapidly recognise specific allergens during later exposures. Basophils, DC and activated monocytes can also express high-affinity receptors for IgE binding. This system of allergen removal by the immune system is over-reactive in allergic individuals causing large quantities of IgE to be produced and as such this contributes to the development of symptoms of allergic disease[11, 16, 18, 22].

Upon re-exposure of the allergen, binding of the allergen to IgE orchestrates the immune system to initiate a more aggressive and rapid memory response[20]. Cross-linking a sufficient number of mast cell/basophil bound IgE antibodies by allergen initiates a process of intracellular signalling, which leads to the degranulation of mast cells, with the release of mediators such as histamine, prostaglandins, heparin and substances that activate blood platelets and attract secondary cells such as eosinophils and neutrophils[18]. These substances cause both dilation and increased "leakiness" of the surrounding capillaries. The resulting dilated, blood filled capillaries cause redness, while plasma leaking into surrounding tissues causes swelling, itching and discharge[6, 7]. Release of histamine and leukotrienes accounts for most of the early and late phase responses, although the cellular origin of these mediators may differ – mast cells in the early phase and eosinophils, basophils and macrophages in the late phase. Although mast cells contribute to chronic airway inflammation, it is activated eosinophils that are considered to mediate most of the disordered airway function in asthma. Their selective recruitment and activation is a characteristic feature of asthma, whether allergic or non-allergic[14].

Although IgE production is associated closely with allergic hypersensitivity responses, a wide range of cellular responses underlies chronic allergic disease, including the

production of inflammatory mediators[18]. Allergic symptoms can occur in a variety of locations, because mast cells can be located in such scattered areas of the body as the respiratory tract, the skin and the digestive tract. Thus allergic mast cell degranulation can lead to such widely varying symptoms as coughing and wheezing, hives, nasal congestion, nausea and itchy watery eyes[4, 6, 10].

The immune system's response to allergen exposure can be divided into two phases. The first is immediate hypersensitivity, or the early phase reaction, that occurs within 15 minutes of exposure to the allergen[20]. After the chemical mediators of the acute response subside, late phase responses often occur. This is due to the migration of other leukocytes such as neutrophils, lymphocytes, eosinophils and macrophages to the initial site of infection. The late phase is usually seen 4-6 hours after the original reaction and can last from 1-2 days. Cytokines from mast cells may also play a role in the persistence of long-term effects. During the early phase reaction chemical mediators released by mast cells including histamine and prostaglandins, produce local tissue responses characteristic of an allergic reaction[20, 27]. For example in the respiratory tract, these include sneezing and mucus secretion, with vasodilation in the nose, leading to nasal blockage, and bronchoconstriction in the lung, leading to wheezing. During the late phase reaction in the lung, cellular infiltration, fibrin deposition and tissue destruction resulting from the sustained allergic response lead to increased bronchial reactivity and further inflammatory cell recruitment[21].

There is still uncertainty as to why certain individual's immune response to allergens is dominated by a Th2 response and others by a Th1 response[21]. The key elements that determine the cytokine phenotype of allergen-specific Th memory cells seems to occur very early in life with initial priming of the Th cells possibly occurring *in utero*[12, 19].

The *in utero* environment is distinctly Th2 biased[17], and the immune response to allergens at birth are dominated by the same Th2 cytokines that are associated with the expression of atopy later in life[18, 19]. It is postnatal exposure to inhaled allergens that results in either redirection of these Th2 responses towards a regulatory or Th1 like cytokine pattern found in non-atopic individuals – a process called immune deviation – or in further boosting fetally primed Th2 polarized immunity in potential atopic individuals[20-22]. In some individuals there is a tendency for this allergen driven immune deviation to fail such that allergies and atopy occur. Exactly why this failure is occurring is the matter of great debate. However it is increasingly more apparent that exposure to microbial stimulation early in life is pivotal for the normal postnatal maturation of healthy immune function[23, 24]. This may partly explain the inverse relationships reported between childhood infections and atopy development as discussed in more detail later[25, 26]. It would also appear that the rate of postnatal transition from the Th2 skewed state characteristic of fetal life towards the relatively Th1 polarised adult state occurs significantly slower in children with genetic predisposition to atopy[28]. It has been hypothesized that this may compromise capacity to develop protective Th1 like immunity against inhalant allergens in infancy[20, 27, 28]. In further support of this Prescott *et al* have shown that there is an imbalance in Th1 and Th2 cytokines in potentially allergic infants[21].

If allergy development is dependent on the lack of exposure to microbial infection, early in life, this would suggest that there is a strong association between the skewing of the adaptive immune response towards a Th1 phenotype, and the innate immune response related to the effective removal of microbes[23, 26, 30, 34].

## 1.4 Innate immunity and host defence

Innate immunity provides a mechanism whereby the host immediately recognises invading microorganisms or foreign antigens. The innate immune system is equipped with specialised cells that detect, and often eliminate, the invader before it is able to reproduce and cause potentially serious injury to the host. As such the innate immune system provides a non-specific and rapid response to invading pathogens[31].

The cells of the innate immune system include the phagocytic cells such as macrophages, neutrophils and DC and natural killer cells (NK), mast cells, basophils and eosinophils[31].

Phagocytes have the function of engulfing and destroying invading microbes. In order for this to happen the phagocyte must first be able to recognise the microbe. This is achieved through either the recognition of surface microbial sugar residues or complement/antibody, which is bound to the pathogen[37].

Macrophages are the most efficient of the phagocytes and can ingest substantial numbers of bacteria or dead and dying host cells. Neutrophils are the most abundant type of phagocyte representing 50-60% of total circulating leukocytes. Neutrophils destroy foreign substances of pathogens by activating a “respiratory burst”; releasing strong oxidising agents including hydrogen peroxide, free oxygen radicals and hypochlorite – all of which are required for successful elimination of the unwanted microbe. Pathogens also stimulate macrophages and neutrophils to produce chemokines, which summon other cells to the site of infection[30].

DC are specialised phagocytic cells that are present in tissues that are in contact with the external environment, mainly the skin and the inner lining of the nose, lungs, stomach and intestines. They can also be found in low numbers in the circulating blood and secondary lymphoid organs such as the spleen and lymph nodes. DC are very important in the process of antigen presentation and together with macrophages serve as a link between the innate and adaptive immune systems[34, 37]. When activated by toll-like receptors (TLR), APC's release elevated levels of pro-inflammatory cytokines, chemokines, and nitric oxide and show increased expression of co-stimulatory molecules[29]. All these changes in APC function facilitate and shape the induction of the adaptive immune response, where both T and B lymphocytes play a crucial role[37].

NK cells attack host cells that have been infected by microbes, but do not attack microbes themselves. Destruction of infected cells is achieved through the release of perforins and granzymes, which induce apoptosis. NK cells are also able to secrete large amounts of IFNγ. The main function of NK cells is to survey the host for cells that are infected by intracellular pathogens. NK cells can be activated by DC's, either directly via specific cell surface molecules or indirectly through cytokines. This in turn can lead to production of IFNγ by NK cells, which serves two purposes, first, to prevent healthy host cells from becoming infected by a virus, and second, to augment the T-cell response to other virally infected cells[23]. In addition NK cells have the ability to eliminate immature DC's, thereby inhibiting T-cell responses. These NK functions indicate that the interaction between NK cells and DC's represents an intricate balance between enhancement and repression of DC function that could be decisive for the development of tolerance versus T-cell effector function[20, 22].

Mast cells and basophils are usually associated with allergy and anaphylaxis, and are instrumental in initiating the acute inflammatory response[14]. However mast cells can also respond directly and rapidly to pathogens in the absence of surface antibody, indicating that they also contribute to innate immune function[31]. When activated, these cells rapidly release characteristic granules, rich in histamine and heparin, along with various hormonal mediators, and chemokines, or chemotactic cytokines into the environment. Histamine dilates blood vessels, causing the characteristic signs of inflammation, and also recruits neutrophils and macrophages. Activated eosinophils secrete a range of highly toxic proteins and free radicals that are highly effective against bacteria and parasites[31].

Activation of the complement system is another pathway able to modulate B-cell and/or T-cell responses. This activation can be triggered either by a target-bound antibody (the classical pathway), recognition of microbial polysaccharide structures (lectin pathway) or recognition of other foreign surface structures as yet uncharacterised (the alternative pathway). The complement pathway participates in humoral immunity through the ligation of C3 with the CD21-CD35 receptor expressed on B-cells, resulting in regulation of B-cell responses. In addition, the complement system is essential for the efficient opsonisation of microbes and subsequent uptake and antigen processing by DC's, which results in an efficient T-cell response[31].

In some cases the innate immune system is not able to eliminate infection and requires the induction of the adaptive immune response[31]. Adaptive immunity has an important role in immune memory so that the response to a specific pathogen is quicker on second exposure[20-22].

The adaptive immune response may tend toward activation of T-helper (Th) cells, or "active immunity", or it may tend towards tolerance[20]. Active immunity is triggered by a specific attack by a specific pathogen. It results in eliminating or neutralising the pathogen in one of three ways: 1) Ingestion and destruction of pathogens; 2) Antibody mediated killing; 3) Production of antitoxins, which neutralize toxins made by the invaders.

The activation of the Th cells can result in either a Th1 or Th2 response[24]. The Th1 response is characterised by the production of IFNγ, which activates the bactericidal activities of macrophages and induces B-cells to make opsonizing antibodies, leading to cell-mediated immunity. In contrast the Th2 response is characterised by the release of IL-4 and IL-13, which results in the activation of B-cells to make neutralising antibodies such as IgE, leading to humoral immunity, and IL-5, which is necessary for the development, differentiation, recruitment and survival of eosinophils[18].

Generally Th1 responses are involved in eliminating intracellular pathogens, while Th2 responses are more effective against pathogens present outside of cells, such as extracellular bacteria, parasites and toxins. However this is not always the case. LPS for example can alter the Th response depending on the dose administered: low doses of LPS favour a Th2 response whereas high doses of LPS facilitate a Th1 response[29]. Helminthic infection favours a Th2 response[30] whereas mycobacterial infection favours a Th1 response[31].

Tolerance can be defined as a selective block in the immune response to particular antigens[20]. Methods of tolerance induction include: 1) The deletion or specific inactivation (anergy) of antigen-reactive lymphocytes; 2) Altering profiles of cytokine

secretion to prevent inflammation and injury; and 3) Preferential induction of regulatory T cells or cytokines that inhibit destructive T cell functions.

A third class of T lymphocyte, known as regulatory T-cells (Tregs) limit and suppress the immune system, and may control aberrant immune responses to self-antigens; an important mechanism in controlling the development of autoimmune and allergic diseases. Tregs are classified as being CD4+CD25+ cells that express Foxp3 and lack CD127[32, 33]. Classical Tregs suppress T effector cells via a contact dependent mechanism. While Tregs usually secrete IL-10 and TGFβ, the extent to which these cytokines are responsible for Treg activity remains a matter of debate. TLR also play a role in the regulation of immune response via direct or indirect influence on the function of Tregs, which results in their induction and subsequent suppression of the immune response or a reversal of suppression.

## 1.5 Hygiene Hypothesis

The rising incidence of allergic disease and atopy in the western world cannot be completely explained by changes in genetic factors or by improvements in diagnostic procedures. Environmental factors, particularly those associated with a westernised lifestyle, are considered to be involved in this increase. In the late 1980s Strachan put forward a theory that has since colloquially been named the "hygiene hypothesis"[25]. He stated, in summary:

> *"These observations … could be explained if allergic diseases were prevented by infection in early childhood, transmitted by unhygienic contact with older siblings, or acquired prenatally … Over the past century*

*declining family size, improved household amenities and higher standards of personal cleanliness have reduced opportunities for cross-infection in young families. This may have resulted in more widespread clinical expression of atopic disease."*

Until this report it had been widely accepted that infection was a trigger of allergic sensitization rather than a protective influence[34]. However, since this first report by Strachan, many other studies, mainly epidemiological, have suggested that development of asthma and allergies is favored by a reduction of naturally occurring infections in infancy and a decrease in microbial exposure.

There is still intense interest, even now, more than 15 years after this initial observation, in the complex interactions between exposure to microbial products and the clinical expression of human allergic diseases such as asthma, allergic rhinitis and eczema. On the one hand microbial infections are frequently implicated in exacerbations of asthma and eczema for example. In contrast, epidemiological studies have suggested that children growing up in microbial rich environments may be protected from the development of allergic sensitization[35, 36] . This protective effect was originally attributed to endotoxin (LPS) derived from Gram-negative bacteria, though more recent studies have suggested that exposure to components of Gram-positive bacteria may also protect against the development of allergic disease[37].

The manner in which the microbial burden affects allergy and asthma outcomes can be considered under the following broad categories;

### 1.5.1 Living environment: farm exposure versus urban living

Farms provide an environment rich in microbial compounds. Many studies have shown a protective element of children raised on farms and the development of atopy or allergic disease. However it is clear that living in a rural area is not enough, protection is most apparent where there is contact with farm animals and possibly drinking unpasteurised milk[36, 38]. Studies have also found that maternal exposure to an environment rich in microbial compounds, in particular to farm animals, might protect against the development of atopic sensitisation and lead to upregulation of receptors of the innate immune system[39]. The strongest protective effect has been reported from areas of central Europe where animals are kept in close proximity to humans, especially over winter.

### 1.5.2 Family size, Birth order, Day care attendance

There have been many reports that have shown a relationship between family size, birth order and day care attendance, linking these with a decreased risk of allergic disease. Reductions in hay fever[25, 40-44], eczema[41, 42, 45-48], atopy[25, 40, 49-54] and asthma or wheeze[40, 42, 44, 47, 55-57] have all been reported in association with increasing birth order or sibling numbers[58].

Day care attendance has also been shown to reduce the risk of asthma[59], allergic sensitisation[54, 60] and hayfever[60]. The exact causation has not yet been determined, however it has been hypothesised that there is an increased transmission of childhood infections in larger families and by contact with other children at day care[61], which may drive the immune response away from the allergic phenotype.

### 1.5.3 Infections: bacterial, parasitic, viral

As Strachan first hypothesised, infections in early childhood may prevent the development of allergic diseases[25]. Certain viral and bacterial exposure (measles and tuberculosis) during childhood appear to be protective against allergic asthma and atopy[62-64]. In the large Tucson Cohort study, children who had pneumonia and tracheobronchitis in their first three years of life, subsequently had reduced skin test reactivity and lower levels of IgE at the age of six years[65]. Matricardi *et al* have shown that exposure to foodborne and orofecal microbes, such as *Toxoplasma gondii* and *Helicobacter pylori* has an inverse relationship to hay fever, allergic asthma and atopy[66].

It is thought that repeated infections involving the activities of Th1-like cytokines (which is seen with measles and tuberculosis infections[67, 68]), particularly early in life, would help T-cell immune responses to mature into a "balanced" phenotype, which would be less likely to favour allergen sensitisation and manifestation of atopic illness in childhood[69, 70]. However only certain infections convey protection or risk depending on the nature of the infectious agent or the host's predetermined immune response (Outlined in Table 1.2).

### 1.5.4 Reduced microbial burden: antibiotic use, immunizations, public and personal hygiene measures

The use of antibiotics and increasing rates of immunisation have also been linked to the increase in allergy and asthma. Public and personal hygiene has greatly improved over the years and as such this results in lower levels of infection. It has been proposed that the use of antibiotics and immunization against many diseases, as well as improved

personal hygiene, may be preventing the immune system from developing a natural resistance and switching to Th2 rather than a Th1 or tolerant immune response to innocuous compounds. [71-73]. However it should be stressed that no studies have shown that childhood vaccinations are a risk factor for the development of allergic disease[74].

| Type of infection | Protection | Refs | Exacerbation or induction | Refs |
|---|---|---|---|---|
| Bacteria | Bacillus Calmette-Guérin (BCG) | 75, 76 | *Bordetella pertussis* | 84 |
| | *Bifidobacterium lactis* | 77 | *Chlamydia pneumoniae* | 85 |
| | *Chlamydia trachomatis* | 78 | *Mycoplasma pneumoniae* | 85-87 |
| | Lactic acid bacteria | 79 | *Staphylococcus aureus* | 80 |
| | *Lactobacillus rhamnosus* | 77 | | |
| | *Listeria monocytogenes* | 75, 80 | | |
| | *Mycobacterium tuberculosis* | 75 | | |
| | *Mycobacterium vaccae* | 75, 81-83 | | |
| Virus | Hepatitis A virus | 66 | Influenza A virus | 80, 89, 92-94 |
| | Influenza A virus | 88, 89 | Metapneumovirus | 95 |
| | Respiratory syncytial virus | 90, 91 | Rhinovirus | 80 |
| | | | Respiratory syncytial virus | 80, 96-99 |
| Parasites | *Heligmosomoides polygyrus* | 100 | *Anisakis simplex* | 106 |
| | Hookworm species | 101 | Ascaris species | 80, 103 |
| | *Nippostrongylus brasiliensis* | 102 | *Fasciola hepatica* | 107 |
| | *Schistosoma haematobium* | 101, 103 | *Nippostrongylus brasiliensis* | 80 |
| | *Strongyloides stercoralis* | 104 | *Strongyloides venezuelensis* | 105 |
| | *Strongyloides venexuelensis* | 105 | Toxocara species | 80, 103 |

**Table 1.2. Effects of infection on allergic disorders.**
(Modified from Kamradt *et al*, Trends Immunol. 26, 5, 2006[108])

The study into the hygiene hypothesis has been biased towards the protective outcomes of microbial exposures, however it is useful to remember that microbial exposures can potentially cause both healthful and harmful outcomes (Outlined in Table 1.2). The multidimensional determinants of health or disease outcomes relative to microbial exposures include differences in the following: 1) Microbes or their components – some are protective, some are causal and others are both in relation to disease; 2) Disease phenotype – for example asthma phenotypes with different biological underpinnings (atopy-associated asthma, transient wheeze, nonatopic wheeze, and endotoxin-hypersensitive asthma). Particular microbial exposures that exacerbate one type of asthma might protect against another; 3) Time elements – microbial exposures at an early age, before disease onset, and exposure persistence are believed to influence

outcomes; 4) Dosage and combination of exposures – the relationship of a select microbial exposure with disease outcome might not be unimodal, co-exposure of multiple microbial components is more realistic; 5) Genetic contributions to the variance in response to these exposures; and 6) Routes of exposure – exposure through the skin, gastrointestinal tract, and/or the upper or lower airways may have differing effects on the incidence of allergic diseases.[109]

Thus the essence of the hygiene hypothesis is the idea that exposure to specific infections, and or microbial components, drives the maturing immune system in infancy/childhood towards a tolerogenic phenotype, and away from the Th2 phenotype associated with atopy.

## 1.6 Microbial recognition by the innate immune system

Microbes are recognised by the innate immune system using pattern recognition receptors (PRRs), with the Toll-like receptor (TLR) family being the best characterised. Currently there are 13 known mammalian TLR's, 10 of which are functional in humans (TLR1-10). TLR's recognise different pathogen-associated molecular patterns (PAMP's), such as lipopolysaccharide (LPS; TLR4) and double stranded RNA (TLR3), while TLR2 is the receptor involved in the recognition of lipoproteins and lipoteichoic acid (LTA) from Gram-positive bacteria (see Table 1.3). Although it was originally thought that signalling through TLR's is required for adaptive T-helper cell type 1 (Th1) responses, more recent studies suggest that the relationship between TLR stimulation and Th1/Th2 polarisation is more complex, varying with both the concentration and type of TLR ligand studied.

The recognition of invading microbes by TLR's on DC's induces proinflammatory cytokine production and enhanced antigen presentation to naïve T-cells, and finally activates antigen-specific adaptive immune responses. Distinct TLR ligands provide distinct activation status and cytokine production patterns for APC, resulting in the induction of differential immune responses. Thus, TLR's are critical molecules to induce not only inflammatory responses but also fine-tuned adaptive immune responses depending on the invading pathogen[110].

| TLR | Mouse/Human | Ligand | Origin of Ligand | Ref |
|---|---|---|---|---|
| TLR1 | Mouse & Human | Triacyl lipopeptides | Bacteria and Mycobacteria | 111 |
| | | Soluble factors | *Neisseris meningitidis* | 112 |
| TLR2 | Mouse & Human | Lipoproteins/lipopeptides | Various pathogens | 113 |
| | | Peptidoglycan | Gram-positive bacteria | 114, 115 |
| | | Lipoteichoic acid | Gram-positive bacteria | 115 |
| | | Lipoarabinomannan | Mycobacteria | 116 |
| | | Phenol-soluble modulin | *Staphylococcus epidermidis* | 117 |
| | | Glycoinositolphospholipids | *Trypanosoma cruzi* | 118 |
| | | Glycolipids | *Treponema maltophilum* | 119 |
| | | Porins | *Neisseria* | 120 |
| | | Atypical lipopolysaccharide | *Leptospira interrogans* | 121 |
| | | Atypical lipopolysaccharide | *Porphyromonas gingivalis* | 122 |
| | | Zymosan | Fungi | 123 |
| | | Heat-shock protein 70* | Host | 124 |
| TLR3 | Mouse & Human | Double-stranded RNA | Viruses | 125 |
| TLR4 | Mouse & Human | Lipopolysaccharide | Gram-negative bacteria | 126 |
| | | Taxol | Plants | 127 |
| | | Fusion protein | Respiratory syncytial virus | 128 |
| | | Envelope protein | Mouse mammary-tumour virus | 129 |
| | | Heat-shock protein 60* | *Chlamydia pneumoniae* | 130, 131 |
| | | Heat-shock protein 70* | Host | 132 |
| | | Type III repeat extra domain A of fibronectin* | Host | 133 |
| | | Oligosaccharides of hyaluronic acid* | Host | 134 |
| | | Polysaccharide fragments of heparan sulphate* | Host | 135 |
| | | Fibrinogen* | Host | 136 |
| TLR5 | Mouse & Human | Flagellin | Bacteria | 137 |
| TLR6 | Mouse & Human | Diacyl lipopeptides | *Mycoplasma* | 138, 139 |
| | | Lipoteichoic acid | Gram-positive bacteria | 115 |
| | | Zymosan | Fungi | 140 |
| TLR7 | Mouse & Human | Imidazoquinoline | Synthetic compounds | 141 |
| | | Loxoribine | Synthetic compounds | 142 |
| | | Bropirime | Synthetic compounds | 142 |
| | | Single-stranded RNA | Viruses | 143, 144 |
| TLR8 | Mouse & Human | Imidazoquinoline | Synthetic compounds | 145 |
| | | Single-stranded RNA | Viruses | 143 |
| TLR9 | Mouse & Human | CpG-containing DNA | Bacteria and viruses | 146 |
| TLR10 | Human | N.D. | N.D. | |
| TLR11 | Mouse | N.D. | Uropathogenic bacteria | 147 |
| | | Profilin | *Toxoplasma gondii* | 148 |
| TLR12 | Mouse | N.D. | N.D. | |
| TLR13 | Mouse | N.D. | N.D. | |

**Table 1.3. Toll-like receptor ligands.**

* It is possible that these ligand preparations were contaminated with LPS and/or other potent microbial components, so more precise analysis is required to conclude that TLR's recognise these endogenous ligands. N.D. not determined.
(Modified from Akira and Takeda, Nat. Rev. Immunol. 4, 499, 2004[149])

TLR activation is a double-edged sword. It is essential for provoking the innate response and enhancing adaptive immunity against pathogens[150-152]. However, members of the TLR family are also involved in the pathogenesis of autoimmune, chronic inflammatory and infectious diseases[153].

There are some ligands, which may signal through more than one TLR, or the exact signalling had not yet been determined. Heat shock proteins (HSP) fall into this category, with the studies to date implicating TLR2 and TLR4 in the recognition of these proteins. In order to tease out the exact receptors involved, studies need to be performed using knockout mice, or *in vitro* studies using cell lines, which do not express TLR's. Interpreting the findings of studies using TLR ligands can be complex, as commercial preparations of TLR ligands may sometimes be contaminated with endotoxins or other microbial components.

TLR's can also be broadly divided into receptors that are predominantly extra-cellular (TLR 1, 2, 4, 5 and 6) which bind bacterial components versus those that are located within cells and bind viral nucleic acids (TLR3, 7, 8, 9)[154-156]. The extracellular receptors have different dimerisation requirements for signalling. For example TLR2 only signals as a heterodimer with either TLR1[112, 140] or TLR6[140]. Similarly TLR10 can form a heterodimer with either TLR1 or TLR6, or can homodimerise[157]. TLR4 also forms a homodimer but requires the presence of CD14 to recognise its ligand LPS.

After ligand binding, TLR's dimerize and undergo the conformational change required for the recruitment of downstream signalling molecules. There are four main adapter molecules – MyD88, MAL/TIRAP, TRIF and TRAM - which help transduce signals from the TIR (Toll/IL-1 receptor homologous region) domains, activating protein

kinases and then transcription factors that cause inflammatory effects. While most TLR's are dependent on the adapter molecule MyD88 to be able to induce inflammatory cytokines, there is another pathway independent of MyD88. TLR4 is able to signal via both pathways, whereas TLR3 only signals through the MyD88 independent pathway. All other TLR's appear to only signal through the MyD88 dependent pathway (see Figure 1.1). Both pathways lead to NF-κB activation. A common property of TLR pathogen recognition is subsequent NF-κB activation and induction of proinflammatory cytokines as well as upregulation of co-stimulatory molecules to initiate immune responses.

### 1.6.1 Specific discussion of TLR2

Cells expressing TLR2 in combination with TLR1 or TLR6 can respond to a large variety of microbial ligands. The variety of TLR2 ligands is the greatest among all the TLR's and this is probably due to the heterodimerization needed for TLR2 mediated responses. TLR2 recognises lipoproteins/peptides on the surface of Gram-positive bacterial walls. The immune system is able to distinguish between triacyl and diacyl lipopeptides through the use of TLR1 and TLR6 respectively in association with TLR2[158, 159]. Triacylated lipopeptides are recognised by TLR2/TLR1[111], whereas diacylated lipopeptides are recognised by TLR2/TLR6[138, 139, 160]. Buwitt-Beckmann *et al* found that the structure of the lipopeptide determines TLR2 dependent cell activation level, with both the ester bound acyl chains and the acyl chain length affecting TLR2 dependent cellular recognition of lipopeptides, and hypothesised that this might provide a basis for design of TLR2 specific adjuvants[161].

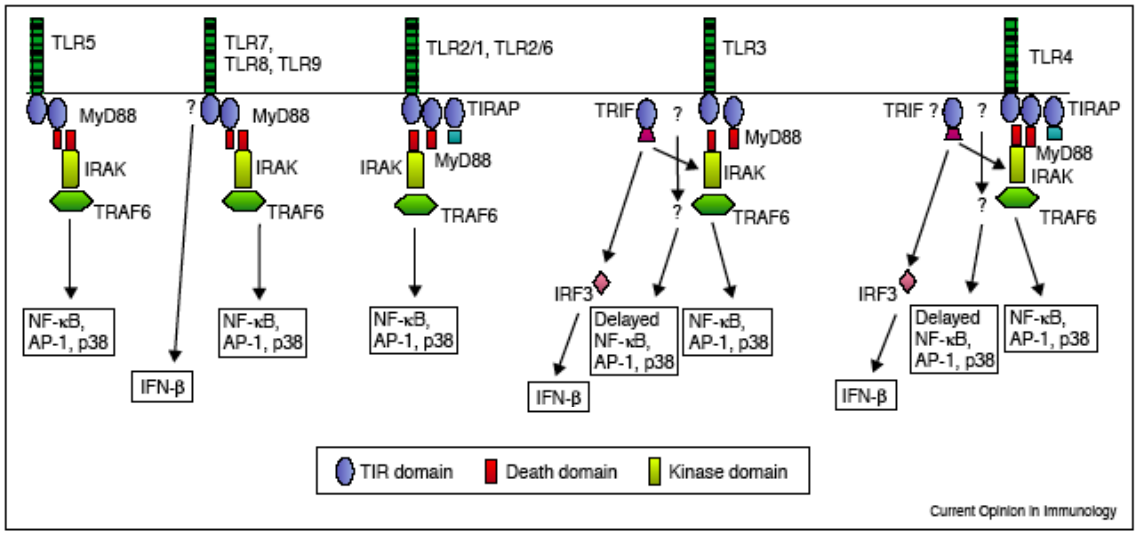



**Figure 1.1. Schematic of mammalian TLR signalling pathways.**

All TLR's are thought to signal through a MyD88–IRAK–TRAF6 pathway to induce NF-kB and MAP kinases. The MyD88-dependent pathway downstream of TLR4 and TLR2 also requires TIRAP. TRIF interacts with TLR3 and induces IFN-b by activating IRF3. Ligands for TLR7, TLR8, TLR9 and TLR4 also induce IFN-b, although it is unclear whether TRIF is involved in these pathways. The TLR3 and TLR4 pathways can induce NF-kB and MAP kinases in the absence of MyD88 with delayed kinetics. Question marks indicate the possible presence of additional signalling molecules.
(Taken from Kopp Curr Opin Immunol 2003, 15:396-401[162])

Like other TLR's, TLR2 signalling induces antigen presenting cell activation, pro-inflammatory cytokine production and increased expression of co-stimulatory ligands. These events are important for induction of the innate immune responses and improved acquired immunity. There is strong suggestive evidence that alteration or lack of TLR2 function *in vivo* may correlate to decreased immune protection from pathogens that contain TLR2 ligands[163].

TLR2 has been shown to be expressed by a variety of circulating cell types within the mononuclear component of circulating leukocytes. TLR2 mRNA has been found in monocytes[164, 165], B-cells[165, 166], NK cells[165, 166] and T-cells[166], as well as mDC[164] but not pDC[164]. Surface expression has been found on monocytes[166], activated T-cells and CD4+CD45RO+ memory T-cells[167]. In all cases the expression is greatest in

monocytes. Tregs from mice express TLR2[168-170], though whether human Tregs express TLR2 has not yet been determined[171].

TLR2 stimulation has been shown to promote the survival, expansion and function of Tregs[169, 171, 172]. The first evidence that TLR2 played a function in Tregs, came from studies in mice that showed TLR2-/- mice have less CD4+CD25+ Tregs than WT mice[173]. This led to the exploration of the effects of TLR2 ligands on the numbers and function of Tregs. Although TLR2 stimulation was directly involved in the expansion of Tregs, the surprising finding was that these expanded Tregs did not result in enhancement of Treg suppression, but rather the opposite. The Tregs were unable to suppress the immune response until the TLR2 stimulation was removed. Once the signal was removed the Tregs regained their suppressive capabilities[169]. This abrogation of suppression by Tregs was associated with a decrease in the level of Foxp3 expression. This downregulation of Foxp3 by TLR2 stimulation may account for the direct transient abrogation of the suppressive functions of Tregs[174]. In addition TLR2 stimulation also promotes the expansion of CD4+CD25- effector T-cells. It has been proposed that this would allow the host to mount an effective immune response to an infection and once that infection subsides the Tregs may then regain their suppressive effect and thus prevent potential autoimmune disease.[169, 171, 174]

With the wide range of cell types expressing TLR2 it is clear that TLR2 signalling can affect a number of cellular functions, thereby modulating innate as well as adaptive immunity. In relation to allergy it is therefore important to consider whether or not TLR2 stimulation can modify allergic responses *in vitro* and *in vivo*.

## 1.7 How TLR ligands affect allergy

Aeroallergen exposure is a prerequisite for the development and persistence of respiratory allergic disease. High exposure to some allergens (such as house dust mite; HDM), has traditionally been linked with increased rates of sensitisation, whereas for some other allergens (dogs, cats and mould) this correlation has not been found[175, 176]. For allergens associated with animals, increased levels of exposure have actually been associated with decreased atopic risk[176, 177], though this issue remains controversial. Woodcock *et al*, designed a study where two groups of pregnant women were placed into either a stringent environmental control group where allergen exposure was avoided as much as possible, or into a non-intervention group. When followed up at the age of 3 years the children in the strict allergen avoidance group had significantly *higher* levels of allergic sensitisation than those in the non-intervention group[178]. This report supports the hygiene hypothesis in that exposure to allergens and microbial compounds early in life may actually protect against the development of allergic disease or sensitisation.

Interpreting the relationship between allergen dose and the incidence of atopy is complex however, especially as exposure to allergens often occurs in conjunction with other immunologically active molecules. House dust for example contains not only allergens from *Dermatophagoides pteronyonissinus* and *D. farinae*, but also endotoxin and fungal elements. Exposure to domestic animals involves not just cat and dog allergens, but also endotoxin. These observations suggest that aside from allergens themselves, other immunomodulatory elements, such as endotoxin, within living environments influence the balance between immune homeostasis and dysregulation.

As discussed previously in this chapter, an emerging body of literature has convincingly revealed an inverse relationship between atopy and asthma and early childhood exposure to rural farm environments. Asthma and allergies are consistently reported to be less prevalent in children raised in a farm environment especially traditional farms in central Europe where the animals are kept in a barn that forms part of the farmhouse[44, 179-181]. Such early exposure in barns, presumably to microbial pathogens and their products, has been associated with protection against development of atopy. One study has shown a relationship between maternal exposure to an increasing number of animal species, and protection against allergic disease or atopy in the offspring, by upregulating receptors (TLR2, TLR4 and CD14) of the innate immune system[39]. Studies have shown that in the blood cells of farmers children there are increased levels of TLR2 and CD14 compared to non-farmers[182].

Genetic association studies have found associations between single nucleotide polymorphisms in TLR2[183], TLR4[184-186], TLR6[187, 188] and TLR10[189] and asthma and atopy. For example studies have shown that genetic variation in TLR2 is a major determinant of the susceptibility to asthma and allergies in children of farmers but not in non-farmers children in a rural setting[183]. Yet other studies have shown no association[190-193]. This discordance in association studies may be due to insufficient power, type 1 error, population heterogeneity and different phenotypes studied. Therefore further studies of innate immunity in asthma and allergy are required, using rigorous study design, measurement of environmental exposure and intermediate phenotypes to demonstrate the functional significance of single nucleotide polymorphisms.

These outcomes are often associated only with individuals raised in a farming environment, or urban environments not both. These results would suggest that there is a gene/environment component to the development of allergic disease. For example the TLR2 polymorphism is only associated with allergic disease in children raised on farms, and not in non-farming children, suggesting the there is a large role of the microbial rich environment of the farm in the development of disease.

In recent years numerous epidemiological studies have examined the relationships between microbial exposure and the development of allergy and asthma. Knowledge of these interactions is now being used to devise novel strategies for the primary prevention of allergic disease[194], or the treatment of established disease. Laboratory based studies have focused on TLR signalling and the mechanisms by which this might modify the pathogenesis of allergic diseases. The following paragraphs summarise current knowledge of the effects of various TLR ligands on allergy and asthma.

### 1.7.1 TLR4

The role of endotoxin (LPS) exposure in allergy and asthma has generated conflicting results, and may reflect differences in dose and timing of exposure. These two factors may help explain the conflicting data in that endotoxin has been found to both exacerbate[195-197] and diminish asthma[35, 198, 199].

Endotoxin has been reported to be present at higher concentrations in homes with regular exposure to animals[35, 200], and infants raised in homes with high endotoxin levels have been found to be at lower risk of subsequently developing allergic disease[35, 201]. It has also been shown that ongoing exposure to low levels of endotoxin can protect

against allergic asthma and that the level of endotoxin associated with an allergen is critically important to the magnitude of ensuing allergic responses. Low doses appear to attenuate allergic airway inflammation while high doses lead to airway inflammation[202].

It would appear that the pro-inflammatory effect of high levels of endotoxin, override the attenuating effect of low levels of endotoxin. This is supported by epidemiological studies showing that low, but not high, levels of endotoxin can attenuate asthma. However, this is further complicated by the findings in mice models that low dose of endotoxin primes the Th2 immune response, whereas high doses promote a Th1 response to inhaled allergen[29].

Prenatal exposure to endotoxin through maternal exposure to stables is associated with less allergy and asthma in infants[36]. Mice models have also shown that maternal LPS exposure has a stimulatory effect on neonatal IFNγ production and protects against allergic sensitisation[203]. Another study has shown that LPS exposure initiated during pregnancy and sustained into the post-natal period increased endotoxin susceptibility and prevented later allergen sensitisation in offspring through inhibition of the Th2 immune response[204].

Early life exposure to household endotoxin protects against the development of allergies in childhood[200, 201, 205-208]. However household endotoxin exposure is a significant risk factor for increased asthma prevalence[209, 210], and endotoxin is a risk factor for wheezing in infancy[195, 211-213]. Endotoxin levels in dust from mattresses are inversely associated to the occurrence of hay fever, asthma and atopic sensitisation[35]. High levels of house dust endotoxin are associated with higher levels of IFNγ producing T-cells[201, 214]. Therefore chronic endotoxin exposure during infancy may provide the stimulus toward enhanced

production of Th1 type cytokines, and thus may provide protection against allergen sensitisation later in life[201]. The Th1 promoting effect of endotoxin is considered to be potentially protective, if the exposure happens to occur very early in life and before the inception of asthma and allergies.

There is a low prevalence among adult or adolescent farmers of allergic symptoms, atopic sensitisation or both[215-218]. Exposure to endotoxins in house dust, later in life appears to have a protective effect on atopic asthma, but may induce non-atopic asthma in farmers[219]. This protective effect could be due to childhood exposures, but one study also showed a decrease in the prevalence of atopic sensitisation and disease among adolescent farmers who were not raised on a farm[220].

In non-farming adults, a study reported trends between high endotoxin levels in house dust and decreased prevalence of atopy and hay fever[186]. Yet another has shown that current exposure to higher levels of house dust endotoxin is associated with reduced odds of allergic sensitisation in adults[221]. These studies however did not take into account the role of endotoxin exposure early in life in these individuals. It is likely that the current level of exposure to endotoxin in the homes of adults are similar to those in their childhood. It may be that the levels of endotoxin exposure during childhood shaped the immune system and that current exposure as adults may be having a lesser effect.

However, Werner and colleagues reported that asthma and wheeze were more prevalent in adult individuals with high endotoxin exposure[186]. Portengen *et al* have also shown that endotoxin exposure in adults is a risk factor for increased airway hyperresponsiveness and low lung function[218]. Endotoxin has a harmful effect in

chronically exposed adults or asthmatic individuals[222]. Patients with asthma are hypersensitive to endotoxin compared with non-asthmatic individuals, and the severity of disease is positively correlated with the amount of endotoxin in their home environment[197]. Endotoxin has strong pro-inflammatory properties, and when inhaled it elicits bronchoconstriction, neutrophilic airway inflammation and systemic responses, such as blood leukocytosis with neutrophilia, in otherwise healthy individuals[223], all of which may play a role in exacerbating established allergic disease in susceptible individuals.

In animal models, the order of endotoxin exposure relative to the allergen/adjuvant is important in determining atopic outcome. When endotoxin exposure occurs immediately before or shortly after allergen sensitisation, inhibition of allergen-specific IgE results. If endotoxin exposure is delayed several days after allergen sensitisation, then endotoxin actually promotes a more robust IgE response [224, 225]. Evidence that endotoxin might reverse atopic disease has been presented in some animal models[224, 226, 227]. Moreover in a mouse model, cockroach antigen induced asthma was ameliorated by the addition of LPS, and this was independent of IL-12[226].

It is important to note that endotoxin exposure is not limited to just LPS, and consequent signalling via the TLR4 pathway. LPS from different species of pathogens can signal via the TLR2 pathway. For example studies comparing lipopolysaccharides from distinct pathogens showed that different immune responses were induced *in vivo*. LPS from *E. coli* (TLR4) differed from that of LPS from *P. gingivalis* (TLR2) in promoting Th1 and Th2 responses respectively[228].

### 1.7.2 TLR9

Endotoxin exposure, as discussed above, appears to play an important role in the development (or inhibition) of allergic diseases. However environmental endotoxin has been shown to be a marker for the presence of bacterial DNA (which signals through TLR9), which is also higher in locales of low asthma and allergy prevalence. DNA from farm barn dust augments the immune modulating effects of endotoxin and may combine with exposure to other such naturally occurring microbial components to mitigate allergy and asthma development[229].

Bacterial and viral DNA causes activation of B cells, NK cells and plasmacytoid DC but not myeloid DC, and the secretion of Th1 cytokines. These effects result from the presence of specific immunostimulatory DNA sequences (ISS) comprising unmethylated CpG dinucleotides (CpG motifs), which are found in abundance in bacterial and viral DNA but are rare in mammalian DNA. These ISS can be mimicked with synthetic oligonucleotides (ODN). These ISS-ODN activate immune cells that express TLR9. Activation through this receptor triggers cellular signalling that leads to the production of a pro-inflammatory and a Th1 type response[230]. This Th1 response is dominated by cytokines (eg IL-12, type 1 IFN's, IFNγ and IL-10) that are known to inhibit the allergic phenotype[231-233]. Therefore, the Th1 response to bacterial DNA is noteworthy in light of the finding that childhood bacterial or mycobacterial infection protects against asthma and other allergic diseases. These data support the hypothesis that during childhood, repeated antigen exposures in the presence of ISS DNA may bias immune responses to Th1 and protect against Th2 type responses such as asthma[234]. As well as providing insight into the pathogenesis of allergy, these studies have now led to attempts to use TLR9 stimulation to prevent or treat allergy in experimental animals and more recently in humans. With this in mind there have been numerous studies using

vaccines, which are ISS DNA-based. These DNA-based therapeutics elicit immunity in part by mimicry of natural infections.

DNA-based therapeutics have shown great promise in rodent models of allergic disease. Presently, four basic DNA based strategies have been utilised in the modulation of allergic hypersensitivities. These include gene vaccination, ISS-ODN immunomodulation, protein (allergen)/ISS vaccination and allergen-ISS-conjugate (AIC) vaccination, summarised in Table 1.4 and discussed below.

| Vaccine type | Effect |
|---|---|
| ISS-ODN | Allergen independent immunomodulator<br>Attenuates hypersensitivity responses<br>Immunomodulation is relatively short lived |
| Protein/ISS-ODN cocktail | Induces Th1 immune response<br>Reverses Th2 biased immune profiles |
| AIC | More immunogenic than protein/ISS-ODN cocktails<br>Less allergenic than allergen extracts |

**Table 1.4. Summary of allergy vaccination strategies.**

### 1.7.2.1 GENE VACCINES

Immunisation with plasmid DNA (pDNA) which encodes allergens may provide a novel method of immunotherapy for allergic disorders[235-237]. Although pDNA encoding allergens appear to show considerable promise in allergy vaccination, pDNA probably does not act via TLR9 and will not be discussed further. However there is emerging interest in combining pDNA immunotherapy with ISS.

### 1.7.2.2 ISS-ODN ALONE

The antigen independent (innate) immune response induced by ISS-ODN is characterised by the production of IL-12, IL-18, type 1 IFN's, IFNγ, IL-6 and IL-10[231, 235, 238-242]. In addition, ISS-ODN induces the expression of a number of costimulatory

molecules on the surface of B cells and APC's[243]. Finally, it has been demonstrated that ISS-ODN inhibits IL-4 dependent IgE synthesis[231]. As such it was hypothesised that by administering ISS-ODN as a vaccine, it's Th1 promoting capabilities would protect against the development or inhibit the Th2 response seen in allergic disorders.

Although allergen-independent ISS-ODN therapy can potently attenuate the allergic phenotype in Th2 sensitised mice, the effect is only temporary as the underlying allergen hypersensitivities are not irreversibly extinguished[244]. For this reason, researchers have examined whether ISS-ODN is more effective when given in conjunction with allergen immunotherapy.

#### 1.7.2.3 ISS-ODN AND ALLERGEN COCKTAIL

Initial reports suggest that ISS-ODN when combined with allergen is an effective way of treating allergy[234, 245-247]. It is more effective than traditional allergen immunotherapy where the allergen is given alone and is more allergen-specific compared to ISS-ODN given alone[234, 239, 248]. By combining ISS-ODN with allergen the immune response to the allergen is reversed from the Th2 allergic response to a Th1 immune response. Several studies have highlighted this effect in mouse models as outlined in the following paragraphs.

Studies using ISS-ODN as a vaccine adjuvant have shown that it elicits a robust, long-lasting adaptive immune response to co-administered proteins[232, 239, 249]. The Th1 biased immune effect of ISS-ODN has been applied to the development of allergy vaccines, which in mice are able to redirect the allergic Th2 response and prevent inflammatory disease manifestations such as asthma, allergic conjunctivitis and allergic rhinitis[244, 250],

even in mice with established allergic disease[234, 251]. In some cases this has been shown to be dependent on Th1 cytokines (IFNγ or IL-12)[252, 253], whereas others show it is independent of a Th1 response[254, 255]. In all of these studies the Th2 immune response was inhibited. As a consequence of preventing and reversing underlying allergic sensitivities, these ISS-ODN vaccination strategies have been further shown to attenuate the immediate hypersensitivity response of anaphylaxis[245, 256, 257] and the late phase allergic response of asthma [235, 248].

The allergen-specific response induced by immunization with ISS-ODN prevents and reverses Th2 biased immune deviation and asthma[234, 245-247, 258], and more importantly this effect seems to be long-lived and allergen specific[244, 245].

#### 1.7.2.4 ALLERGEN-ISS-CONJUGATE (AIC)

As the combination of allergen and ISS-ODN administered together elicits a fairly robust inhibition of Th2 responses, studies next looked at whether conjugating the two together could enhance the effect. A potential or theoretical problem with administering the ISS-ODN and the allergen separately is that the two compounds might be taken up by separate APC's, so that any beneficial effect would rely on cross talk between separate APC's. It was hypothesized that by physically linking ISS-ODN to allergens (AIC), that would colocalise the ISS-ODN and the allergen, and that this would lead to allergen presentation on ISS-ODN activated APC's. It has been shown that ODN conjugation facilitates APC uptake and presentation of antigens[259]. Further to this it was hypothesised that AIC might be more immunogenic than a cocktail of ISS-ODN and allergen, and that AIC might be less allergenic than allergen/ISS-ODN cocktail.

Several groups of investigators have shown that AIC vaccination is more effective than allergen/ISS-ODN co-administration[260-263] in preventing Th2 biased immune deviation and the early phase hypersensitivity response in a model of anaphylaxis[264]. The available evidence suggests that AIC is more immunogenic than native antigens and antigen/ISS-ODN cocktails across species, and is more effective in the prevention of allergic hypersensitivity responses, as detailed below.

It has been shown that injection of ragweed allergen (Amba1) linked to an ISS-ODN led to a robust Th1 biased response against Amba1 in mice, rabbits and monkeys while Amba1 alone induced a Th2 biased immune response. In addition the conjugate induced immune response was significantly stronger than the immune response elicited after injection of Amba1 mixed with ISS-ODN or a plasmid encoding Amba1[261]. This Amba1-ISS-ODN-conjugate has been evaluated as an allergy vaccine in human clinical trials, and is providing encouraging evidence for a selective and specific redirection of the allergic Th2 response towards a non-allergic and non-inflammatory Th1 response, and has shown a significant clinical benefit with reduced allergic symptoms[262].

The principles of vaccination and immunomodulation represent two distinct yet interrelated strategies for the utilisation of ISS DNA-based reagents in the treatment of allergic diseases. With DNA vaccination, a long-lasting Th1 biased adaptive immune response develops. This Th1 biased immune profile both prevents and reverses allergen specific Th2 biased responses, and consequently, animals vaccinated with DNA-based vaccines are protected from allergic hypersensitivity responses for an extended period of time. In contrast ISS-ODN induced immunomodulation appears to depend on the antiallergic innate cytokine response, which is generated upon exposure to ISS-ODN. It is likely that ISS-ODN induced immunomodulation requires repeated exposures to

allergen in the context of ISS induced innate immune activation in order to imprint a Th1 bias on the adaptive immune system[265].

The therapeutic goal of allergen vaccination is the induction of a protective immune response to an allergen to which a clinical hypersensitivity pre-exists by affecting a change in the allergen specific adaptive immune response. The encouraging results obtained with immunotherapy with TLR9 ligands conjugated to allergens raises important questions about the mechanisms involved and provides an impetus for similar studies of other TLR ligands.

### 1.7.3 TLR2

Previous reports looking at the interaction between TLR2 stimulation and allergy have provided contradictory data. Initial studies using murine models of allergic asthma reported that TLR2 ligands administered during the sensitisation period led to an enhancement of Th2-mediated allergic inflammation[252, 266]. Studies using Pam3CSK4, showed increased airway hyperresponsivness, induction of IL-13 and other Th2 markers, in animal models of OVA induced asthma when the Pam3CSK4 was given at the same time as the allergen[252, 266]. In contrast to these studies Velasco *et al*[199] also used Pam3CSK4 and another TLR2 ligand peptidoglycan, and when these ligands were given either during allergen sensitisation or at the time of challenge resulted in decreased allergen-induced pulmonary recruitment of eosinophils and overall a decrease in allergic responses. Similarly, another report looking at the TLR2 ligand lipoprotein Opr1, given in combination with allergen at the time of sensitisation also found an inhibition of Th2 responses[267]. These two studies are in agreement with Patel *et al*[268] who found that Pam3CSK4, when given to previously sensitised mice, caused a

decrease in total inflammatory cell infiltrate and eosinophilia. Further, Pam3CSK4 resulted in a decrease in allergen-specific IL-4 and IL-5. However Patel *et al* also noted an increase in allergen induced IFNγ, IL-12 and IL-10, indicative of an enhanced Th1 response[268]. The conflicting results between these studies could be due to experimental design and/or the dose and timing of ligand administration compared to allergen sensitisation. Whether these results are indicating a redirection of the immune response to Th1 response as Patel *et al*[268] suggests, or whether it is just a switching off of the Th2 response[267] is still the mater of debate.

The data in published human studies are also just as conflicting as those into the mice models with several papers showing an increase in Th2 response and others a decrease in Th2 response. In human *in vitro* cultures TLR2 stimulation is reported to enhance mast cell degranulation and Th2 cytokine production[269-272]. For example, Re and Strominger[270] demonstrated with several TLR2 ligands that stimulate human DC, resulted in cytokine production which favoured a Th2 response, but did not promote a Th1 response. These studies were supported by Agrawal *et al*[272], who found that Pam3CSK4 also induced DC's to promote Th2 biased responses. However, Weigt *et al* have shown that MALP-2 a TLR2/6 ligand shifts a Th2 skewed immune response to Derp1 towards a Th1 response *in vitro*, but this response is only seen when MALP-2 is combined with IFNγ[273].

Further studies have found that cord blood monocytes from children with allergic mothers had lower TLR2 expression compared to maternal monocytes, in contrast to children with non-allergic mothers who showed no difference[274]. The authors speculate that this result suggests that monocytes from children with allergic mothers have reduced capacity to respond to microbial stimuli[274]. However, Ege and colleagues have

shown that maternal exposure to farm animals during pregnancy caused an increase in TLR2 gene expression, and provided a protective effect for allergic disease in the offspring[39]. Furthermore they also highlighted a dose response relationship between the extent of upregulation of TLR2 genes and the number of different farm animal species the mother had encountered during pregnancy[39]. This is supported by a study by Lauener *et al*, who also found farmers children have more TLR2 expression than non-farmers children, and that this is associated with a decreased risk of developing allergies[182]. Suggesting that maternal exposure to microbe enriched environments (ie farms) during pregnancy, or the early exposure of children to these environments, may protect against the development of atopic sensitisation.

Eder and colleagues have also shown that TLR2 is a major gene for asthma in children of European farmers[183]. They found that genetic variation in TLR2 is a major determinant of the susceptibility to asthma and allergies in children of farmers, but this was not demonstrated in their urban counterparts. Several studies testing this association between genetic differences and allergy have not confirmed this association[191-193]. However, these other studies were relatively small and used a different population compared to the study by Eder.

The above research highlights the need for further studies into the effect of TLR2 ligands and to define the mechanisms by which they exert their effects in established human allergic disease.

### 1.7.4 Other TLR's

It remains to be determined whether TLR3, TLR5, and TLR7/8 ligands can significantly influence the allergic phenotype, though their immunomodulatory influence on adaptive immunity has been investigated to some extent. TLR3 ligands have been found to serve as Th1 biasing adjuvants[275], similarly, the TLR5 ligand flagellin was initially described as a Th1 adjuvant[276], but more recently studies have suggested that flagellin favours a Th2 response[277]. Ligands for TLR7 and TLR8 have consistently been described as Th1 polarizing adjuvants. R848 (TLR7/8) has been reported to inhibit Th2 cell cytokine production while promoting production of IFNγ by human Th2 cells[278].

It is likely that both innate and adaptive immune mechanisms contribute to the initiation and maintenance of asthma. Although not well defined, the potential links are intriguing both in the pathogenesis of the disease and for its treatment. A large component of the discussion in TLR and asthma revolves around endotoxin, which contains many microbial compounds. LPS can both exacerbate established asthma, by increasing airway inflammation as well as prevent asthma development, through early exposure. Although the mechanisms remain to be defined, it is clear that TLR triggering can either protect or exacerbate the severity of asthma, likely depending on the timing and dose of exposure. The challenge of current research is to reconcile these paradoxic observations. Furthermore the majority of our current knowledge focuses on LPS and TLR4 interactions, while a newer body of literature would seem to suggest that other TLR ligands and receptors might have similar effects and warrant further investigation.

## 1.8 Project Aims

There has been a large increase in allergic disease worldwide, resulting in a large burden to the individual and society. This increase has been linked to a cleaner and more "healthy" environment as outlined in the *hygiene hypothesis*. From epidemiological studies it has become apparent that exposure to microbial compounds during the early years of life can decrease the risk of allergic disease. Numerous studies utilising mouse models have enhanced our knowledge of how microbial compounds (mainly endotoxin, TLR4) can affect the onset or reversal of allergic disease. However few studies have looked at the same effects in humans, and none have looked at the effects of TLR2 stimulation on established allergic responses in humans.

The studies outlined in this thesis aim to investigate the effect of TLR2 ligands on the immune response to allergen in adults with and without established allergies. In particular, I sought to:

a. Examine the effects of various components of gram-positive bacteria, acting through TLR2, on T-cell responses to HDM allergens in adults with established allergic sensitisation, and
b. Determine the mechanisms by which TLR2 ligands exert their effects.

# 2 METHODS

## 2.1 Subjects

Forty-five laboratory volunteers aged between 19 and 63 years were recruited for the study. All subjects underwent blood sampling and skin prick testing (SPT) to common environmental allergens including HDM, domestic animals, grass pollens, cockroach and staple foods, and completed a questionnaire detailing symptoms of asthma, allergic rhinitis and eczema. Based on SPT results, subjects were classified into those who were HDM allergic (n=23, mean wheal size 7±2mm) and those who were not sensitised to HDM (n=22, negative SPT). Subjects with weakly positive SPT reactions to HDM (wheal size 1-3mm) were not included. No subjects were taking inhaled or systemic corticosteroids or antihistamines. The Human Research Ethics Committee, Sir Charles Gairdner Hospital, Perth, approved the study and all subjects provided written informed consent to participate in the study. For mechanistic experiments requiring large numbers of cells, buffy coats were also obtained from the Red Cross blood transfusion centre from healthy adult volunteers, whose atopic status was unknown.

## 2.2 Reagents

The following ligands for Toll-like receptor (TLR) 2 were purchased: heat-killed *Staphylococcus aureus* strain Cowan I (Calbiochem, California, USA under the brand name Pansorbin (PS)), *Staphylococcus aureus* lipoteichoic acid (LTA; InvivoGen, San Diego, USA), and the synthetic lipopeptide Pam3CSK4 (certified endotoxin free; InvivoGen, San Diego, USA). An aqueous extract of lyophilised *Dermatophagoides pteronyssinus* (HDM) was provided courtesy of Professor PG Holt. The HDM extract was prepared 'in-house' using mites purchased from CSL (Parkville, Australia), and has been extensively used in studies from the Division of Cell Biology, Telethon Institute of Child Health Research, over the last decade[21, 279-281]. Prior dose response experiments

have shown that a concentration of 10μg/mL provides optimal discrimination between mite allergic and normal individuals. The extract contains the two major allergens Der p 1 and Der p 2, but does not contain some of the minor lipid soluble allergens found in *Dermatophagoides* species (B. Hales and W. Thomas, unpublished communication). Though the HDM extract contains trace amounts of endotoxin, removal of endotoxin from the allergen preparation (Acrodisc® Chromatography Unit, Pall Corporation, Ann Arbor, MI) has no effect on IL-5 and IL-13 synthesis by HDM-stimulated PBMC (B. Hales, personal communication). Biotinylated anti-human TLR2 mAb (clone TL2.1) was obtained from eBioscience (San Diego, USA), while streptavidin allophycocyanin (APC), anti-CD3 peridinin chlorophyll protein (PerCp), anti-CD14 fluorescein isothiocyannate (FITC), anti-CD19 phycoerythrin (PE), anti-CD56 phycoerythrin (PE), and isotype matched control mAbs were purchased from BD Biosciences (San Jose, CA, USA).

## 2.3 Cell preparation and culture

All laboratory experiments were performed and analysed without knowledge of the atopic status of the subjects. Peripheral blood was collected into an equal volume of RPMI-1640 (Gibco BRL, Melbourne, Australia) containing preservative-free heparin (20U/mL). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation and cryobanked in liquid nitrogen as previously described[282]. Previous studies from others within the Division of Cell Biology and elsewhere have demonstrated that this procedure does not distort cellular immune responses[282-284].

Allergen-specific lymphocyte responses were determined as previously described[21, 280]. Briefly, PBMC were thawed and resuspended in AIM-V medium (Gibco Life

Technologies, New York) supplemented with 1% autologous plasma and 2-mercaptoethanol (final concentration, 4 x $10^{-5}$M; Sigma, Sydney, Australia). Cell viability was >90%. Cytokine production and lymphoproliferation were assessed by culturing PBMC in round bottom 96-well plates at 2.5 x $10^5$ cells/well, in duplicate for cytokine production, for 24hrs, 48hrs or 5 days, and in triplicate for proliferation, for 5 days. Cells were cultured in medium alone or with optimal stimulating concentrations of HDM (10μg/mL) in the presence or absence of Pansorbin 0.01% and 0.0001%, LTA 20μg/mL or Pam3CSK4 300ng/mL. In some experiments, PBMC were also cultured with a control antigen, tetanus toxoid (TT, CSL Ltd., Melbourne, Australia; 0.5 Lf/mL) in the presence or absence of TLR2 ligands. Optimal concentrations of all three TLR2 ligands were determined in preliminary dose-response experiments (data not shown). Proliferation was measured by incorporation of $^3$H-thymidine for 16-18hrs before harvest. After subtraction of results obtained from unstimulated control cultures, data are presented as Δ disintegrations per minute (dpm) per culture.

In experiments designed to look for LPS contamination, cells were cultured as stated previously with the addition of polymyxin B (PmB, Sigma-Aldrich, Castle Hill, NSW, Australia) at a final concentration of 30μg/mL. This concentration was able to inhibit the response to 10ng/mL LPS.

In some experiments, PBMC were cultured for 24 hours in the presence or absence of TLR2 ligands, and HLA-DR expression determined by flow cytometry, as a measure of the activation status of antigen presenting cells (APC).

## 2.4 T-cell activation

In some experiments it was necessary to examine T-cell activation in the absence of APC's. This was achieved using a T-cell activation kit from Miltenyi Biotec (Auburn, CA, USA). This kit comprises beads, which are labelled with CD2, CD3 and CD28 antibodies (hereafter referred to as TBds), and are used to mimic antigen-presenting cells and activate resting T-cells from PBMC. T-cells were isolated from PBMC's by positive CD4 selection using CD4 MACS beads (Miltenyi Biotec, Auburn, CA, USA). Cells were resuspended to $1x10^6$ cells/mL in AIM-V medium supplemented with 1% fetal calf serum and 2-mercaptoethanol (final concentration, $4 \times 10^{-5}$M). Cells were cultured in round bottom 96-well plates at $2.5 \times 10^5$ cells/well, in duplicate. The TBd to T-cell ratio used was 1:10.

In other experiments APC activation was promoted with the use of CD40 Ligand (CD40L; Alexis, San Diego, CA, USA). PBMC's were cultured at $1x10^6$ cells/ml in AIM-V medium (Gibco Life Technologies, New York) supplemented with 1% autologous plasma and 2-mercaptoethanol (final concentration, $4 \times 10^{-5}$M). Cells were cultured in round bottom 96-well plates at $2.5 \times 10^5$ cells/well, in duplicate, in medium alone or with optimal stimulating concentrations of HDM (10μg/mL) in the presence or absence of CD40L (100ng/mL) and enhancer (1ug/mL) for 5 days.

## 2.5 Quantitative Real-Time PCR - Foxp3

Foxp3 expression in cultured cells was determined by RT-PCR as a surrogate marker of Tregs. PBMC's were cultured as stated previously for 48hs. Cell pellets were resuspended in RNAlater and stored at -20˚C until used. Cell pellets were spun out and resuspended in lysis buffer and total RNA was isolated using the Qiagen RNeasy 96

well Kit (Qiagen, VIC, Australia) according to the manufacturer's directions. Reverse transcription was performed using the Omniscript kit (Qiagen, VIC, Australia) according to the manufacturer's protocol with oligo-dT (Promega, NSW, Australia) and Superasin (Promega, NSW, Australia). Foxp3 intron-spanning primers were based on previously published sequences[285]. Reverse transcribed cDNA samples were diluted 1/5 and quantitated by real-time PCR using QuantiTect SYBR Green Master Mix (Qiagen, VIC, Australia) on the ABI PRISM 7900HT (Applied Biosystems, CA, USA). Melting curve analysis was used to assess the specificity of the assay. Copy numbers were determined by 10-fold serial dilutions of plasmid standards and normalised to the reference gene UBE2D2[286].

## 2.6 Cytokine protein detection

The levels of IL-5, IL-6, IL-10, IL-13, TNFα and IFNγ were measured in culture supernatants by time resolved fluorometry (TRF), as previously described[279]. Capture and detection antibody pairs were from BD Pharmingen: rat $IgG_1$ anti-mouse/human IL-5 (clone TRFK5) and biotinylated rat $IgG_{2a}$ anti-human IL-5 (clone JES1-5A10), rat $IgG_1$ anti-human IL-6 (clone MQ2-13A5) and biotinylated rat $IgG_{2a}$ anti-human IL-6 (clone MQ2-39C3), rat $IgG_1$ anti-human/viral IL-10 (clone JES3-9D7) and biotinylated rat $IgG_{2a}$ anti-human/viral IL-10 (clone JES3-1298), rat $IgG_1$ anti-human IL-13 (clone JES10-5A2) and biotinylated mouse $IgG_{1,\kappa}$ anti-human IL-13 (clone B69-2), mouse $IgG_1$ anti-human TNFα (clone MAb1) and mouse $IgG_1$ anti-human TNFα (clone MAb11), mouse $IgG_{1,\kappa}$ anti-human IFNγ (clone NIB42) and biotinylated mouse $IgG_{1,\kappa}$ anti-human IFNγ (clone 45.33). IL-12 was measured by DuoSet ELISA Development System for human IL-12p70 (R&D systems) as per manufacturers instructions. The lower limit of detection of the IL-12p70 assay was 50pg/mL and for all other cytokines

10pg/mL. Levels of cytokine synthesis are presented as Δ above unstimulated control cultures. Preliminary experiments showed that IL-6, IL-10 and TNFα synthesis was maximal at 24hrs, whereas IFNγ, IL-5 and IL-13 were maximal at day 5 (data not shown).

## 2.7 Cellular expression of TLR2

Constitutive TLR2 expression on cell subsets of PBMC was determined by flow cytometry. Cells were washed in PBS supplemented with 0.1% sodium azide and 1% normal human serum, and incubated for 30 minutes on ice with anti-TLR2 in combination with anti-CD3 (T-cells), anti-CD14 (monocytes), anti-CD19 (B-cells), anti-CD56 (NK-cells) or corresponding isotype matched control mAbs, followed by streptavidin APC. Dendritic cells (DC) were identified using a commercial kit (BD Biosciences, California, USA) as HLA-DR+ cells lacking CD3, CD14, CD16, CD19, CD20 and CD56 expression, and further divided into myeloid DC and plasmacytoid DC based on differential expression of CD11c (myeloid) or CD123 (plasmacytoid). Analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson) and FlowJo software to determine the percentage of various cell populations being positive for TLR2.

## 2.8 Antigen uptake/processing

Antigen uptake was determined using dextran fluorescein (Molecular Probes, Eugene, OR) while antigen processing was evaluated using DQ-Ova (Molecular Probes, Eugene, OR). This latter compound is a self-quenched conjugate of ovalbumin that only exhibits bright green fluorescence upon proteolytic degradation within antigen presenting

cells[287]. These studies employed monocytes purified by adherence that had been cultured for 48hrs in RPMI supplemented with IL-4 (10ng/mL), GM-CSF (100ng/mL) and 5% FCS, and hereafter referred to as Mono48 cells. This short period of culture induces monocytes to become non-adherent and highly endocytic, and therefore ideally suited to studies of antigen uptake and processing, though they have not fully differentiated into DC.

Mono48 cells were pulsed with PS 0.01% for 45 min at 37°C and then washed extensively with RPMI + 5% FCS at 4°C. Cells were transferred to 37°C, and 0.25μg/mL dextran fluorescein or 5μg/mL DQ-Ova added. Excess dextran fluorescein was removed by washing with PBS supplemented with 0.1% sodium azide and 1% normal human serum and quenched with 0.25μg/mL trypan blue. The uptake of dextran fluorescein and the processing of OVA into peptide were assayed by measuring the increase in mean fluorescence intensity (MFI) over time. Dextran fluorescein and DQ-conjugated OVA peptide were quantified using the FITC channel of the flow cytometer.

## 2.9 Statistical analysis

Group data were expressed as median & interquartile ranges unless otherwise stated. Comparison between different groups was performed using the Mann-Whitney U test and within groups using the Wilcoxon Signed Ranks test. Spearmans Correlation was used for the RT-PCR Foxp3 data. Values of $p \leq 0.05$ were considered significant. Antigen uptake and processing were expressed as the percentage MFI, relative to the untreated media control at the final time point.

# 3 TLR2 LIGANDS INHIBIT TH2 RESPONSES IN VITRO

## 3.1 Introduction

There is intense interest in the interaction between exposure to microbial products and the clinical expression of human allergic diseases such as asthma, allergic rhinitis and eczema, as discussed in chapter 1. Microbial infections are frequently implicated in exacerbations of asthma and eczema for example[288, 289]. In contrast, epidemiological studies have suggested that children growing up in microbial rich environments may be protected from the development of allergic sensitization[35, 36]. This protective effect was originally attributed to endotoxin (LPS) derived from Gram-negative bacteria, though more recent studies have suggested that exposure to components of Gram-positive bacteria may also protect against the development of allergic disease[290].

TLR2 is the receptor involved in the recognition of lipoproteins and lipoteichoic acid (LTA) from Gram-positive bacteria. Previous reports looking at the interaction between TLR2 stimulation and allergy have provided contradictory data. Initial studies using murine models of allergic asthma reported that TLR2 ligands administered during the sensitisation period led to an enhancement of Th2-mediated allergic inflammation[252, 266]. Conversely, other studies have found that synthetic lipopeptides administered immediately prior to airway allergen challenge inhibit Th-2 type responses and IgE production[291]. Recent studies also suggest that TLR2 stimulation inhibits established allergic airway inflammation, an effect which is mediated by an augmented Th1 response[268]. Notably, these studies have all used murine models of asthma, and none have looked at the effects of TLR2 stimulation on established allergic responses in humans.

In this chapter I investigate the effect of microbes on the immune response to allergens in humans. In particular, I sought to examine the effects of various components of Gram-positive bacteria, acting through TLR2, on the established allergic immune response to HDM. My studies show that all three TLR2 ligands examined, including a heat killed preparation of *Staphylococcus aureus* (Pansorbin), staphylococcal lipoteichoic acid (LTA) and a synthetic lipopeptide (Pam3CSK4) were able to inhibit Th2 cytokine secretion by HDM stimulated PBMC from mite allergic individuals, but had no effect on immune responses from non-allergic individuals. The effects of the three TLR2 ligands did not involve induction of an allergen-specific Th1 (IFNγ) response.

## 3.2 Results

### 3.2.1 Subjects

Twenty-three subjects (6 men and 17 women; mean age 42yrs) were classified as being HDM-allergic by having a positive HDM SPT (mean wheal size 7mm, range 4-11) and twenty-two (6 men and 16 women; mean age 37yrs) as non-HDM allergic (negative SPT). Positive SPT (wheal size >3mm) to other allergens (Grass, cat, dog, peanut, mould, ova and milk) were noted but not used to distinguish between groups. In the HDM-allergic group, 87% (n=20) had a positive SPT to one or more of the other allergens tested, whereas only 27% (n=6) of the non-HDM allergic group had a positive SPT to any allergen. There were no significant differences in age or gender between groups. (Table 3.1)

| Atopy status | HDM Mean wheal size | Sex M/F | Age Mean in yrs | Other Positive SPT |
|---|---|---|---|---|
| HDM-allergic | 7 (4-11mm) | 6/17 | 42 | 20 (87%) |
| Non-allergic | 0 | 6/16 | 37 | 6 (27%) |

**Table 3.1. Subject statistics.**

### 3.2.2 TLR2 ligands produce varying patterns of cytokine synthesis

Prior to determining the effects of TLR2 ligands on allergen-specific T-cell responses, it was important to examine the effects of TLR2 ligands in the absence of allergen. PBMC were therefore stimulated with PS, LTA or Pam3CSK4, and supernatants collected at 24hrs and 5 days.

Although all three ligands stimulate through TLR2, they produced varying patterns of cytokine synthesis. Whereas all three stimuli induced IL-6 synthesis, only the higher concentration of PS (0.01%) produced substantial amounts of IFNγ or TNFα (Figure 3.1). PS and LTA, but not Pam3CSK4, induced IL-10 synthesis. Production of the Th2 cytokines IL-5 and IL-13 was minimal with all ligands (Figure 3.1b).

### 3.2.3 TLR4 signalling/LPS contamination

To rule out the possible effect of LPS contamination, PBMC were cultured with TLR2 ligands, with and without polymyxin B (PmB) to determine if the same cytokine patterns were seen. PmB is a cyclic fungal peptide that binds to LPS with high affinity, this neutralises the LPS, making it unable to signal via TLR4[115]. The addition of PmB to the TLR2 ligands did not significantly alter the IL-6, IL-10 and TNFα cytokine production by the TLR2 ligands at 24hrs (Figure 3.2). It did however inhibit the cytokine production by the control ligand LPS, indicating that the concentration of PmB

used can efficiently reduce the effect of LPS up to a concentration 10μg/mL. This confirms that the effect of the three ligands (PS, LTA and Pam3CSK4) is occurring through TLR2 activation and not through signalling of TLR4 by LPS contamination of these reagents.

### 3.2.4 TLR2 ligands decrease Th2 cytokine response to HDM

As expected, PBMC from HDM-allergic subjects produced more IL-5 and IL-13 than PBMC from non-allergic subjects when stimulated with HDM *in vitro* ($p<0.001$, $p=0.014$ respectively; Figure 3.3). HDM-stimulated IFNγ production was similar in both groups of subjects (Figure 3.3). When HDM and TLR2 ligand stimulation were combined, all three TLR2 ligands induced a significant inhibition of IL-5 (40-80%) and IL-13 (40-75%) synthesis in the HDM-allergic subjects. In contrast, the addition of TLR2 ligands had only a minor effect on HDM-stimulated IL-5 and IL-13 synthesis in the non-allergic group, aside from a small reduction associated with LTA ($p=0.016$ IL-5, $p=0.006$ IL-13).

I next asked whether the ability of TLR2 ligands to inhibit Th2 cytokine production might be associated with deviation towards a Th1 immune response. The different TLR2 ligands showed markedly different effects on IFNγ production, which was mirrored in both the HDM-allergic and non-allergic groups. High dose PS (0.01%) combined with HDM was associated with high levels of IFNγ production (Figure 3.3), though this appeared to be due to the direct effects of PS 0.01% *per se*, as shown in Figure 3.1. However, low dose PS (0.0001%) did not change IFNγ production, while the more highly purified TLR2 ligands LTA and Pam3CSK4 led to a small reduction in HDM stimulated IFNγ production in the allergic group (Figure 3.3a). IL-12 is a potent

Th1 associated cytokine, and was measured to determine if the TLR2 ligands were inducing it. IL-12p70 was not detected (limit of detection 50pg/mL) under any condition. IL-10 is a regulatory cytokine and has been measured to see if the TLR2 ligands induced it. IL-10 production in TLR2 stimulated PBMC was examined and all TLR2 ligands except Pam3CSK4 induced IL-10. IL-10 production in stimulated PBMC was examined in a small subset of subjects, no TLR2 ligand altered the level of IL-10 when cultured with HDM, from that observed with HDM alone (Figure 3.4). IL-12p70 was not detected under any condition (<50pg/mL, data not shown). Thus it appears that the mechanism(s) by which all three TLR2 ligands inhibit allergen-induced Th2 cytokine production is unlikely to involve IFNγ, IL-12p70 or IL-10.

### 3.2.5 Inhibition of Th2 response is allergen specific

To test whether the inhibition of Th2 response was allergen specific, experiments were also conducted with a control antigen tetanus toxoid (TT) instead of HDM. In both allergic and non-allergic subjects, TLR2 ligands had no significant effect on PBMC responses to the control antigen TT. This data suggests that the inhibition of Th2 cytokine production is allergen specific (Figure 3.5).

## 3.3 Discussion

The key finding to emerge from the studies in this chapter is that TLR2 ligands have the capacity to inhibit Th2 cytokine production by blood mononuclear cells stimulated with mite allergen. All TLR2 ligands studied induced significant and substantial reduction in IL-5 and IL-13 synthesis by mononuclear cells from mite allergic adults. The lack of a similar effect in the non-allergic subjects can probably be explained by the relatively small responses to mite allergen in this group.

LPS contamination and subsequent TLR4 signalling has been addressed and appears not to be the cause of the inhibition of Th2 cytokine synthesis by the addition of the TLR2 ligands. LPS may be present in small quantities in the PS and LTA preparations, but this is not the main route of signalling for these ligands. Strengthening the argument that TLR2 stimulation is the main factor for the inhibition of Th2 response to HDM in this system.

Increasing evidence suggests that exposure to microbial stimuli, acting via the innate immune system, can influence adaptive immune responses to allergens and the development of asthma and other allergic diseases[35, 36, 289]. While most experimental studies have examined the TLR4 ligand LPS in this context, recent studies in mouse models of asthma have suggested that TLR2 ligands can also enhance or inhibit Th2 immune responses[266, 268, 291]. In the current study I have shown in humans that TLR2 ligands inhibit recall/memory responses to HDM allergen. Murine models investigating the effect of LPS and TLR4 signalling have suggested divergent outcomes depending on the timing of allergen challenge and TLR ligand administration. This may explain the different responses seen in relation to TLR2 ligands and allergen induced Th2 responses. The studies presented within this thesis have not only investigated TLR2 ligands in relation to allergen outcomes in a human population, but also showed that TLR2 ligands can reduce allergen induced Th2 responses in sensitised individuals, and is specific to allergens and not other antigens.

The inhibition of Th2 cytokine production was observed with a variety of TLR2 ligands, including high and low concentrations of heat killed *Staphylococcus aureus*, lipoteichoic acid and Pam3CSK4. The fact that both the heat killed *Staphylococcus*

*aureus* and both the purified TLR2 ligands inhibit allergen-specific Th2 cytokine production is consistent with the notion that the findings described in Figure 3.3 are mediated via a TLR2 dependent mechanism. Further confirmation of this would require the use of blocking antibodies against TLR2, and this is an issue worthy of future study.

It does not appear that this involves deviation to an allergen specific Th1 response pattern, as induction of IFNγ production was not consistently observed with the various TLR2 ligands and no IL-12 was detected. While the higher dose of PS did induce substantial IFNγ synthesis, this appeared to be due to direct effects of PS on PBMC, as shown in Figure 3.1, and may reflect a Th1 response to staphylococcal proteins. Importantly, the lower dose of PS had no effect on IFNγ production, while the purified TLR2 ligands LTA and Pam3CSK4 actually inhibited HDM stimulated IFNγ production in the allergic subjects (Figure 3.1b). The apparent lack of involvement of IFNγ in mediating these effects is in contrast to the findings of Patel *et al* who recently reported in a mouse model of asthma that the ability of Pam3CSK4 to inhibit allergic airway inflammation was IFNγ dependent, but did not involve the induction of regulatory T-cells[268]. A previous study in humans reported that the lipopeptide LP40 enhances IFNγ and IL-12p70 production in humans[291], though in that study LP40 also induced a residual cytokine response in TLR2 knockout mice, suggesting that the LP40 lipopeptide may also activate other receptors in addition to TLR2. If these TLR2 ligands are going to be used in a clinical setting, further studies are needed to examine a wide range of lipoproteins and lipopeptides in order to determine those that exhibit the most favourable down-regulation of an immune response to allergens, without inducing the potentially deleterious pro-inflammatory cytokines seen with heat killed *Staphylococcus aureus*.

This chapter has shown that TLR2 ligands have the ability to inhibit the Th2 response to mite allergen in previously sensitized individuals by a mechanism yet to be determined. I next sought to find the mechanism by which this inhibition of Th2 response was mediated, and to determine which cells express TLR2 in order to determine which cells are targeted by TLR2 ligands.

## 3.4 Figures

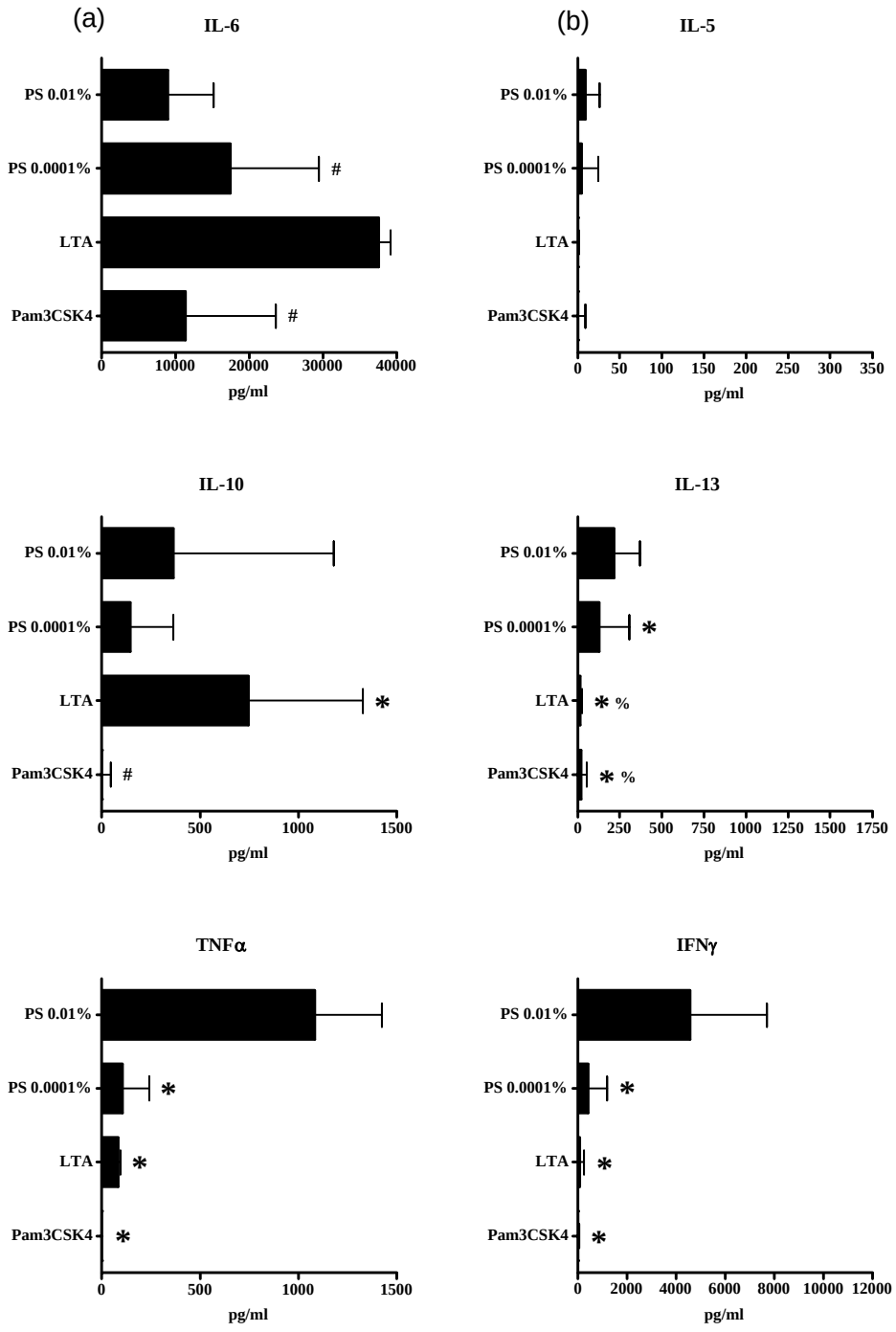
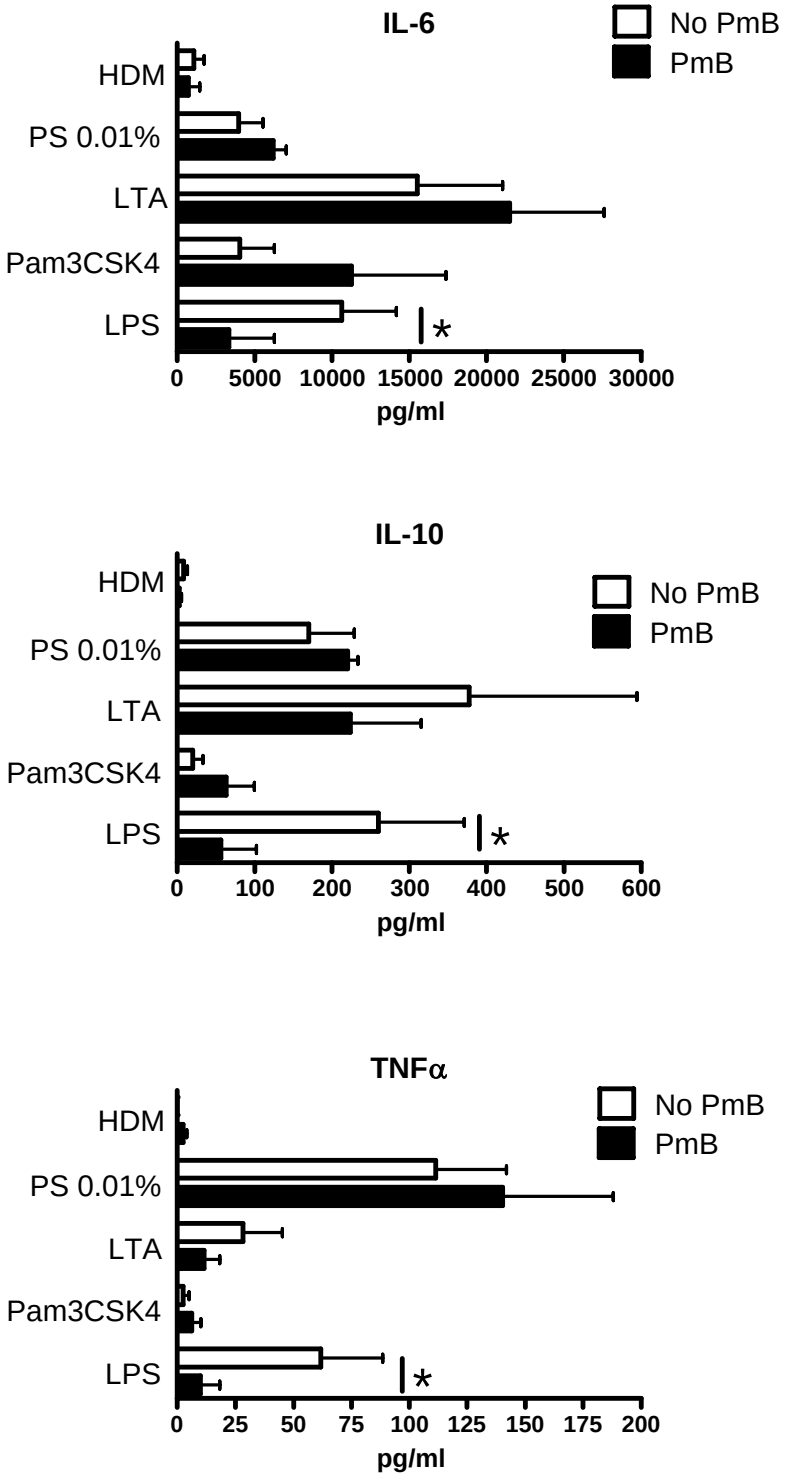


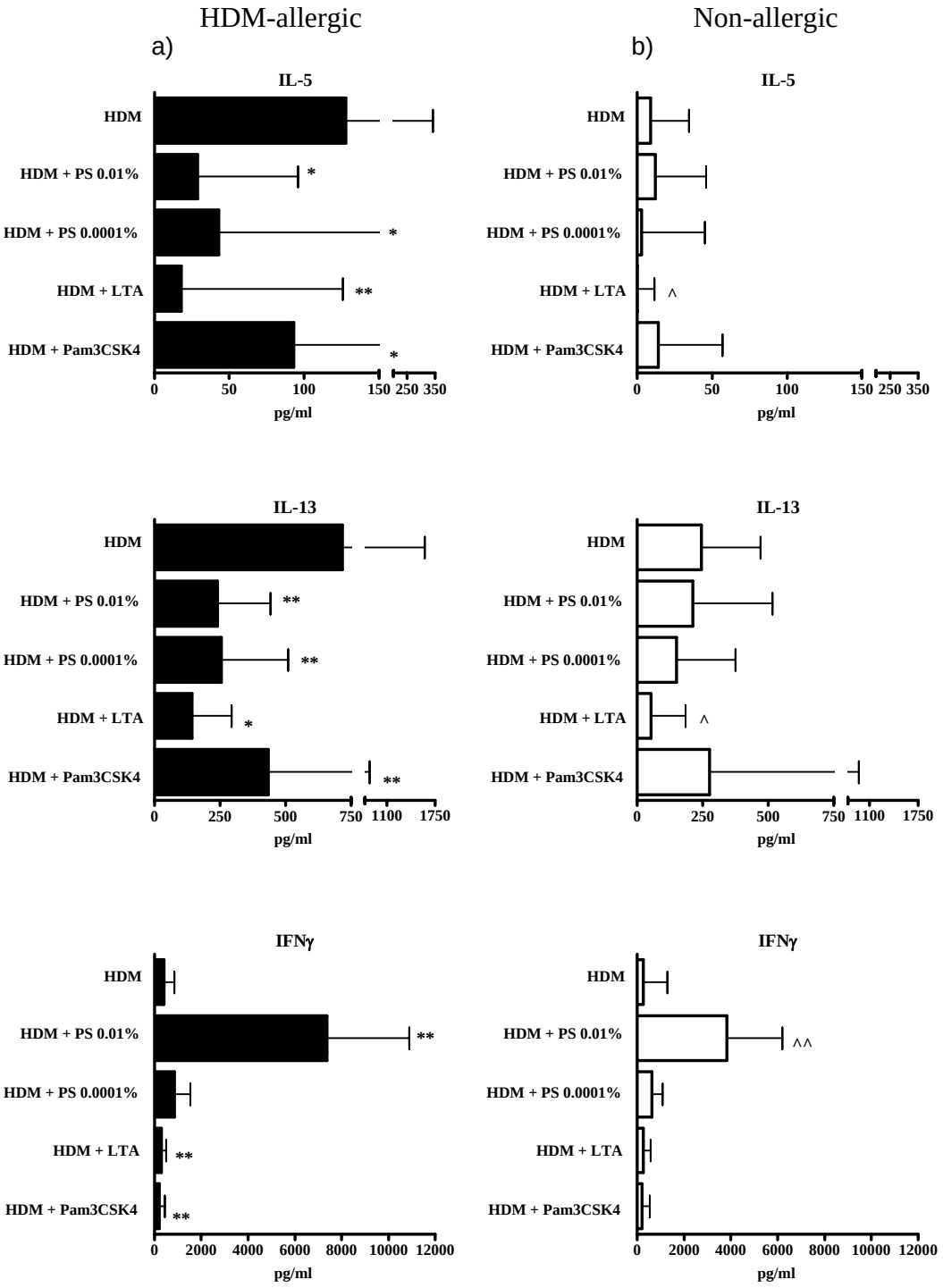

**Figure 3.1. Effects of TLR2 ligands on PBMC in the absence of allergen.**

PBMC (HDM-allergic and non-allergic) were cultured with optimal concentrations of PS, LTA and Pam3CSK4, for 24hrs and 5 days. Concentrations of cytokine synthesis were measured in the supernatant by TRF. (a) Concentrations of IL-6, IL-10 and TNFα measured at 24hr post-stimulation. n=4. (b) Concentrations of IFNγ, IL-5 and IL-13 measured at day 5 post-stimulation; n=45. $^{*/\%/\#}$ $p<0.05$ compared to *PS 0.01%, $^{\%}$PS 0.0001% and $^{\#}$LTA.



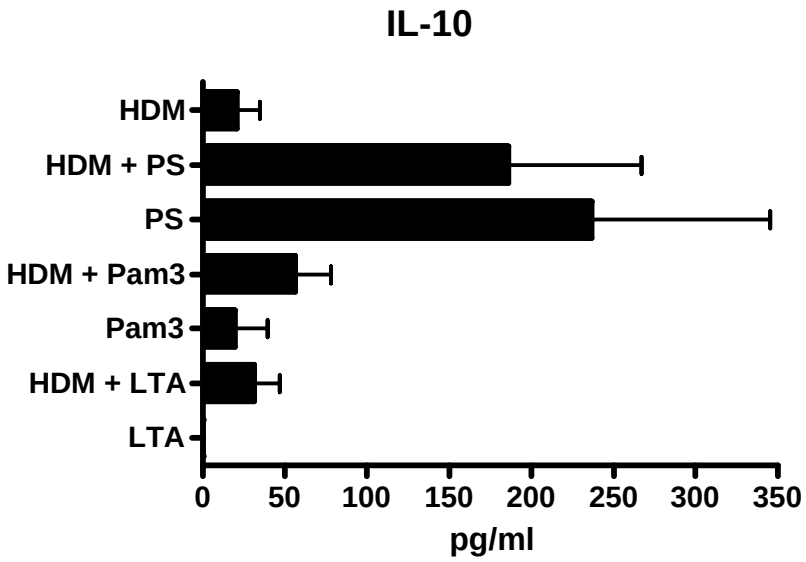

**<u>Figure 3.2.</u> Effect of PmB on cytokine synthesis by TLR2 ligands.**

PBMC (HDM-allergic and non-allergic) were cultured with TLR2 ligands and control ligand LPS, with and without PmB for 24hrs, to determine the presence of LPS contamination. Concentrations of cytokine synthesis were measured in the supernatant by TRF. Graphs show the concentration of IL-6, IL-10 and TNFα. n=4. *p≤0.05.




**<u>Figure 3.3.</u> Effect of TLR2 ligands on cytokine synthesis in the presence of HDM allergen.**

PBMC were cultured with HDM with and without TLR2 ligands for 5 days. Concentrations of cytokine synthesis were measured in the supernatant by TRF. Graphs show the concentrations of IL-5, IL-13 and IFNγ. a) HDM-allergic, b) Non-allergic. */^p<0.03 **/^^ p<0.003 compared to HDM alone (*HDM-allergic, ^non-allergic).




**<u>Figure 3.4.</u> Effect of TLR2 ligands on IL-10 synthesis in the presence of HDM allergen.**

PBMC cultured with HDM with and without TLR2 ligands for 5 days. Concentration of IL-10 synthesis was measured in the supernatant by TRF. n=4 HDM-allergic. PS= high dose 0.01%.

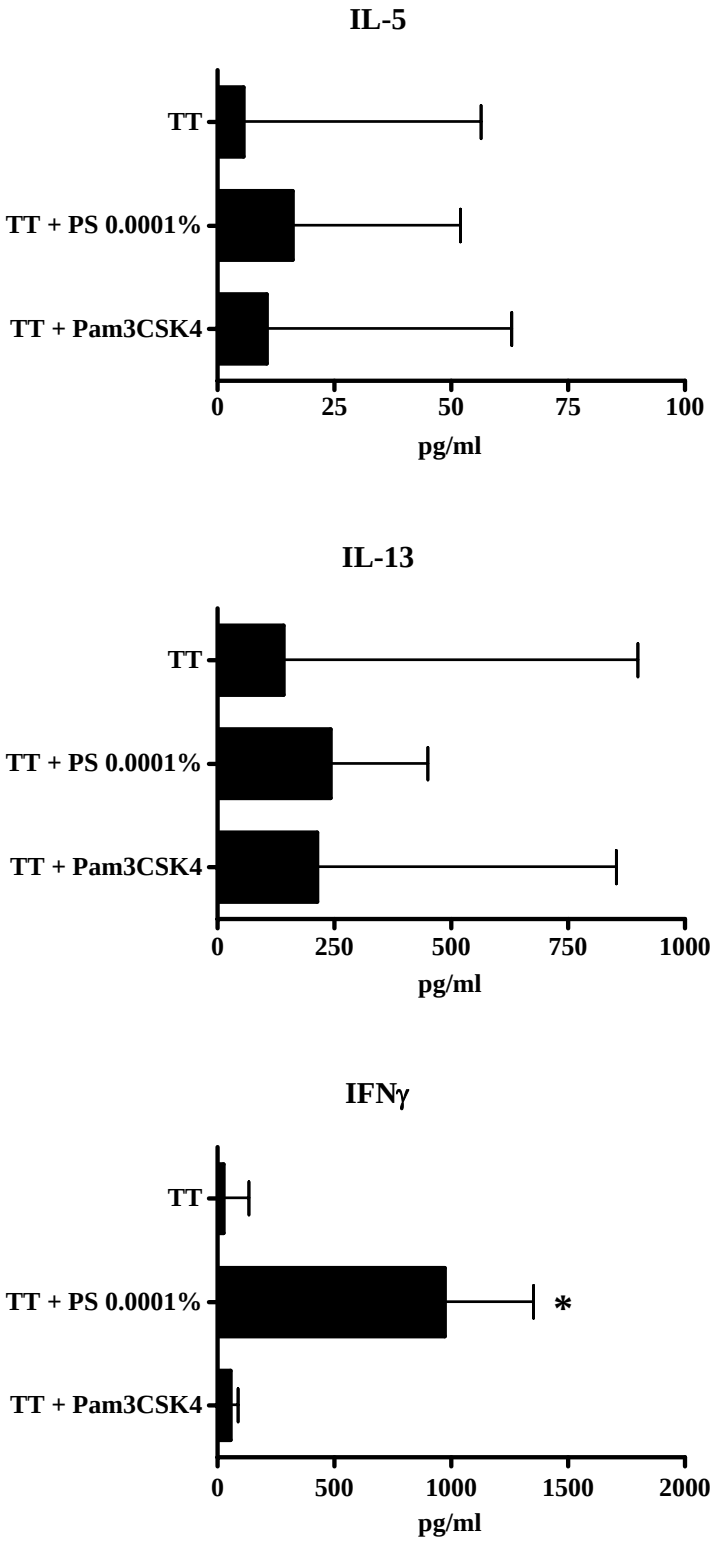



**Figure 3.5. Effect of TLR2 ligands on cytokine synthesis in the presence of tetanus toxoid (TT) antigen.**

PBMC were cultured with TT and with and without TLR2 ligands for 5 days. Concentrations of cytokine synthesis were measured in the supernatant by TRF. Graphs show the concentrations of IL-5, IL-13 and IFNγ. n=16. *$p<0.05$ compared to TT alone.

# 4 CELLULAR MECHANISMS: WHAT IS THE ROLE OF APC'S AND T-CELLS?

## 4.1 Introduction

Having found that the TLR2 ligands Pansorbin (PS), lipoteichoic acid (LTA) and Pam3CSK4 are able to inhibit the Th2 immune response to HDM in allergic subject, it was necessary to identify which cells might be involved in this process. To start with I needed to identify which cells express TLR2 on their surface and whether TLR2 expression differed between HDM-allergic and non-allergic subjects.

Previous studies looking at mRNA expression in humans have shown TLR2 to be predominantly expressed in monocytes and myeloid, but not plasmacytoid, DC[164] while low levels of TLR2 mRNA were expressed in T-cells[292] and B-cells[165]. Studies looking at the surface expression of TLR2 have shown that TLR2 is present on the surface of monocytes, myeloid DC and activated T-cells[167, 168, 292], though there is disagreement as to whether TLR2 is present on naïve T-cells[166]. In mice TLR2 has also been found to be expressed on Tregs[168-170] though this has not yet been determined in humans[171]. It thus appears that APC and T-cells, both of which play a key role in the immune response to allergens, might express TLR2 and might therefore be mediating the responses to TLR2 ligands described in the previous chapter. In this chapter I used flow cytometry to look at the surface expression of TLR2 on cellular subsets of PBMC, in order to identify and confirm the expression pattern of TLR2.

Next I examined the effect of the TLR2 ligands on the APC and T-cells. Possible mechanisms by which the TLR2 ligands could influence the activity of the APC include inhibition of antigen uptake, processing or presentation to the T-cells and changes in APC maturation.

As the processing of antigen occurs, DC mature as evidenced by the change in expression of co-stimulatory markers such as CD80/86, upregulation of MHC class II and their endocytic activity is reduced. There is very little information available on how these processes are affected by TLR2 stimulation though Hertz *et al* have shown that microbial lipopeptides stimulate DC maturation via TLR2, and that this is associated with decreased endocytic activity of these cells[293].

I hypothesised that the TLR2 ligands alter the ability of APC's to take up or process antigen, and that this would lead to a reduction in the amount of antigen processed and presented to the T-cells. This might partly explain the reduction of allergen specific T-cell responses, presented in the previous chapter. Another possibility is that the TLR2 ligands are inducing APC maturation such that antigen presentation by activated APC alters the T-cell response to allergen. As a control for APC activated via TLR2 I also examined APC activation induced by CD40L, to determine if non-specific APC activation would also lead to the decrease in TH2 response to HDM.

Upon exposure to allergen the immune system is activated. The allergen is first taken up by the APC which process and display allergen peptides on its surface ready for presentation to the T-cells. T-cells respond to the peptide presented by the APC. Part of this response is proliferation of the T-cells and secretion of cytokines IL-5 and IL-13. I hypothesised that there is a difference in proliferation when TLR2 ligands are added to HDM stimulated PBMC.

As previously mentioned[170], TLR2 is expressed by T-cells, and therefore it was important to determine whether the TLR2 ligands could be directly signalling the Th2

cells. Alternatively, the TLR2 ligands could be activating Tregs, which in turn might suppress the production of IL-5 and IL-13 by effector T-cells.

Tregs are specialised T-cells which are thought to regulate the Th response to allergens. It has been shown that CD4+CD25+ (Tregs), expressing Foxp3, may prevent the inappropriate immune response to allergens (as seen in allergic disease) in healthy non-atopic individuals. Foxp3 is a Treg transcription factor and is the regulator of the development and function of Tregs[294-296]. It has been proposed that the number and function of Tregs or IL-10 producing T-cells may be impaired in allergic or atopic individuals[172, 297, 298]. I wanted to measure Foxp3 expression in my system to determine effect TLR2 ligands have any effect on the expansion of Tregs.

In this chapter I look at TLR2 surface expression on subsets of PBMC to determine the candidate cells involved in inhibiting the Th2 response to HDM. I looked at the effect of TLR2 ligands on the ability of APC to take up and process antigens, and I measured HLA-DR expression as a marker of APC maturation induced by the TLR2 ligands. I used lymphoproliferation to check that the TLR2 ligands are not inhibiting the ability of T-cells to proliferate. I also determined whether the inhibition of Th2 response is mediated by TLR2 stimulation. Finally the role of T-cells is examined by investigating direct stimulation of T-cells by the TLR2 ligands. Preliminary experiments were performed, focussing on the level of Foxp3 expression and how this was altered by the TLR2 ligands.

## 4.2 Results

### 4.2.1 TLR2 expression on PBMC: which cells are targeted by the TLR2 ligands?

In order to examine the cellular mechanisms by which TLR2 ligands exert their effects, the pattern of TLR2 expression within resting unstimulated PBMC was examined by flow cytometry. PBMC were double-stained with TLR2 mAb in combination with cell-specific mAbs for B-cells, T-cells, NK-cells, monocytes and DC subsets. TLR2 expression was prominent on CD14+ monocytes (88%) but few CD19+ B-cells (7%), and CD3+ T-cells (5%) were TLR2+ (Figure 4.1a). Overall 17% of PBMC expressed TLR2 receptor on their surface. HDM allergic and non-allergic subjects had similar expression of TLR2 regardless of whether the data was analysed according to the proportion of TLR2 positive cells (Figure 4.1a) or the mean fluorescence intensity (data not shown). TLR2 was also detected on myeloid DC (Lin-/HLA-DR+/CD11c+), but not on plasmacytoid DC (Lin-/HLA-DR+/CD123+) (Figure 4.1b and c) or on NK-cells (CD56) (data not shown).

### 4.2.2 Effects of TLR2 ligands on antigen presenting cell function

Having confirmed that TLR2 was predominantly expressed on monocytes and myeloid DC, I next examined the effects of TLR2 ligands on APC function, including antigen uptake, antigen processing and HLA-DR expression.

As a measure of antigen uptake I used the model antigen dextran fluorescein, which fluoresces brightly and can be detected by flow cytometry. This method provides a measure of the amount of dextran endocytosed by the APC, in order to determine

whether the TLR2 ligands might be inhibiting antigen uptake. To determine what effect the TLR2 ligands have on the ability of cells to process antigen, I used DQ-OVA. This molecule only exhibits fluorescence upon proteolytic degradation within the APC, enabling me to determine if the processing by APC is inhibited by the presence of TLR2 ligands.

I used monocytes cultured for 48hrs in GM-CSF and IL-4 (Mono48) for the antigen uptake and processing experiments. The complete transition of monocytes into DC usually requires culture in GM-CSF and IL-4 for 5-7 days. Mono48 cells are non-adherent and highly endocytic, and therefore ideally suited to studies of antigen uptake and processing, even though they have not fully differentiated into DC, and still bear some features of monocytes, such as low-level expression of CD14.

Using unstimulated Mono48 cells I saw a progressive rise in uptake over time of the model antigen dextran fluorescein by the cells at 37˚C (Figure 4.2a). This is active endocytosis rather than passive diffusion of dextran because there is virtually no uptake of dextran by cells kept at 4˚C. The ability of Mono48 cells to take up the dextran was not inhibited by the TLR2 ligand PS, but rather there was a trend that the uptake of dextran was actually increased at all time points (Figure 4.2a). Similar results were obtained with the synthetic lipopeptide Pam3CSK4 (Data not shown). This result shows that there is no inhibition of antigen uptake by cells stimulated with TLR2 ligands.

Mono48 cells exposed to DQ-OVA at 37˚C in the absence of stimulation showed a time dependent increase in fluorescence, corresponding to intra-cellular processing of this model antigen (Figure 4.2b). This increase was not seen with the unstimulated cells at 4˚C. Exposure of cells to the TLR2 ligand PS did not significantly alter the appearance

of the fluorescent signal (Figure 4.2b). Antigen processing is therefore not inhibited by stimulation with TLR2 ligands.

Activation of PBMC with all three TLR2 ligands induced upregulation of HLA-DR expression at 24hrs (Figure 4.3), indicative of APC activation. This upregulation was significantly higher in TLR2 stimulated cultures than the unstimulated media control ($p \leq 0.05$).

During APC/T-cell interactions, CD40L expression on activated T-cells feeds back on APC to enhance their interaction. This process can be modelled using recombinant CD40L. When PBMC were cultured with HDM, the addition of CD40L did not alter IL-5 or IL-13 responses at day 5 (Figure 4.4). This suggests that non-specific activation of APC is not sufficient to alter the Th2 responses to an allergen and that the inhibitory effects of TLR2 ligands described in Chapter 3 are operating by distinct mechanisms.

### 4.2.3 Effect of TLR2 ligands on T-cell function

The various TLR2 ligands had no consistent effect on HDM-induced lymphoproliferation (Figure 4.5). While PS 0.0001% and Pam3CSK4 combined with HDM increased $^{3}$H-thymidine incorporation and proliferation above that seen with HDM alone, PS 0.01% and LTA did not significantly alter $^{3}$H-thymidine incorporation. There was no difference in lymphoproliferation between HDM-allergic and non-allergic subjects (data not shown). It thus appears unlikely that the ability of all TLR2 ligands to inhibit Th2 cytokine production could be attributed to changes in lymphoproliferation.

By using T-cell activation beads (TBds), T-cells can be directly activated without the need for APC's. This experimental system provides a means to determine whether TLR2 ligands can directly signal T-cells and alter their responses. Purified CD4+ T-cells were positively selected from PBMC's and were subsequently cultured with TBds in the presence or absence of the three TLR2 ligands. T-cell activation was determined by measuring synthesis of IFNγ, IL-5 and IL-13 at day 5 (Figure 4.6). The IL-5 and IL-13 response was not diminished with the addition of the TLR2 ligands PS 0.01% and 0.0001%, LTA or Pam3CSK4 (Figure 4.6), and in some cases the response was actually increased. This suggests that the TLR2 ligands are not acting directly on the T-cells but rather they are acting through the APC's to elicit the down regulation of Th2 responses to HDM.

### 4.2.4 Effects of TLR2 ligands on Foxp3 expression #

In order to have a preliminary assessment of the possible role of Tregs, mRNA was obtained from 48hr cultured cell pellets and tested for expression of Foxp3, a Treg marker, by real-time (RT)-PCR. Results were normalised against a stable housekeeping gene UBE2D2[286]. Figure 4.7 shows the ratio of Foxp3 to UBE2D2. The expression of Foxp3 is upregulated by the stimulation of PBMC's with HDM ($p<0.001$). This induction of Foxp3 expression is significantly increased ($p<0.05$) with the addition of all three TLR2 ligands (Figure 4.7). While enhancement of Foxp3 expression tended to be associated with inhibition of IL-5 and IL-13 production, this was not statistically significant when assessed using Spearmans Correlation (Table 4.1).

---

# RT-PCR was kindly performed by Dr Stephanie Yerkovich

| | | Spearmans r / p-value | Significance |
|---|---|---|---|
| **1** | **Foxp3 v IL-5 production** | | |
| | HDM, | 0.4123 / 0.0565 | ns |
| | HDM+PS 0.0001%, | 0.0669 / 0.7731 | ns |
| | HDM+LTA, | 0.3732 / 0.0871 | ns |
| | HDM+Pam3CSK4 | 0.1661 / 0.4600 | ns |
| **2** | **Foxp3 v IL-13 production** | | |
| | HDM, | 0.2739 / 0.2175 | ns |
| | HDM+PS 0.0001%, | 0.3143 / 0.1653 | ns |
| | HDM+LTA, | 0.3086 / 0.1623 | ns |
| | HDM+Pam3CSK4 | 0.0717 / 0.7511 | ns |
| **3** | **ΔFoxp3 v ΔIL-5** | | |
| | HDM+PS 0.0001%, | 0.03897 / 0.8633 | ns |
| | HDM+LTA, | 0.0401 / 0.8593 | ns |
| | HDM+Pam3CSK4 | -0.1339 / 0.5525 | ns |
| **4** | **ΔFoxp3 v ΔIL-13** | | |
| | HDM+PS 0.0001%, | 0.1124 / 0.6186 | ns |
| | HDM+LTA, | 0.0954 / 0.6727 | ns |
| | HDM+Pam3CSK4 | -0.2163 / 0.3336 | ns |
| **5** | **%Foxp3 v %IL-5** | | |
| | HDM+PS 0.0001%, | -0.2299 / 0.3034 | ns |
| | HDM+LTA, | 0.1951 / 0.3842 | ns |
| | HDM+Pam3CSK4 | -0.2576 / 0.2471 | ns |
| **6** | **%Foxp3 v %IL-13** | | |
| | HDM+PS 0.0001%, | -0.2716 / 0.2214 | ns |
| | HDM+LTA, | 0.0392 / 0.8626 | ns |
| | HDM+Pam3CSK4 | 0.0090 / 0.9682 | ns |

**Table 4.1. Correlation of Foxp3 mRNA expression and IL-5 or IL-13 cytokine synthesis.**

PBMC were cultured with HDM with and without TLR2 ligands for 48hrs (mRNA) or 5 days (cytokine synthesis). RNA was extracted from cell pellets and Foxp3 determined by RT-PCR. Concentrations of IL-5 and IL-13 synthesis were measured in culture supernatant by TRF. In sections 3 and 4 of the table, data were expressed as the Δ value, representing the difference between cultures stimulated with HDM and TLR2 ligand versus those stimulated with HDM alone. In sections 5 and 6 of the table, data were expressed as a percentage of that seen with HDM alone.

## 4.3 Discussion

The key findings to emerge from this chapter were as follows: Firstly TLR2 expression was most prominent on monocytes and myeloid DC, implying that the effects of the TLR2 ligands on Th2 cytokine synthesis are likely to be mediated via these APC. This is further supported by the fact that the TLR2 ligands do not alter the cytokine response to artificially stimulated T-cells. Secondly, there were no differences in TLR2 expression found between the HDM-allergic and non-allergic subjects. This latter finding suggests that the differences in the inhibition of Th2 responses seen in the HDM-allergic subjects compared to non-allergic subjects is not mediated by a difference in TLR2 expression.

I hypothesised that the TLR2 ligands were inhibiting the ability of the APC to take up or process antigen. However as seen in Figure 4.2 there was no inhibition, but rather a trend for an *increase* in the amount of dextran endocytosed by the Mono48 cells. In addition the processing of DQ-OVA by these cells was not diminished with the addition of TLR2 ligands, and remained at the same level as the unstimulated control. Formal examination of the uptake and processing of HDM allergens was not undertaken in the current study because of the lack of fluorochrome-conjugated purified allergens, though at this stage it seems unlikely that major changes in allergen uptake or processing explain the ability of TLR2 ligands to inhibit Th2 cytokine synthesis.

Studies using *Mycobacterium tuberculosis* (Mtb) lipoproteins suggest that prolonged incubation of macrophages with Mtb lipoproteins (TLR2 ligands) inhibits MHC class II expression and antigen processing[299-303]. Whether this is specific for Mtb or for all TLR2 ligands is unknown, though the data presented in this chapter suggests that purified TLR2 ligands are increasing the MHC class II expression, and the processing of allergen is the same or slightly increased. While activation of DC by microbial derived TLR ligands is traditionally thought to shut down antigen uptake, there is probably a transient increase in pinocytosis prior to DC migration to the lymph node and full DC maturation. This may explain my findings in Figure 4.2.

TLR2 ligands did induce APC activation, as shown by increased HLA-DR expression. This in itself may not be sufficient to explain the observed inhibition of Th2 cytokine synthesis, especially as APC activation or maturation usually induces a strong Th1 immune response[304]. I found no evidence of this occurring in my system (see Chapter 3). Furthermore, activating APC via CD40/CD40 ligand interactions did not lead to

changes in allergen specific IL-5 or IL-13 synthesis. Further work looking at the effects of TLR2 ligands on other costimulatory molecules such as CD80, CD86 and CD40 would determine if the APC are matured fully or if they remain in a semi-immature state. Recent studies have also suggested that the interaction between ICOS and ICOS-Ligand on T-cells and APC may regulate immune tolerance and the induction of Tregs in animal models of allergy[305, 306]. It will therefore be important for future studies to examine whether ICOS/ICOS-L expression is altered by TLR2 activation.

Sun and co-workers recently showed that TLR engagement on mouse DC could inhibit the development of Th2 cells via an unidentified pathway[307], and further studies are needed to examine the mechanisms by which TLR2 ligands alter human APC function, including the induction of various co-stimulatory molecules and secreted cytokines, or the generation of regulatory T-cells.

The lymphoproliferation data shows that there is no inhibition of cell proliferation following TLR2 stimulation. Further work using CFSE labelling and flow cytometry is required to determine if the same subsets of cells are proliferating in the HDM stimulated versus the HDM and TLR2 ligand stimulated cells.

Direct T-cell signalling by the TLR2 ligands does not seem sufficient by itself for the reduction in Th2 cytokine production seen in whole PBMC cultures. These results indicate that there is a role for the APC's and TLR2 signalling of these cells.

The role of Tregs has been addressed in preliminary experiments, and although the level of Foxp3 increases slightly with stimulation by PS 0.0001%, LTA and Pam3CSK4, the level of Foxp3 did not correlate to the reduction of IL-5 and IL-13 cytokine synthesis.

This could be due to the timing of the Foxp3 data. Only 48hr cell pellets were available for this experiment, which is why this time point was chosen, and further studies need to be carried out to determine the optimal time to measure Foxp3 expression. However these findings are consistent with the notion that TLR2 signalling modulates the function of CD4+CD25+ regulatory T-cells [169, 174] in that Foxp3 expression is upregulated with the addition of TLR2 ligands. Whether this is sufficient to explain the observations presented in chapter 3 is not clear at this stage. Further studies are needed looking at the numbers of Tregs using flow cytometry, and the activity of Tregs in suppression assays where Tregs are co-cultured with effector T-cells, and then stimulated with HDM in the presence or absence of TLR2 ligands.

Having found no inhibition of antigen uptake or processing I could not attribute these mechanisms as the cause of the inhibition of Th2 cytokine production to HDM by the TLR2 ligands, as described in the previous chapter. Nor could I attribute direct T-cell activation by the TLR2 ligands to alter/inhibit the T-cell response to HDM. Therefore at this stage the mechanism by which the TLR2 ligands reduce allergen-specific cytokine secretion remain unclear. In the next chapter I address possible ways in which TLR2 stimulated inhibition of Th2 response to HDM could be enhanced in order to induce a response which is able to confer greater inhibition of the allergic response to HDM.

## 4.4 **Figures**

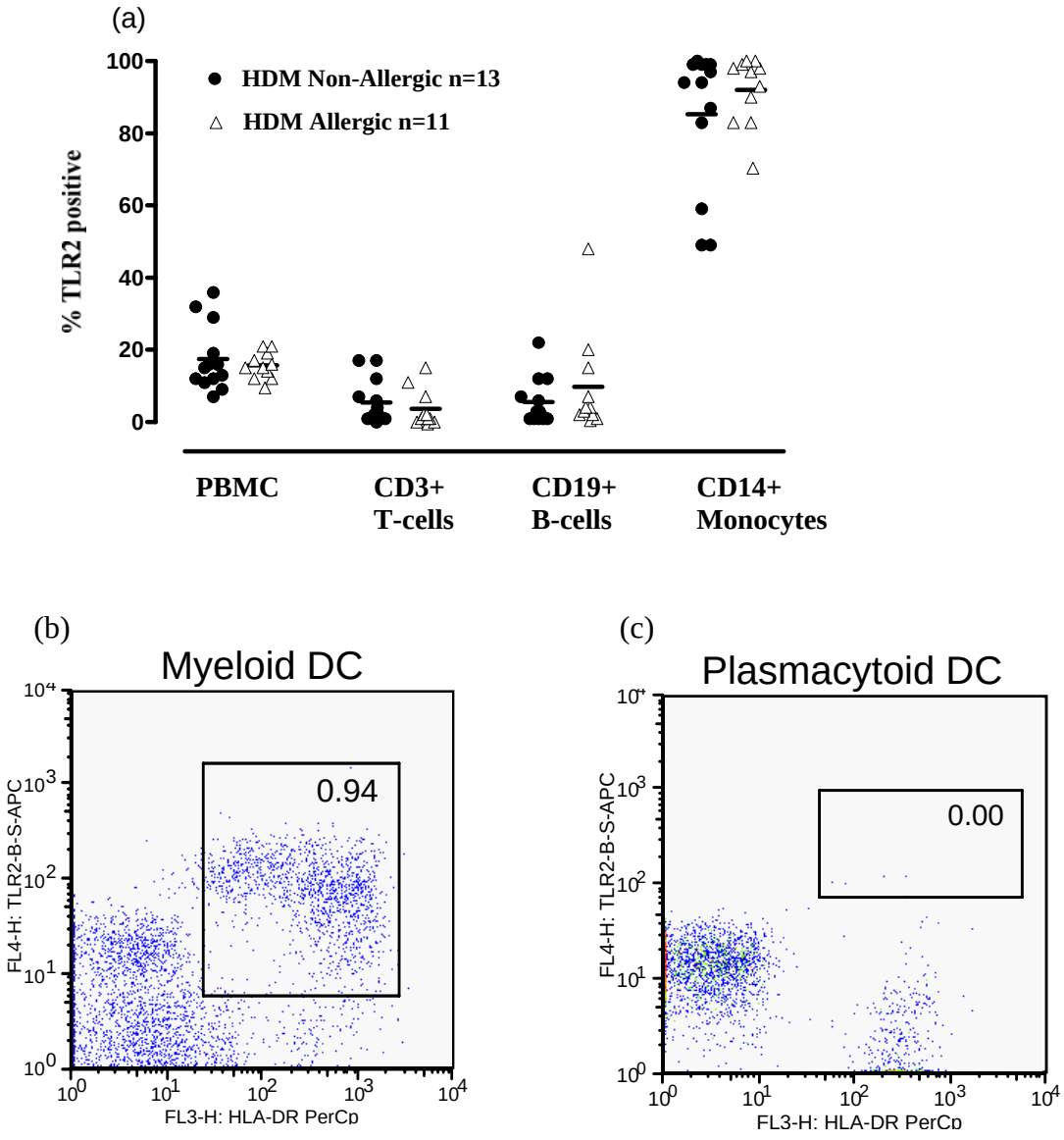



**Figure 4.1. Constitutive TLR2 surface expression on subsets of PBMC**

(a) TLR2 expression on whole PBMC, and further subdivided into CD3+ T-cells, CD19+ B-cells and CD14+ monocytes. Blood DC were defined as HLA-DR+ cells lacking lineage specific markers, and further subdivided into myeloid DC (b) and plasmacytoid DC (c), based on their expression of CD11c and CD123 respectively. Gate frequencies are percentage of PBMC that are DC and are TLR2+. Figure 4.1b and 4.1c are representative of 9 experiments.

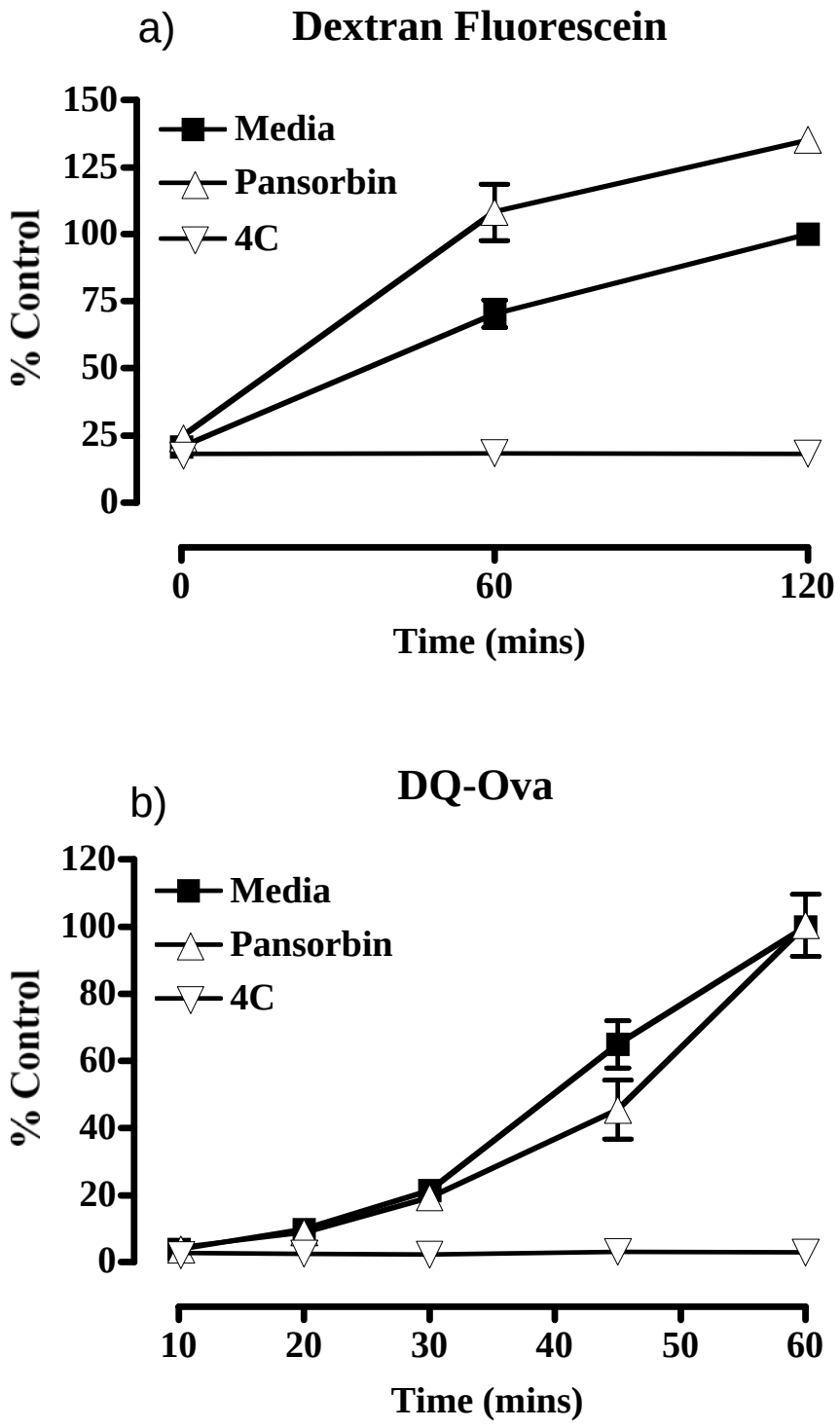



**Figure 4.2. Effects of TLR2 ligands on antigen presenting cell function.**

(a) Antigen uptake by Mono48 cells was determined using dextran fluorescein; n=5. (b) Antigen processing by Mono48 cells was determined using DQ-Ova; n=6. Values are expressed as a percentage of the 37˚C unstimulated control at 120mins (dextran) or 60mins (DQ-OVA).

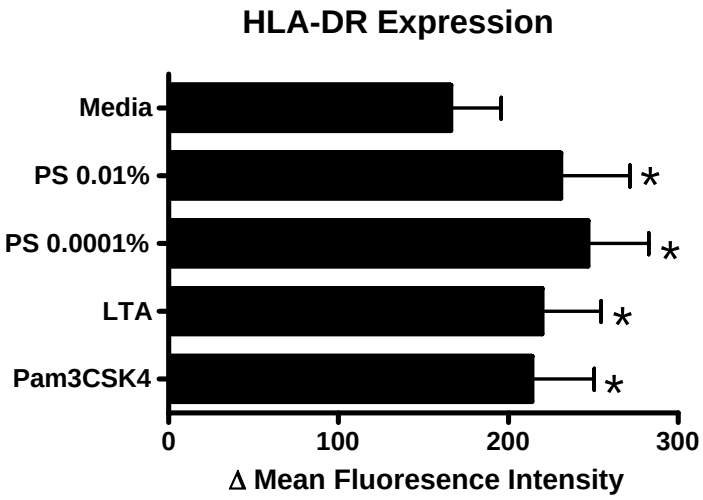



**Figure 4.3. HLA-DR expression on whole PBMC.**

PBMC cultured with TLR2 ligands for 24hrs. HLA-DR expression was measured by flow cytometry. Δ in mean fluorescence intensity is above the level of the baseline control. n=12 (HDM-allergic and non-allergic subjects). *p<0.05 when compared to media control.

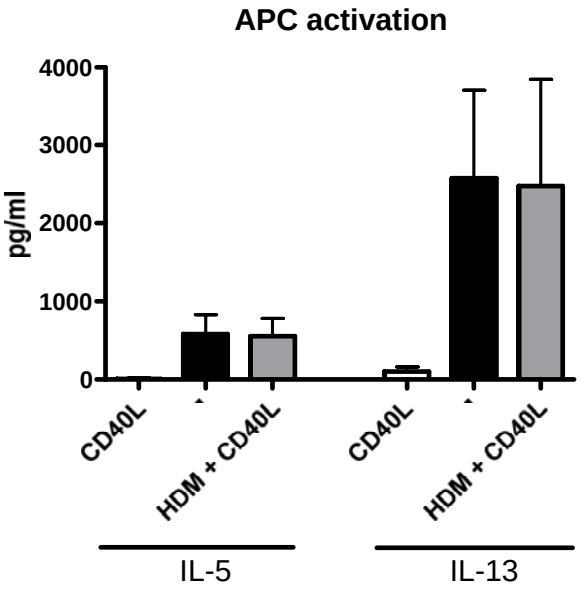
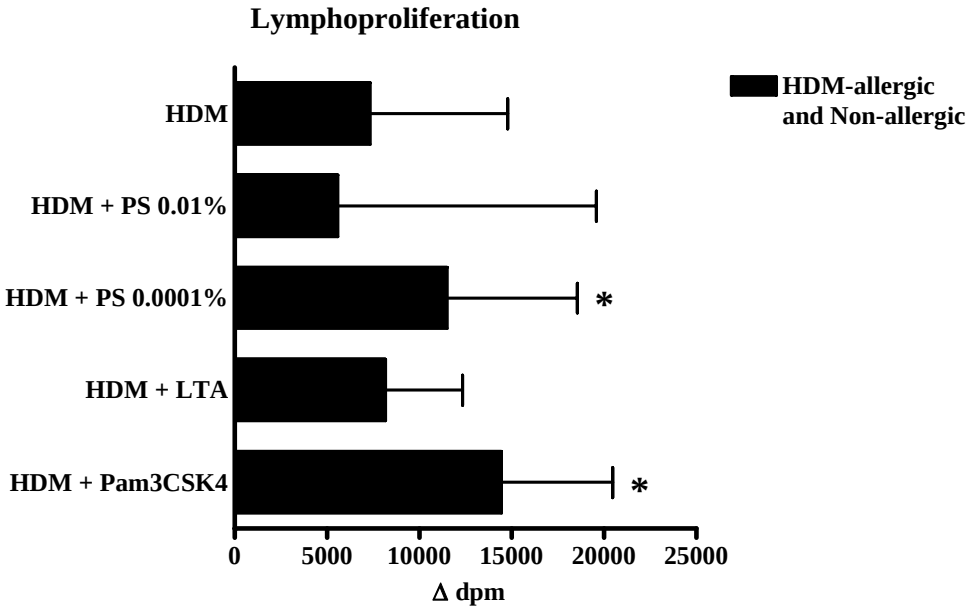


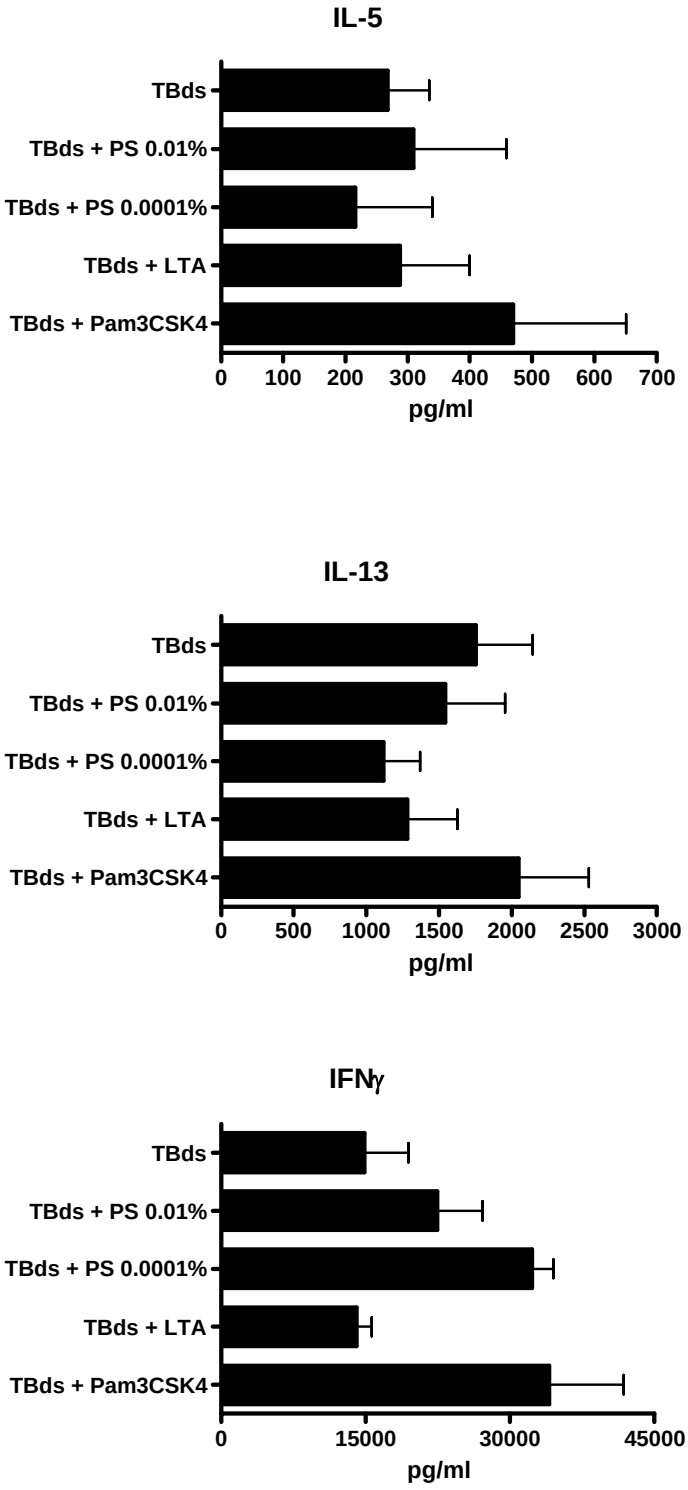

**Figure 4.4. Effect of CD40L on cytokine synthesis by HDM.**

PBMC were cultured with HDM with and without CD40L for 5 days. Concentration of IL-5 and IL-13 cytokine synthesis, were measured in the supernatant by TRF. n=4 HDM-allergic.




**<u>Figure 4.5.</u> Effect of TLR2 ligands and HDM allergen on lymphoproliferation.**

PBMC were cultured with HDM with and without TLR2 ligands for 5 days. $^{3}$H-thymidine was added to the culture for the last 16-18hrs. Proliferation was assessed by measuring the amount of $^{3}$H-thymidine incorporation. Data are expressed as the change in disintegrations per minute above media control (Δ dpm). n=8. * = $p \leq 0.05$ compared to HDM alone.




**<u>Figure 4.6.</u> Effect of TLR2 ligands on cytokine synthesis in the presence of T-cell activation beads (TBds).**

CD4+ T-cells were isolated from PBMC's and cultured with TBds with and without TLR2 ligands for 5 days. Concentrations of cytokine synthesis were measured in the supernatant by TRF. Graphs show the concentrations of IL-5, IL-13 and IFNγ. n=4.

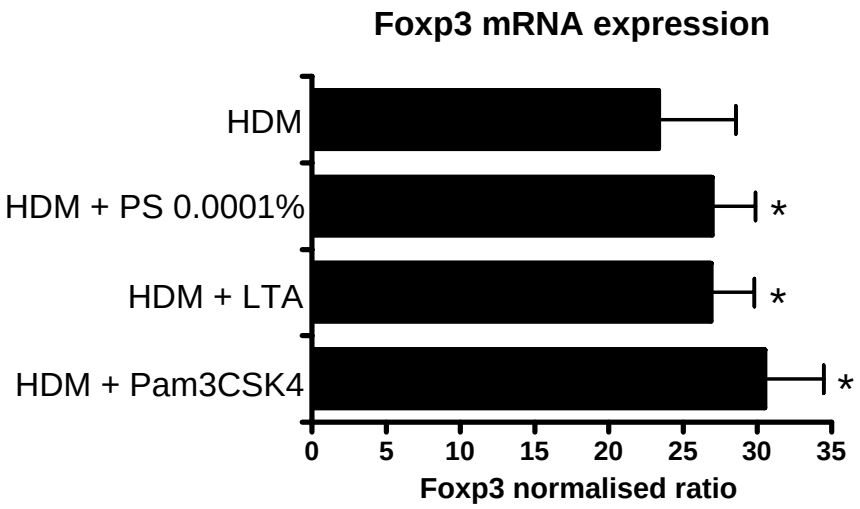



**Figure 4.7. Foxp3 mRNA expression.**

PBMC cultured with HDM with and without TLR2 ligands for 48hrs. RNA was isolated, reverse transcribed and Foxp3 measured by real-time PCR. The level of Foxp3 was normalised to UBE2D2 and presented as arbitrary units n=22 HDM-allergic. *p≤0.05 compared to HDM alone.

# 5 DO DIFFERENT TLR2 LIGANDS SYNERGISE WITH EACH OTHER, OR WITH GLUCOCORTICOIDS?

## 5.1 Introduction

As discussed in the previous chapters, the Th2 response to HDM in HDM-allergic individuals is inhibited with the addition of TLR2 ligands, and this is not mediated by inhibition of antigen uptake or processing or by non-specific activation of APC. It is also not mediated by direct inhibition of the ability of Th2 cells to respond to stimulation. In order to look at ways of more completely inhibiting Th2 response using TLR2 ligands, I studied the potential synergism between different TLR2 ligands, as well as with a synthetic glucocorticoid known to inhibit Th2 responses, which could be important in a clinical setting..

The preparation of Pansorbin (PS) used in these studies is crude, being made up of a whole heat killed *Staph. aureus*, which has the potential to signal through other receptors including other TLR's and other pathogen recognition receptors. The other two ligands used in this study however, are purer and as such will only signal through their specific receptors. LTA signals through TLR2 in conjunction with CD14[115, 308, 309], whereas Pam3CSK4 signals through TLR1 in conjunction with TLR2[310]. Because LTA and Pam3CSK4 have slightly different signalling receptors, I wanted to determine if combining the two ligands could enhance the reduction in Th2 response shown in chapter 3.

Glucocorticoids (GC) are believed to be immunoregulatory[311], and are physiological inhibitors of inflammatory responses and are widely used as anti-inflammatory and immunosuppressive agents in the treatment of many autoimmune and allergic diseases[312]. Dexamethasone is a synthetic glucocorticoid widely administered in human inflammatory pathologies[311], and is known to exhibit immunomodulatory effects on

cytokines *in vitro*[312-316]. It has been shown that dexamethasone inhibits the expression of both Th1 (IL-2, IL-12, IFNγ and TNFα) and Th2 cytokines (IL-4, IL-5, IL-6, IL-10 and IL-13), at the level of message transcription and secreted protein[312, 317-319]. Human studies have shown that dexamethasone induced inhibition of cytokine production may be through the inhibition of human leukocyte activation, as well as a decrease in the frequencies of cytokine producing cells[317].

In addition to being able to inhibit Th2 cytokine production, dexamethasone has been shown to increase the expression of TLR2 in epithelial cells[320, 321] and in cell lines[322], and more recently on DC[323]. I wanted to determine if dexamethasone was able to increase TLR2 expression on PBMC's and if so, what affect this would have on the ability of TLR2 ligands to inhibit Th2 responses to HDM. If dexamethasone were able to increase TLR2 expression on APC or T-cells, then the combination of dexamethasone, with its cytokine suppressive ability, and TLR2 ligands might enhance the inhibition of Th2 responses seen with either agent used alone.

## 5.2 Results

### 5.2.1 Lack of synergistic effects with the combination of TLR2 ligands

I wanted to determine if there was an additive or synergistic effect between LTA and Pam3CSK4. PBMC were cultured with HDM, in conjunction with LTA, Pam3CSK4 or a combination of both and the production of IL-5 and IL-13 and IFNγ was determined. Despite the fact that LTA and Pam3CSK4 utilise slightly different receptors, there were no additive or synergistic effects seen when LTA and Pam3CSK4 were added into culture together, compared to LTA alone (Figure 5.1).

### 5.2.2 Synergistic effects between TLR2 ligands and Glucocorticoids (GC)

Dexamethasone is a GC, which has been shown to enhance TLR2 expression in epithelial cells and DC. Firstly, I wanted to see what effect if any it had on TLR2 expression on PBMC. I found a dose dependent increase in surface TLR2 expression on PBMC after 24hr culture with dexamethasone (Figure 5.2), with significant enhancement of TLR2 MFI at $10^{-6}$M and $10^{-5}$M dexamethasone. However, $10^{-10}$M dexamethasone did not significantly alter TLR2 expression.

The ability of dexamethasone to inhibit cytokine production is well established. In order to determine if dexamethasone has additive or synergistic effects when used in combination with TLR2 ligands, it was important to first find a dose which was suboptimal in inhibiting the cytokine response to HDM. To this extent I titrated the dexamethasone in the presence of HDM stimulated PBMC (Figure 5.3). The results show that there was a dose dependent inhibition of cytokine (IL-5, IL-13 and IFNγ) response to HDM, with doses $10^{-6}$-$10^{-8}$M dexamethasone causing significant inhibition, whereas the lower doses $10^{-10}$M and $10^{-11}$M dexamethasone did not significantly inhibit the cytokine response to HDM. The dose chosen for the remainder of the experiments was $10^{-10}$M. This dose did not significantly inhibit the cytokine production by HDM stimulated PBMC and importantly it did not increase the surface expression of TLR2.

Next I looked at the effect of low dose dexamethasone in combination with LTA and Pam3CSK4. As shown in Figure 5.4, LTA inhibited IL-5 and IL-13 production whereas dexamethasone $10^{-10}$M had no effect on these cytokines when used alone. However, the combination of LTA and dexamethasone $10^{-10}$M markedly inhibited IL-5 and IL-13 production, further than that seen with LTA alone but had no effect on IFNγ. In contrast, while Pam3CSK4 also inhibited IL-5 and IL-13 production by HDM

stimulated PBMC, the combination of Pam3CSK4 and dexamethasone $10^{-10}$M did not lead to any greater inhibition of cytokine production (Figure 5.5).

## 5.3 Discussion

Pansorbin (PS) is a whole heat killed *Staph. aureus* preparation. As such it is reasonable to assume that it might signal through other TLR's, or other pathogen recognition molecules. PS caused the greatest reduction in Th2 cytokine production to HDM. I hypothesised that the purer TLR2 ligands LTA (TLR2) and Pam3CSK4 (TLR1/2) could cause a more significant reduction in Th2 response to HDM if they were added into culture together with HDM. The results did not show an enhancement in Th2 cytokine reduction above that seen with LTA alone. This may be because the TLR2 receptors were saturated at the concentrations of LTA and Pam3CSK4 used in the experiments leading to maximal TLR2 signalling. Another possibility is that there is a limiting factor in the level of TLR1 expression, thus further response by Pam3CSK4 which requires TLR1 dimerization with TLR2 to signal, cannot be induced. It may be possible to see an additive effect if lower concentrations of LTA and Pam3CSK4 were used in future studies.

Glucocorticoids are commonly used as anti-inflammatory and immunosuppressive agents in the treatment of allergic diseases. They have been shown to inhibit cytokine responses and cell activation, and may suppress CD4+ and CD8+ T-cells. As expected I found a dose dependant inhibition of cytokine secretion by HDM stimulated PBMC (Figure 5.3).

Dexamethasone ($10^{-6}$M) has also been shown to regulate TLR2 expression, particularly in airway epithelial cells[320, 324], but also more recently on dendritic cells[323]. I found a dose dependent increase in TLR2 cell surface expression with dexamethasone treated PBMC. However the concentration of dexamethasone chosen for the experiments described in Figures 5.4 and 5.5 was $10^{-10}$M, which did not increase TLR2 surface expression on the PBMC. The effects of dexamethasone induced TLR2 was not examined within the subpopulations of PBMC, so it remains to be seen if others can replicate the effect of dexamethasone inducing TLR2 expression on DC as seen by Rozkova *et al*[322].

Low dose dexamethasone ($10^{-10}$M) when combined with LTA significantly enhanced the inhibition of Th2 cytokine production by HDM stimulated PBMC. I initially proposed that this increase in inhibition might be caused by enhanced TLR2 expression. However the data suggests that this mechanism is unlikely, at least at $10^{-10}$M dexamethasone. Although I did not detect an increase in cell surface expression of TLR2, I did not look at intracellular expression of TLR2, which may be important. It should also be pointed out that the TLR2 surface expression data was obtained after 24hr exposure to dexamethasone, similar to that used in previous studies[320] whereas the cytokines were measured at day 5. It may be possible that dexamethasone could increase TLR2 expression at later times in the culture, thereby increasing the response to LTA, and this should be addressed in future studies. It seems more likely however that the additive effects seen with the combination of LTA and low dose dexamethasone are occurring because they inhibit cytokine production by different intra-cellular mechanisms.

When combined with dexamethasone, LTA showed a further reduction in HDM-stimulated Th2 cytokine synthesis, an effect not observed with Pam3CSK4. The only difference between these ligands is their requirement for other receptors to dimerize with TLR2. Therefore the lack of an effect with Pam3CSK4 may reflect limited or saturated TLR1 usage.

## 5.4 Figures

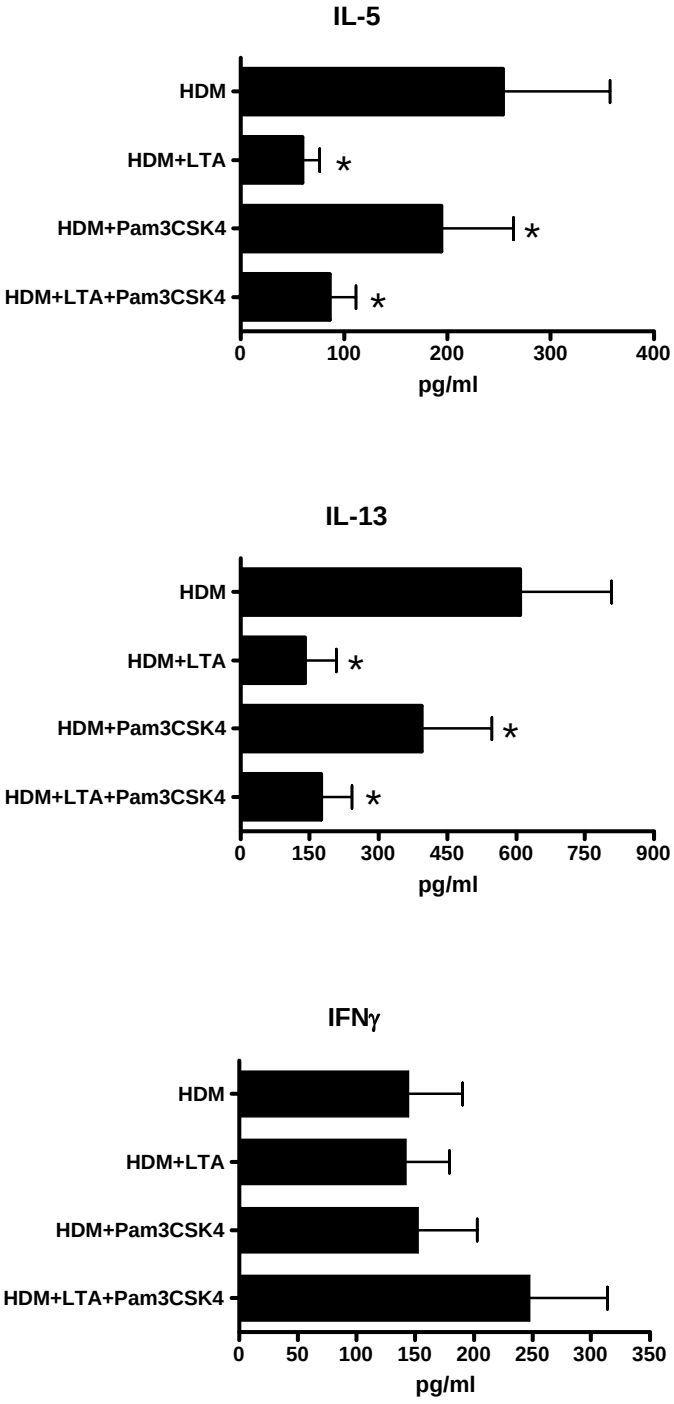



**Figure 5.1. Effects of TLR2 ligands used in combination.**

PBMC's from HDM-allergic subjects were cultured with HDM with LTA and Pam3CSK4 for 5 days. Concentrations of cytokine synthesis were measured in the supernatant by TRF. Graphs show concentrations of IL-5, IL-13 and IFNγ. n=8. *p≤0.05 compared to HDM alone.

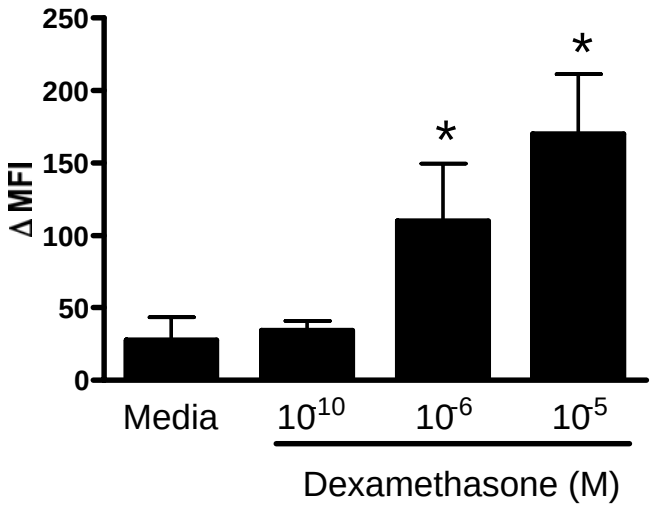
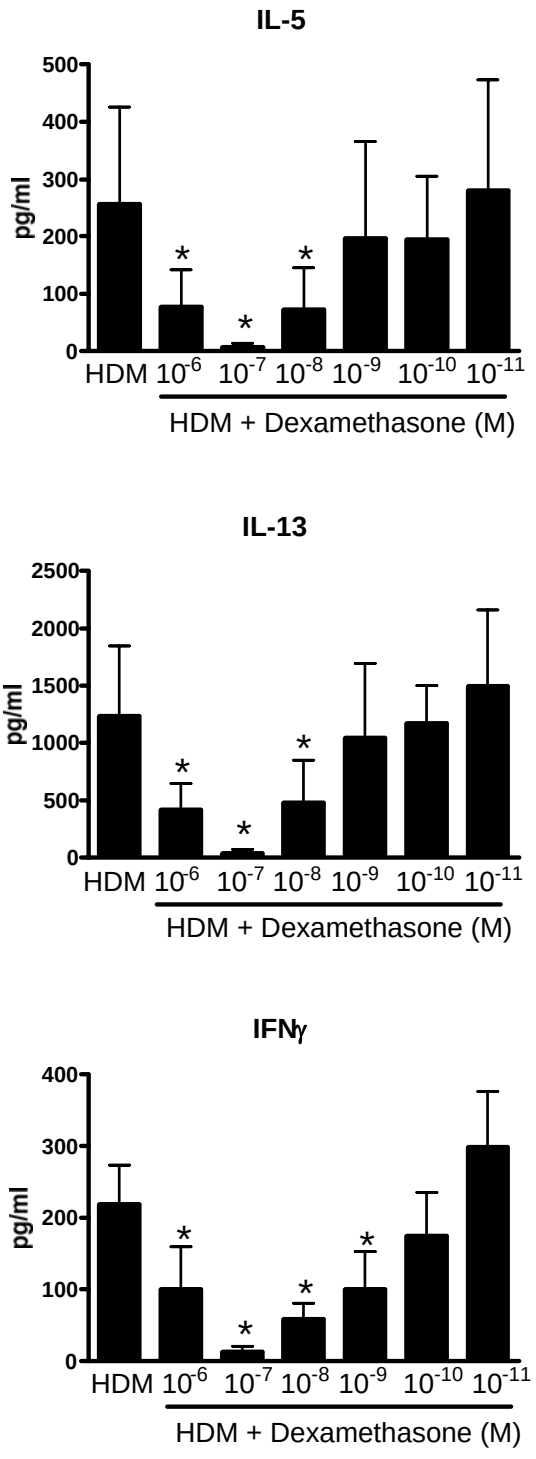


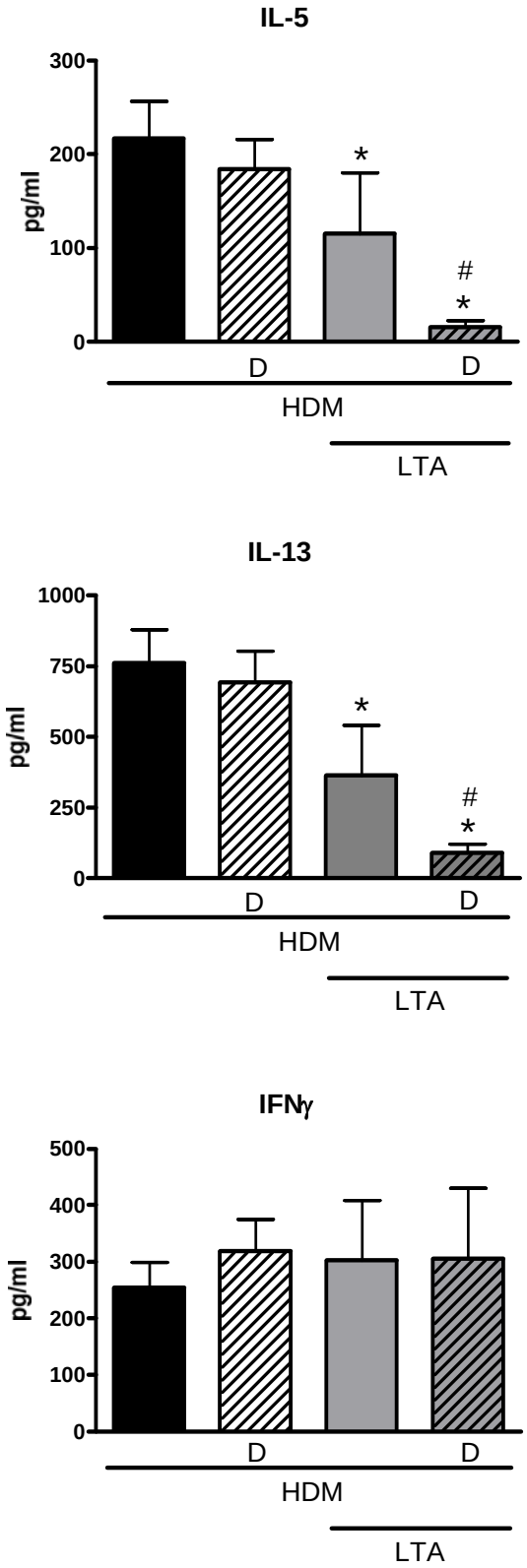

**Figure 5.2. Effect of dexamethasone on TLR2 surface expression on PBMC's.**

PBMC's were cultured with various concentrations of dexamethasone for 24hrs. TLR2 expression was measured by flow cytometry. Graph shows the change in mean fluorescence intensity (MFI) above baseline control. n=8. *$p \leq 0.05$ compared to media control.



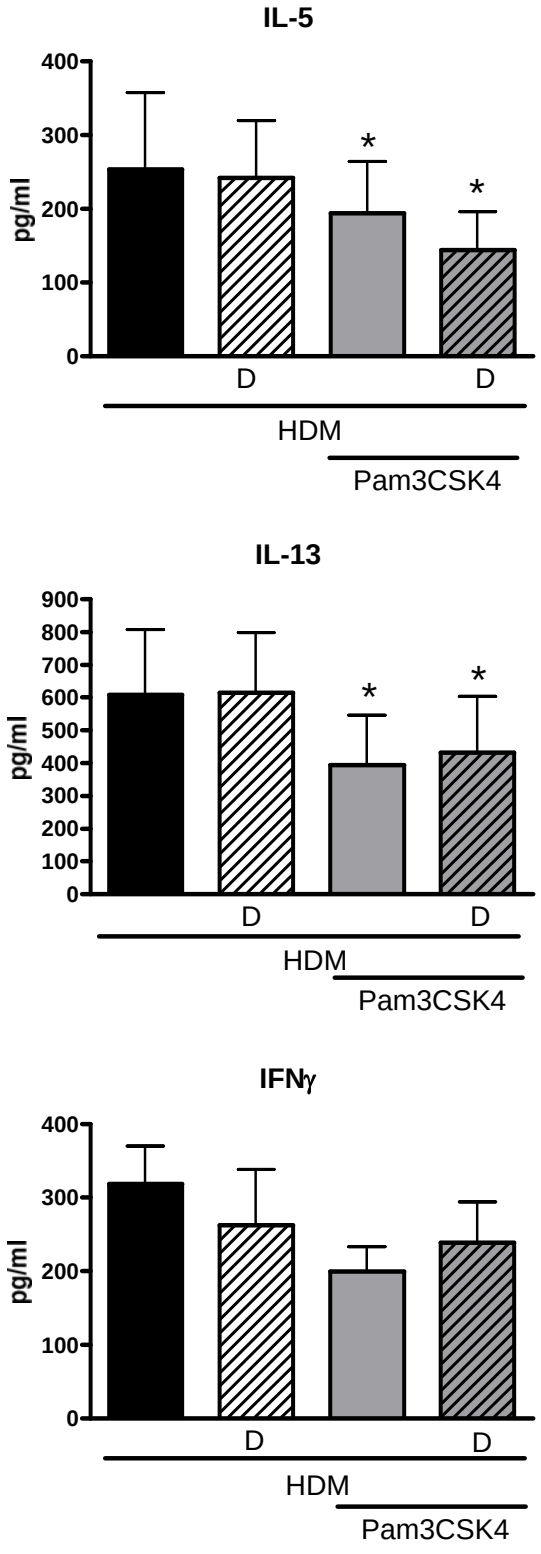

**<u>Figure 5.3.</u> Effect of varying concentrations of dexamethasone on cytokine production by HDM-stimulated PBMC.**

PBMC's were cultured with HDM, with various concentrations of dexamethasone for 5 days. Concentrations of cytokine synthesis were measured in the supernatant by TRF. Graphs show the cytokine concentration of IL-5, IL-13 and IFNγ. n=4 HDM-allergic. *$p \leq 0.05$ compared to HDM alone.




**<u>Figure 5.4.</u> Effect of dexamethasone (D) in combination with LTA.**

PBMC's were cultured with HDM, with and without LTA and dexamethasone $10^{-10}$M for 5 days. Concentrations of cytokine synthesis were measured in the supernatant by TRF. Graphs show concentration of IL-5, IL-13 and IFNγ. n=8 HDM-allergic. *$p \leq 0.05$ compared to HDM alone, # $p \leq 0.05$ compared to HDM with LTA.




**<u>Figure 5.5.</u> Effect of dexamethasone (D) in combination with Pam3CSK4.**

PBMC's were cultured with HDM, with and without Pam3CSK4 and dexamethasone $10^{-10}$M for 5 days. Concentrations of cytokine synthesis were measured in the supernatant by TRF. Graphs show concentration of IL-5, IL-13 and IFNγ. n=8 HDM-allergic. *p≤0.05 compared to HDM alone.

# 6 DISCUSSION

## 6.1 Introduction

The incidence and severity of allergic diseases are increasing and they continue to be a major health and financial burden in the developed nations of the world. With rising costs, it is important to find the underlying mechanism(s) of allergic disease in order to find potential "cures" for these diseases, or at least improved and more cost effective treatments for those already sensitised. This might lead to lower health related costs as well as a better quality of life for allergy sufferers.

The 'hygiene hypothesis' as first proposed by Strachan in the 1980's[25], suggests that exposure to microbial compounds early in life may prevent the onset of atopy and allergic disease. Since then many studies have tried to find the underlying mechanism(s) by which exposure to microbes (studies have primarily focused on endotoxin, TLR4) exert their protective effect. Finding these underlying mechanisms might provide more specific targets for immunotherapy of established disease or facilitate the development of novel preventive strategies. Despite intensive research, no effective prevention measure exists to date. Recent animal experiments, however have shown clearly that it is possible to vaccinate animals with allergens in conjunction with attenuated bacteria, or microbial components such as CpG-DNA, to protect them from developing allergic Th2 responses. This shows promise that with more research it may be possible to develop vaccines for humans which prevent the development of allergic responses.

## 6.2 This Thesis

The aims of this thesis were to examine the effects of TLR2 ligands on T-cell responses to HDM allergen, and to try to determine the mechanisms by which TLR2 ligands exert their effects.

### 6.2.1 Effect of TLR2 ligands on the immune responses to HDM

The key finding to emerge from this thesis is that TLR2 ligands are able to inhibit the memory/recall response to HDM in previously sensitised individuals, and that the reduction in Th2 cytokine production is not mediated by a shift towards an allergen specific Th1 response pattern.

All three TLR2 ligands studied; heat killed *Staphylococcus aureus* (Pansorbin, PS), lipoteichoic acid (LTA) and the synthetic bacterial lipopeptide Pam3CSK4 were able to inhibit Th2 cytokine production. The fact that both the heat killed *Staph. aureus* and the purified TLR2 ligands inhibit allergen-specific Th2 cytokine production is consistent with the notion that the findings in Figure 3.3 are mediated via a TLR2 dependent mechanism. It does not appear that this involves deviation to an allergen specific Th1 response pattern, as induction of IFNγ production was not consistently observed with the various TLR2 ligands, and IL-12 could not be detected under any condition studied. This inhibition of Th2 responses appears to be allergen specific, as I could not replicate the same inhibition of Th2 responses when using tetanus toxoid (TT) as the antigen.

Initial studies by others suggested that TLR2 activation promoted and exacerbated allergic disease[252, 266]. However the inhibition of Th2 responses to HDM found in my study, are in agreement with more recent reports showing that TLR2 ligands are able to inhibit the Th2 responses in mouse models of asthma and allergic disease[273, 291].

#### 6.2.1.1 DIFFERENCES BETWEEN TLR2 LIGANDS

Although all three ligands studied primarily signal through TLR2, they are all inducing different cytokine patterns by PBMC, both alone and in the presence of HDM. All three inhibit the Th2 response to HDM but by varying degrees. Possible reasons for this are the exact signalling pathway each uses:

(a) PS signals through TLR2, but being a crude preparation is likely to signal through other receptors including other TLR's,

(b) LTA signals through TLR2 with dependence on CD14

(c) Pam3CSK4 signals through a dimer of TLR1 and TLR2.

Signalling via these other receptors may cause greater or milder reduction in Th2 response. I have only addressed the addition of these three known TLR2 ligands, and there are many more TLR2 ligands which need to be studied in this system, to determine if the same (or greater) effect is seen. Despite the best reduction in Th2 cytokines being found using a whole bacteria preparation (PS), this can potentially cause deleterious pro-inflammatory effects such as high IFNγ production. There was still partial reduction using the purer preparations LTA and Pam3CSK4, however this reduction may not be enough to confer total inhibition of allergic response, both *in vivo* and *in vitro*.

Varying the ligand used could potentially change the down stream signal within the cells. Burwitt-Beckmann *et al* have shown that changes in the nature of the ligand (in particular changes in the acyl chain) effects TLR2 dependent cellular recognition of lipopeptides[161]. The nature of the ligands might therefore contribute to the level of activation that the cells undergo. Further work to determine the exact signalling cascade each ligand induces, involving studies into the expression of downstream molecules, such as MyD88 or NF-κB, would provide information on the level of activation of these

cells by the different TLR2 ligands. Utilising this information could provide a basis for design of TLR2 specific adjuvants mediating immune activation in order to further optimise the inhibition of allergic inflammation.

Another way of finding an effective adjuvant is to look at possible synergistic effects between ligands, or using ligands for multiple TLR's. Having shown a more effective inhibition of Th2 response using the TLR2 ligand PS, and the nature of that ligand potentially involving multiple receptors in its mediation of Th2 responses, I hypothesised that by combining the two purer adjuvants, LTA and Pam3CSK4, would result in a substantial inhibition of Th2 response compared to the addition of either of these two ligands alone. However, I found no additive or synergistic effect when the two ligands were added into the culture together with HDM compared to the addition of LTA to HDM on its own (Figure 5.1). Despite this finding it is still possible that other combinations of TLR2 or other TLR ligands may result in a more effective treatment of allergic disease.

#### 6.2.1.2 OTHER TLR'S

This study has focused on TLR2, though there are currently 10 TLR's identified in humans. Further studies looking at ligands specific for one or more TLR are needed to determine if the effects seen in this study are TLR2 specific, or if activation of other TLR's can also mediate a similar effect. TLR4, TLR9 and TLR2 are perhaps the best characterised of the TLR's to date, so it is not surprising that studies of interactions between TLR signalling and allergy have mainly focused on these TLR's.

The studies looking at endotoxin (LPS; TLR4) have similar findings as those studies looking into TLR2. Like TLR2 it has been found that endotoxin can have divergent effects on the allergic phenotype, by inhibiting the development of allergen-specific IgE and asthma in some situations[202 227], as well as leading to an exaggeration of allergic inflammation in other circumstances. Studies have shown that LPS can induce either a Th1 or Th2 response depending on the timing of administration of LPS and the dose used[29]. More recently Revets *et al* found that a lipoprotein I (OprI) stimulating the TLR2/4 signalling pathway, can modulate allergen-specific Th2 effector cells in an allergic response *in vivo* for a prolonged period[267]. TLR9 and its recognition of bacterial DNA, is well documented, and is another avenue of potential use as an adjuvant in allergy treatment/prevention (discussed further below).

Most of the work looking into TLR activation and the effect it has on allergic phenotypes has utilised murine models of asthma. This present study looks at the effect of TLR2 ligands on PBMC's from adult subjects with established HDM sensitivity. The individuals studied had only mild clinical disease, and it remains to be determined whether TLR2 ligands will have similar beneficial effects on allergen specific responses from individuals with severe asthma or eczema for example.

Whether or not TLR2 ligands might prevent allergic sensitisation in young children is also an important issue, but was outside the scope of the current study. Replicating this study with children, young infants or even cord blood would help to expand the knowledge of the effect of TLR2 ligands on the developing immune system. This study does however provide more insight into the potential of TLR ligands to alter allergic disease. Some of the mechanisms by which this may occur are addressed and discussed below.

Study design, timing of ligand addition, ligand choice and dose, have all shown to influence the Th1 and/or Th2 allergen-specific response, or the balance between the two in some way. Thus the effectiveness of any treatment using TLR ligands will probably involve a small window of opportunity. The timing, dose and particular ligand used will change depending on the presence of allergic disease, age of recipient, previous exposure and genetic makeup.

### 6.2.2 Possible mechanisms by which TLR2 ligands are exerting their effect on Th2 responses to HDM

Having shown that TLR2 ligands are able to influence the immune response to allergen, I next explored some of the mechanisms by which the TLR2 ligands might be exerting their effects. To narrow down the search for the cell types involved in this process, I performed TLR2 cell-surface staining on subsets of PBMC's, allowing me to focus my attention on the cell types which are the primary sensors for TLR2 ligands.

#### 6.2.2.1 TLR2 CELL SURFACE EXPRESSION

In agreement with other studies[164, 166, 167], I found TLR2 to be predominantly expressed on the surface of monocytes and myeloid DC, as well as low levels on T-cells. TLR2 expression did not differ between allergic and non-allergic groups. Having shown this expression pattern of TLR2, I hypothesised that the ligands were modifying the activity of the APC, or T-cells in some way. Possible mechanisms include inhibition in antigen uptake or processing, maturation of APC or altering the DC-T-cell interaction. To address some of these mechanisms I undertook experiments to allow me to determine

what effect if any TLR2 ligands had on APC maturation, antigen uptake and processing, as well as the examining the direct effects of the TLR2 ligands on T-cell function.

#### 6.2.2.2 APC MATURATION

The addition of all three TLR2 ligands to PBMC induced the maturation of the APC as seen by the increased surface expression of HLA-DR at 24hrs. To determine whether there is an immediate and sustained maturation of the APC by the TLR2 ligands, or whether this effect is transient, a more extensive time course of HLA-DR expression needs to be addressed in future studies. Further to this, other APC maturation markers such as CD80, CD86 and CD40 need to be examined in this system, to determine in more detail the extent to which the ligands are inducing APC maturation. It would also be interesting to investigate whether TLR ligands induce inhibitory molecules on the surface of APC such as programmed death (PD) ligands. When the APC were activated using CD40L this did not lead to the inhibition of the Th2 cytokines IL-5 and IL-13 (Figure 4.4), suggesting that APC activation on its own is not sufficient to inhibit Th2 responses and that the inhibition of Th2 responses are mediated by a TLR2 dependent mechanism. (See Figure 6.1)

#### 6.2.2.3 ALLERGEN UPTAKE/PROCESSING

Experiments looking directly at allergen uptake and processing have shown that neither are inhibited by the presence of TLR2 ligands. However these experiments were based around a short period of TLR2 ligand presence and it remains to be determined if a longer stimulation of Mono48 cells by TLR2 ligands would alter the ability of these cells to take up and process allergen. West *et al* have shown that there is a transient enhancement of antigen uptake followed by longer-term down-regulation of endocytic

activity after TLR stimulation in murine bone marrow-derived and spleen-derived DC's[325]. There is however, no evidence found to date in this system, which would indicate that allergen uptake or processing is inhibited by the TLR2 ligands thus explaining the reduction in Th2 cytokine production. In this study I have not formally looked at HDM uptake due to the lack of a suitable fluorochrome-conjugated purified allergen. (See Figure 6.1)

#### 6.2.2.4 ALLERGEN PRESENTATION

As discussed above, I have shown that pre-treating Mono48 cells with TLR2 ligands, does not inhibit allergen uptake or processing. In this study, I have not formally addressed potential effects of TLR2 ligands on changes in allergen presentation. A study designed to look at the ability of APC to present allergen to the T-cells before and after stimulation with TLR2 ligands, would provide insight into the possible changes in antigen presentation, which may then affect the Th2 response. Similar experiments by Weigt *et al* have shown that pre-treatment of DC's with a TLR2/6 agonist MALP-2 in conjunction with IFNγ followed by co-culture with autologous lymphocytes resulted in a shift in allergen-dependant Th2 reaction toward a Th1-type response[273]. Although the study outlined in this thesis has not demonstrated a swing to Th1 response, this may be a mechanism by which the TLR2 ligands are able to inhibit Th2 responses under some circumstances.

#### 6.2.2.5 TLR2 EFFECT ON T-CELLS

Direct TLR2 signalling of the T-cells does not appear to be responsible for the reduction in Th2 response. When purified CD4+ T-cells, were activated by T-cell activation beads – comprising anti- CD2, CD3 and CD28 antibodies – as well as the TLR2 ligands PS,

LTA and Pam3CSK4, there was no significant reduction of IL-5 or IL-13 cytokine synthesis (Figure 4.6). The effect of the TLR2 ligands is therefore not purely mediated by inhibiting T-cell responses alone. This taken with the data (discussed above) showing that there is no inhibition of antigen uptake or processing suggests that the effect of the TLR2 ligands inhibiting the Th2 response to HDM is mediated by changes in both APC and T-cell responses or interaction with one another. It is possible that induction of inhibitory molecules in APC by TLR2 ligands then has an indirect effect on T-cells to inhibit IL-5 and IL-13 production. (See Figure 6.1)

#### 6.2.2.6 ROLE OF TREGS

A number of subsets of Tregs have been described which regulate the activity and expansion of CD4+ Th cells. This regulation of Th1 and Th2 responses is through contact-dependent mechanisms and/or the production of IL-10 and TGFβ by Treg cells. While several markers have been associated with Treg cells, Foxp3 is the most reliable indicator of Tregs and has been demonstrated as the master control gene of Tregs[296].

Previous studies have shown reduced Treg function in allergic patients[172, 326], which results in increased Th2 cytokine production. Tregs are able to suppress the CD4+ T-cell proliferative response to allergens[327]. One study has shown that Treg suppression of effector Th cells in birch pollen allergic subjects is impaired during the birch allergen season, causing deficient suppression of Th2 cells by the Tregs, however there was no difference in Treg ability between allergic and non-allergic subjects outside the pollen season[326]. Others looking at grass pollen allergy have shown that there is still a difference in Treg suppression of Th cells between allergic and non-allergic subjects even during the off season[172]. Furthermore, it has been shown that TLR2 ligands induce

Treg cells[169, 173]. These observations suggest that Treg action could contribute to the reduced cytokine production observed when HDM stimulated PBMC were cultured with TLR2 ligands.

As a preliminary investigation into a possible role of Tregs in this system, mRNA was isolated from PBMC's cultured with HDM and with or without TLR2 ligands and Foxp3 analysed by RT-PCR. The addition of all three TLR2 ligands to HDM stimulated PBMC lead to increased Foxp3 expression compared to HDM alone. The increase in Foxp3 expression was relatively minor, and due to the short culture period may not be indicative of Treg expansion. Furthermore mRNA expression was normalised in order to estimate the relative expression of Foxp3 on a per-cell basis. It is unknown if this increased level of Foxp3 mRNA is related to increased activity of the Tregs. Previous studies have suggested that an increase in Foxp3 expression seen with the addition of TLR2 ligands was linked to a transient decrease in suppressive activity by these Tregs, which was restored when the TLR2 stimulus was removed[169, 171, 174], suggesting that increased Foxp3 mRNA may not equate to increased suppressive activity.

To determine the role of Tregs in reduced cytokine production by TLR2 ligands in future studies, the number of Tregs should be enumerated following culture using flow cytometry and identified by the markers CD4+CD25+Foxp3+ and $CD127^{lo}$ [32, 33], which collectively appear to be the best markers to define Tregs. This would indicate if TLR2 ligands do induce Treg cells (as reported in previous references[169, 173]). To assess if these Tregs are suppressive, Treg activity could also be assessed by the use of suppressive assays. This would indicate if the TLR2 ligands are inducing Treg activity resulting in the reduced cytokine production observed. It must be noted that the patients used in these studies that showed reduced cytokine production and increased Foxp3

expression were all highly HDM allergic, and this population has previously been shown to have reduced Treg function[172, 326]. Therefore it should also be investigated if the action of TLR2 ligands is to activate Treg function directly. It has been reported that one of the heat shock proteins (HSP60) can act via TLR2 on Tregs resulting in reduced cytokine secretion[328]. However, production of IL-10 by PBMC's stimulated with HDM, did not significantly change with the addition of the TLR2 ligands, compared to HDM alone (Figure 3.4). IL-10 is one mechanism by which Treg cells mediate their suppression, though in many circumstances cell-cell contact is more important than soluble cytokines. As there was no difference in IL-10 levels in my study it suggests that if the Tregs are responsible for the decrease in cytokine production, it is not mediated by IL-10.

Another mechanism by which Treg cells are acting may involve APC and the decrease observed in cytokine production is a result of reduced APC function. Studies have demonstrated that Tregs are able to restrain the maturation and antigen-presenting function of DC's[329], especially in the myeloid DC but not plasmacytoid DC population[330]. I have not observed any inhibition of APC antigen uptake and processing, and in fact HLA-DR is increased following TLR2 stimulation, suggesting that reduced APC function is not responsible for the observed affect. APC have an important role, as the TLR2 ligands do not directly inhibit T-cell responses, however the assessment of APC function was performed on Mono48 cells cultured alone and it is possible that an affect of Tregs cells is still occurring in my model but is not identified when APC are removed from the Tregs. Therefore it is still possible that the TLR2 ligands are stimulating the Tregs, which are then in turn modulating the function of the APC, the results of which are seen in less allergen presented to the T-cells and thus less T-cells are responding to the allergen, hence less IL-5 and IL-13 cytokine is synthesised. Future

studies could look at antigen uptake and processing in whole PBMC cultures rather than isolated APC to identify if Tregs are acting on the APC. (See Figure 6.1)

#### 6.2.2.7 ANTIGEN SPECIFICITY

Having shown that there is a difference in the inhibition of Th2 response from HDM and tetanus toxoid (TT) stimulated PBMC, it would be beneficial to be able to repeat the experiments outlined in this thesis with other allergen peptides to determine if this effect is specific to HDM. If the same response patterns are seen with other allergens (inhibition of Th2 cytokine production), it would widen the scope of potential targets for the future use of TLR2 ligands in allergy treatment or prevention.

#### 6.2.2.8 DIFFERENCES BETWEEN SUBJECT GROUPS

The inhibition of Th2 responses are only significant in the HDM-allergic subjects and not in the control subjects. Possible explanations of this could be the experimental design and the way in which the results are analysed. I have looked at the reduction in IL-5 and IL-13 with the addition of TLR2 ligands compared to HDM alone. As shown in previous reports, the levels of HDM induced IL-5 and IL-13 are much higher in the allergic subjects compared to the controls. I hypothesise that there is in fact, no difference in the way TLR2 ligands are acting on the immune cells in both groups, rather the allergic response is “turned off”, an effect which is more prominent in the allergic subjects.

The difference between the inhibition of Th2 response to HDM compared to no inhibition to tetanus toxoid (TT) could be explained by differences in the memory responses. T-cell responses to HDM are stronger than the TT responses because HDM

exposure occurs naturally every day whereas TT responses are weaker because most of the people in the study had not received a TT vaccine for several years. Therefore the hypothesis is that TLR2 ligands are able to inhibit strong memory responses but have little effect on weaker responses. Secondly, TLR2 ligands may specifically inhibit allergen-specific T-cell responses but have little effect on responses to other antigens. This could be addressed by studying a larger range of allergens (eg pollen, both in and out of season) or by studying people a few weeks after a tetanus vaccine when a much stronger recall would be expected.

In support of this there were no detectable differences in TLR2 expression on circulating leukocytes between allergic and non-allergic subjects. However it is possible that differences in the reduction of Th2 responses seen between the two groups may be caused by differences in TLR2 signalling. If this is so, there might be differences between the two groups in HLA-DR upregulation or the production of innate cytokines (IL-6 and TNFα). However the numbers of subjects analysed for these outcomes within this study were insufficient to detect any significant differences. To address this issue would require a much larger study. There is some data to indicate that polymorphisms in the TLR2 gene may be linked to atopy[183]. In my study there did appear to be variability in the extent to which TLR2 ligands inhibited IL-5 and IL-13 responses, though whether there was a genetic basis to this variability would require a large study including both genetics and cellular immunology.

## 6.3 Future Directions

One area of interest highlighted in the current study is the potential role of Tregs in the ability of TLR2 ligands to inhibit the Th2 response to mite allergens. As outlined in

other studies (discussed above), it appears that Tregs may be deficient in suppressive function in allergic subjects. It remains to be seen in the system, outlined in this thesis, whether Tregs are present in insufficient number or if they are just unable to elicit an effective suppression of Th2 response in allergic subjects. Looking at the number of Tregs in both allergic and non-allergic subjects, as well as their suppressive activity before and after TLR2 ligand stimulation could shed light on whether Tregs are involved in the mechanism(s) by which the TLR2 ligands are inhibiting the Th2 response. The TLR2 ligands may be directing the upregulation of Tregs, these Tregs may then be exerting their effects on the CD4+ T-cells and/or they may be modulating the function of APC. (See Figure 6.1)

#### 6.3.1.1 REGULATORY SIGNALLING MOLECULES: SOCS AND OTHER RELATED MOLECULES

However, there are other inhibitory signalling molecules that might be relevant to the effects of TLR2 ligands described in this thesis. Molecules such as suppressors of cytokine signalling (SOCS) have been implicated in the regulation of Th responses[331, 332]. SOCS1 has been shown to be induced by TLR ligands LPS and CpG DNA, and inhibits signalling of IL-4, IL-6 and IFNγ[332]. SOCS3 is expressed at higher levels in Th2 cells and inhibits the Th1 response[333], and is thought to regulate and maintain Th2 mediated allergic responses[334, 335]. This effect has been highlighted in atopic mice models (asthma and atopic dermatitis) where SOCS3 expression mirrors the severity of disease[334]. SOCS5 is preferentially expressed by Th1 cells, and its expression can result in a reduction of Th2 differentiation as a consequence of inhibiting IL-4 signalling[336]. There is very little information available in regard to TLR2 upregulation of SOCS

family molecules and whether this might explain the inhibition of IL-5 and IL-13 synthesis described in this thesis. Further work is needed to address these issues.

### 6.3.2 TLR's: Therapeutic implications

Ligands that mimic pathogen-associated molecular patterns and activate immune cells through TLR are being developed for therapy against diverse diseases in humans as well as for vaccine adjuvants. In terms of allergy, immunotherapy should ideally have the capacity to produce a long-term, antigen-specific, protective immune response. Immunotherapy has been limited in the past by the potential severe side effects associated with conventional immunotherapy using whole allergen extracts, and by the fact that treatment often needs to be given for a number of years. Although specific allergen immunotherapy is successful for several allergens (primarily in patients suffering from seasonal rhinitis or those with allergies to bee and wasp venom[337, 338]), desensitisation, using intact non-standardised allergen extracts, risks inducing anaphylaxis and is not successful in all patients[339-341]. Thus novel strategies to minimise the side effects and improve the efficacy of immunotherapy are of considerable interest in the treatment of atopic diseases.

The current use of TLR ligands in immunotherapy has mainly been designed around the use of TLR9 stimulation. ISS-ODN has been proven effective in attenuating hypersensitivity responses associated with asthma, allergic conjunctivitis, and allergic rhinitis[244, 250], and TLR9 adjuvants are already in clinical trials for allergic disease[262, 342-344]. One example of the potential use of TLR's in allergic disease treatment in humans, is the vaccine developed for allergic rhinitis using a ragweed-pollen allergen (Amb a1) conjugated to an ISS-ODN (AIC)[345]. This AIC vaccine is showing promise as it appears

to offer long-term clinical efficacy. TLR9 adjuvants have been shown to be safe and are consistently reported as being highly effective in the prevention of allergen sensitisation, despite the evidence showing that TLR9 ligands increase the Th1 immune response.

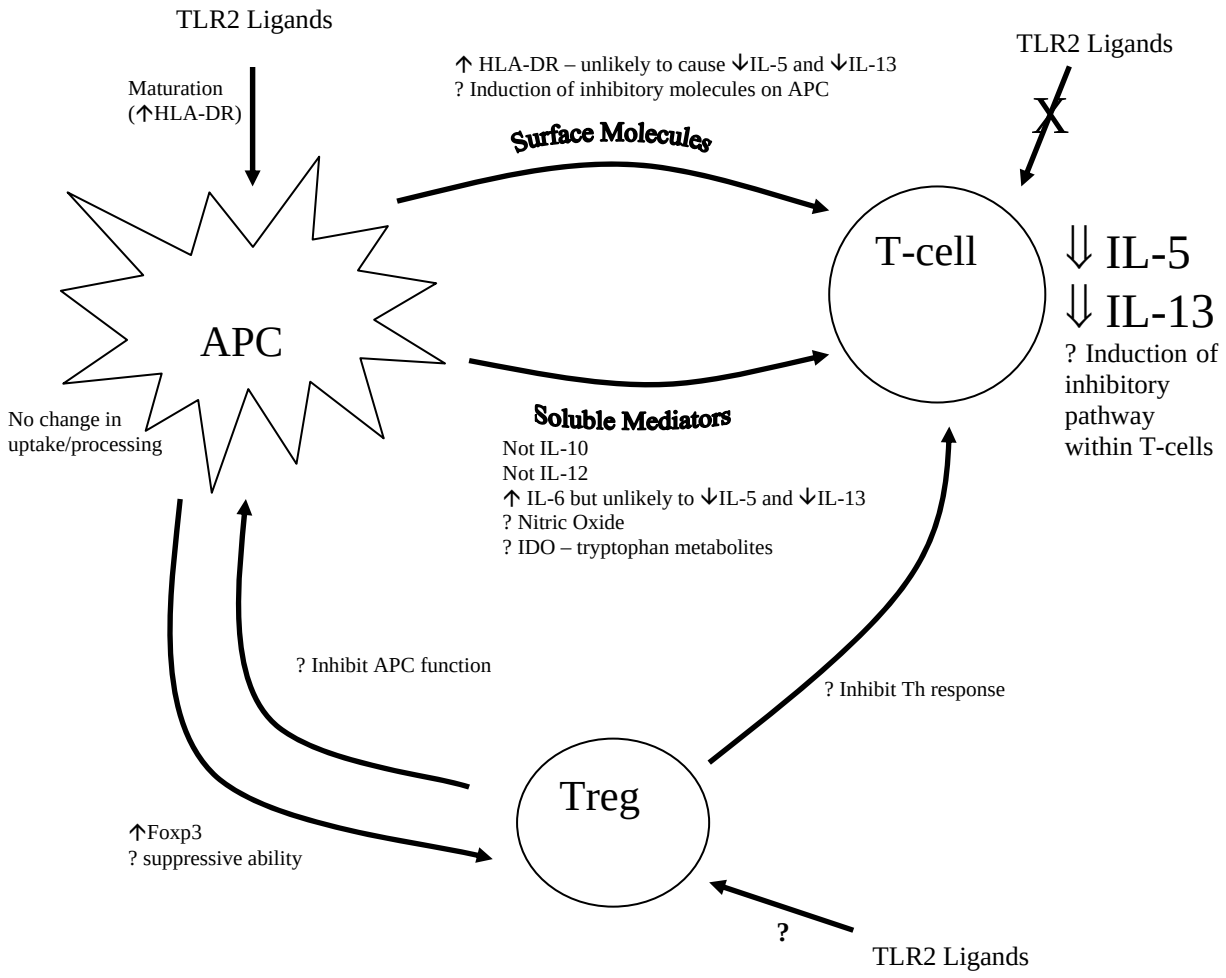
Patel *et al* investigated the therapeutic effect of Pam3CSK4 (TLR2) on established airway inflammation in a murine model of asthma[268]. Pam3CSK4 attenuated an established OVA-specific asthma in mice, this effect was dependent on IL-12, but not on IL-10 or TGFβ, and was accompanied by an enhanced specific Th1 response (IFNγ). Though this study is promising, it is important to note that other studies utilising TLR2 and TLR4 ligands as adjuvants for immunotherapy have provided contradictory reports. Both TLR2 and TLR4 ligands have been shown to inhibit Th2 responses as well as exacerbate them. Until the mechanism(s) involved in these systems are worked out and the potential harmful effects addressed, the use of TLR2 or TLR4 as adjuvants in immunotherapy remains uncertain.

As put forward earlier, the partial inhibition of Th2 response using the purer ligands LTA and Pam3CSK4 may not confer total inhibition of allergic response. To try and induce a more beneficial response it was hypothesised that by combining the ligand together, or with drugs that are known to inhibit Th2 responses that the inhibition of Th2 responses would be enhanced. The addition of the two ligands together did not enhance the inhibition beyond the level that LTA induced on its own. There was however an additive effect when LTA was combined with low dose dexamethasone, this was not replicated with Pam3CSK4. The difference in Pam3 CSK4 and LTA may be explained by the differences in their signalling pathways, Pam3CSK4 may be limited in its signalling by low levels of TLR1 expression. However the results with LTA are

promising in that a TLR2 ligand/drug combination may be able to be developed in the future into an effective therapy for allergic disease. Also it may allow use of lower dose of glucocorticoids and therefore reduce the risk of side effects.

The immune system is balanced between immunity and tolerance, between Th1 and Th2, and between inflammation and unresponsiveness. Thus, inducing a shift from allergen specific Th2 to a Th1 response, might be beneficial in asthma but may lead to other immune problems. For example, many autoimmune diseases are Th1 mediated, and there is the danger of precipitating disease in predisposed individuals by using Th1 inducing agents. Still, the most promising approaches in primary prevention of allergic diseases include the induction of systemic or local allergen-dependent or –independent Th1 immune responses, through the use of killed bacteria (or components derived from them), ISS-ODN or pDNA, and the induction of allergen-specific T-cell tolerance.

The results outlined in this thesis are promising in terms of disease treatment, as they illustrate that TLR2 ligands are able to inhibit the established allergen-specific Th2 immune response without promoting an allergen-specific Th1 response. Thus, treatment with these ligands has the potential to alleviate allergic disease without the risk of developing other immune problems. This study provides promising results that a new generation of immunotherapeutics can be developed, and encourages the thought that TLR based immunotherapy might prevent or even cure allergic diseases in the future.




**<u>Figure 6.1.</u> Overview of main cell types involved in the Th2 response to allergen and potential action of TLR2 ligands.**

# 7 KEY FINDINGS FROM THIS THESIS

- TLR2 ligands have the ability to influence and inhibit Th2 responses to HDM in sensitised individuals. (Chapter 3)
- This effect is TLR2 dependent. (Chapter 3, 4, 5)
- This inhibition is not mediated by IFNγ, IL-12 or IL-10. (Chapter 3)
- This inhibition is not caused by changes in APC activity such as antigen uptake and proliferation, or APC maturation. (Chapter 4) However the presence of APC's is needed for the inhibition of Th2 cytokine secretion to occur. (Chapter 5).

In conclusion, this study shows that TLR2 ligands have the ability to inhibit the Th2 response to mite allergen in previously sensitized individuals by an as yet unknown mechanism. However the findings described herein do provide an impetus for future studies designed to uncover novel mechanisms by which allergic responses can be ameliorated, and may open new treatment modalities.

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# 9 APPENDIX: ABSTRACTS AND PUBLICATIONS

**Abstract: American Thoracic Society 2006 San Diego**

HOW DO TOLL-LIKE RECEPTOR 2 LIGANDS INHIBIT ALLERGEN SPECIFIC TH2 RESPONSES?

R.C. Taylor[1]. P Richmond[2] and J.W. Upham,[1]. [1]Institute for Child Health Research, Perth, WA, Australia and [2]University of Western Australia, Perth, WA, Australia.

Increasing evidence suggests that exposure to microbial stimuli can influence adaptive immune responses to allergens and the development of asthma and other allergic diseases. We have previously shown that toll-like receptor 2 (TLR2) ligands are able to influence the response to House dust mite (HDM) in allergic individuals. This is viewed as a change in Th2 response, as seen by a decrease in the levels of IL-5 and IL-13 produced in response to HDM when the ligands are present. The aim for this study was to characterise this altered response.

Endocytosis of dextran fluorescein and DQ-Ova by 48hr monocyte derived DC's with/without prior TLR2 stimulation, with heat-killed Staph. aureus, indicated that the reduction in Th2 response is not due to an inhibition of antigen uptake or processing. A lack of IFNg and IL-12 cytokine synthesis by mononuclear cells (MNC) stimulated with HDM in conjunction with TLR2 ligands, indicates that a switch to a Th1 response is not occurring. Purified T-cells stimulated with TLR2 ligands in the presence or absence of T-cell activation beads indicates that TLR2 stimulation of antigen presenting cells is required to obtain a reduction in Th2 response.

TLR2 ligands have the capacity to inhibit allergen-specific Th2 responses in previously sensitized individuals. This effect appears to be mediated by the actions of TLR2 ligands on antigen presenting cells, but does not involve the inhibition of antigen uptake or processing, or the induction of a strong Th1 immune response.

**Abstract: American Thoracic Society 2005 San Diego**

TOLL-LIKE RECEPTOR 2 AGONISTS INHIBIT TH2 RESPONSES TO MITE ALLERGEN IN ATOPIC INDIVIDUALS

R.C. Taylor, BSc[1]. P Richmond, FRACP[2] and J.W. Upham, FRACP PhD[1]. [1]Institute for Child Health Research, Perth, WA, Australia and [2]University of Western Australia, Perth, WA, Australia.

There is intense interest in the interaction between microbial compounds and the immune response to allergens. While toll-like receptor 2 (TLR2) ligands may enhance allergic sensitisation in animal models, it is not clear what effect TLR2 ligands would have on allergen-specific T-cell memory in humans with established allergic sensitisation. Our aim was thus to determine whether exposure to TLR2 ligands would modify the immune response to house dust mite allergen (HDM).

Mononuclear cells (MNC) were obtained from both HDM sensitive (n=23) and non-atopic (n=22) individuals. Flow cytometry was used to determine cell surface expression of TLR2. Production of interferon gamma (IFNg), IL-5 and IL-13 were measured after stimulation of MNC with HDM in the presence or absence of TLR2 agonists. In HDM-allergic subjects, IL-5 and IL-13 responses to HDM were significantly inhibited with the addition of the TLR2 agonists, heat-killed Staph. aureus (Pansorbin), Staphylococcal lipoteichoic acid (sLTA) and the synthetic lipoprotein Pam3CSK4 ($p<0.005$ for all stimuli). The IFNg response remained unchanged (sLTA and Pam3CSK4) or was increased (Pansorbin). In contrast, TLR2 agonists had no significant influence on cytokine responses to HDM in non-atopic subjects. FACS analysis indicated that TLR2 was expressed predominantly on monocytes and myeloid dendritic cells.

These findings indicate that TLR2 agonists have the ability to markedly alter the T-cell responses to HDM in sensitised individuals, possibly through their effects on monocytes and myeloid DC.

**Abstract: Thoracic Society of Australia and New Zealand 2005 Perth**

[1]TOLL-LIKE RECEPTOR 2 AGONISTS INHIBIT TH2 RESPONSES TO MITE ALLERGEN IN ATOPIC INDIVIDUALS

Rebecca C. Taylor[1], Peter Richmond[2], and John W. Upham[1].

*[1]Institute for Child Health Research and [2]School of Paediatrics and Child Health, University of Western Australia, Perth, Australia*

It is not clear whether exposure to microbial compounds will modify the immune response to house dust mite (HDM). Our aim was to determine whether exposure to toll like receptor 2 (TLR2) ligands would modify the immune response to HDM. Mononuclear cells (MNC) were obtained from both HDM sensitive (n=23) and non-atopic (n=22) adult individuals. Production of interferon gamma (IFNγ), IL5 and IL13 were measured after stimulation of MNC with HDM in the presence or absence of TLR2 agonists. Flow cytometry was used to determine expression of TLR2.

In HDM-allergic subjects, IL5 and IL13 responses to HDM were significantly inhibited with the addition of the TLR2 agonists, heat-killed *S. aureus* (Pansorbin), *Staphylococcal* lipoteichoic acid (sLTA), and the synthetic lipoprotein Pam3CSK4 ($p<0.005$ for all stimuli). The IFNγ response remained unchanged (sLTA and Pam3CSK4) or was increased (Pansorbin). In contrast, TLR2 agonists had no significant influence on cytokine responses to HDM in non-atopic subjects. FACS analysis indicated that TLR2 was expressed predominantly on monocytes and myeloid dendritic cells (DC).

These findings indicate that TLR2 agonists have the ability to markedly alter the T-cell responses to HDM in sensitised individuals, possibly through their effects on monocytes and myeloid DC.

**Abstract: Combined Biological Sciences Meeting 2005 Perth**

# Toll-like Receptor 2 Ligands inhibit Th2 Responses to Mite Allergen in HDM Sensitised Individuals

Rebecca C. Taylor[1], Peter Richmond[2], and John W. Upham[1]

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There is intense interest in the interaction between microbial compounds and the immune response to allergens. While toll-like receptor 2 (TLR2) ligands may enhance allergic sensitisation in animal models, it is not clear what effect TLR2 ligands would have on allergen-specific T-cell memory in humans with established allergic sensitisation. Our aim was thus to determine whether exposure to TLR2 ligands would modify the immune response to house dust mite allergen (HDM).

Mononuclear cells (MNC) were obtained from both HDM sensitive (n=23) and non-allergic (n=22) individuals. Flow cytometry was used to determine cell surface expression of TLR2 and for antigen uptake analysis. Production of interferon gamma (IFNg), IL-5 and IL-13 were measured after stimulation of MNC with HDM in the presence or absence of TLR2 ligands. In HDM-allergic subjects, IL-5 and IL-13 responses to HDM were significantly inhibited with the addition of the TLR2 ligands, heat-killed Staph. aureus (Pansorbin), Staphylococcal lipoteichoic acid (sLTA) and the synthetic lipoprotein Pam3CSK4 ($p<0.005$ for all stimuli). The IFNg response remained unchanged (sLTA and Pam3CSK4) or was increased (Pansorbin). In contrast, TLR2 ligands had no significant influence on cytokine responses to HDM in non-allergic subjects. FACS analysis indicated that TLR2 was expressed predominantly on monocytes and myeloid dendritic cells. Endocytosis using Dextran Fluorescein and a self-quenched peptide (DQ-Ovalbumin) showed that there was no effect of the TLR2 ligands on antigen uptake or processing by 48hr monocytes. These findings indicate that TLR2 ligands have the ability to markedly alter the T-cell responses to HDM in sensitised individuals, possibly through their effects on monocytes and myeloid DC. Though this was not due to an inhibition of antigen uptake or processing.

**Abstract: Australasian Society of Immunology 2005 Melbourne**

# TLR2 LIGANDS INHIBIT TH2 RESPONSES TO MITE ALLERGEN IN HDM SENSITISED INDIVIDUALS.

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There is intense interest in the interaction between microbial compounds and the immune response to allergens. While toll-like receptor 2 (TLR2) ligands may alter allergic sensitisation in animal models, it is not clear what effect TLR2 ligands have on allergen-specific T-cell memory in humans with established allergic sensitisation. Previous studies by us (ASI 2004) have shown that the TLR2 ligands heat-killed *Staph. aureus* (Pansorbin), staphylococcal lipoteichoic acid (LTA) and the synthetic lipoprotein Pam3CSK4, have the ability to inhibit the Th2 (IL-5 and IL-13) response to mite allergen in HDM-allergic subjects ($p<0.005$ for all stimuli). This reduction is enhanced with certain combinations of TLR2 ligands. Our focus is now on finding potential mechanisms by which this is occurring.

Peripheral blood mononuclear cells (PBMC) were obtained from both HDM sensitive (n=23) and non-allergic (n=22) individuals. Production of interferon gamma (IFNg), IL-5 and IL-13 were measured after stimulation of PBMC with HDM in the presence or absence of TLR2 ligands. Flow cytometry was used to determine cell surface expression of TLR2 and for analysis of antigen uptake.

FACS analysis indicated that TLR2 was expressed predominantly on monocytes and myeloid dendritic cells. Endocytosis using dextran fluorescein and a self-quenched peptide (DQ-Ovalbumin) showed that there was no effect of the TLR2 ligands on antigen uptake or processing by antigen presenting cells.

The three TLR2 ligands did not induce consistent changes in IFNg secretion and we were unable to detect IL-12 under any condition. This indicates that the reduction of IL-5 and IL-13 is not due to a deviation to an allergen specific Th1 response.

We have shown that TLR2 ligands have the ability to inhibit the Th2 response to mite allergen in previously sensitized individuals. This effect is likely to be mediated by the action of TLR2 ligands on antigen presenting cells.

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# [2]TLR2 AGONISTS INHIBIT TH2 RESPONSES TO MITE ALLERGEN IN ATOPICS.

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It is not clear whether exposure to microbial compounds will modify the immune response to house dust mite (HDM). Our aim was to determine whether exposure to toll like receptor 2 (TLR2) ligands would modify the immune response to HDM. Mononuclear cells from healthy adults were obtained from both HDM sensitive (n=23) and non-atopic (n=22) individuals. Flow cytometry was used to determine cell surface expression of TLR2. Production of interferon gamma (IFNγ), IL5 and IL13 were measured after stimulation of PBMC with HDM in the presence or absence of TLR2 agonists or LPS for 2 or 5 days.

After 5 days, the HDM sensitive groups IL5 and IL13 responses to HDM were significantly inhibited with the addition of the TLR2 agonists, heat-killed *Staph. aureus* (Pansorbin), *Staphylococcal* lipoteichoic acid (sLTA) and the synthetic lipoprotein (Pam3CSK4) and LPS ($p<0.005$ for all stimuli). In contrast, the IFNγ response remained the same or was increased with the addition of TLR2 agonists. FACS analysis indicated that TLR2 was expressed predominantly on monocytes and myeloid DC's, and low expression was found on T-cells.

These findings indicate that TLR2 agonists have the ability to influence the cellular responses to HDM, with monocytes and myeloid DC's being candidates for regulating this response.