Dishing the dirt on asthma: What we can learn from poor hygiene

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Abstract: Allergic asthma continues to represent a huge health burden worldwide and is largely treated by non-selective immunosuppressive drugs, which often prove ineffective. The hygiene hypothesis proposes that the increased incidence of allergy and asthma in Western countries observed in the last 50 years is due to environmental changes that include improved hygiene and a lack of infections. The immunological mechanisms that must underpin such an environmental impact on immune regulation remain to be defined, making it difficult to identify specific ways of preventing development of allergy and asthma in early life. In this article we will seek to review some of the pathways that might underlie the hygiene hypothesis in an attempt to provide targets for future asthma prevention.

Keywords: hygiene hypothesis, asthma, allergy, infection

The environment and asthma

Asthma is a chronic, inflammatory disorder of the airways leading to airway remodeling. There are both genetic and environmental influences on asthma development, with sensitization to such factors as house dust mite and maternal smoking appearing to stem from as early as 22 weeks gestation. Most asthma is caused by an allergic response to innocuous antigens leading to Th2 cytokine release, eosinophilic inflammation and IgE mediated mast cell degranulation. It is estimated that around 300 million people in the world currently have asthma, and the incidence has been rising dramatically. In 1989 Strachan postulated the hygiene hypothesis, in which he suggested that in the past allergy would have been prevented by early childhood infections but this protection had been diminished by changes in lifestyle over the past 30 years. Many aspects of modern living may contribute to this increase in asthma including, smaller family size, more urban living including a lower exposure to farm livestock, increasing vaccination and a more hygienic lifestyle.

Asthma is in most cases an allergic condition in which there is an immune response to innocuous environmental antigens leading to a Th2-type CD4 T cell response and an IgE antibody response. Both components are directly involved in the immunopathogenesis of asthma, the latter via triggering of mast cell inflammatory mediators. Asthma is characterized by non-specific airway hyperresponsiveness and hyperreactivity to a range of usually innocuous stimuli such as cold air, mild infection, pollutants and even exercise, in addition to allergen exposure. There is chronic mucosal inflammation (Beasley et al 1989) leading to damage to the lung epithelium, thickening of the subbasement membrane collagen layer, hyperplasia and hypertrophy of bronchial smooth muscle and hyperplasia of goblet cells leading to blockage of the bronchial lumen with mucus (Dunill 1960). It has long been accepted that there is a genetic component to the development of asthma and many studies have attempted to identify the genes involved. Of the many genes associated with asthma, the main chromosomes involved appear to be 5, 6, 11, 12 and 13. The region 5q31 of chromosome 5 contains genes for

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the interleukins IL-4, IL-5 and IL-13. High level expression of these interleukins is classified as a Th2 response, associated with atopy and asthma; polymorphisms in the IL-4 and IL-13 genes are also associated with varying IgE levels (Marsh et al 1994; Wills-Karp et al 1998).

There is also an expanding list of environmental factors thought to be involved in the development of asthma. The most common allergens known to cause asthma are inhaled airborne particles which trigger a cascade of events leading to inflammation and sensitization in the lungs. Exposure in early childhood to house dust mite (HDM) (*Dermatophagoides pteronyssinus*) allergens is an important determinant of the subsequent development of asthma (Sporik et al 1990). Increasing sensitization correlates with increasing exposure to this and other allergens such as cockroach proteins in inner-city children (Rosenstreich et al 1997). Sensitization to HDM can occur from as early as 22 weeks gestation (Jones et al 1996), hence not only quantity of allergen is important as a trigger for asthma but also the timing of that exposure.

Another trigger may be lower respiratory tract virus infection in early life. Respiratory syncytial virus (RSV) is the most frequent cause of lower respiratory tract disease during infancy; although whether RSV bronchiolitis is a cause of later asthma development is unclear. RSV has been linked to recurrent wheeze but not atopic asthma in children up to age 13 (Stein et al 1999), but RSV bronchiolitis severe enough to cause hospitalization may indeed be linked to asthma (Sigurs et al 2000). Murine models have also linked airway inflammation and hyperresponsiveness to acute RSV infection (Schwarze et al 1999), so it may be that severe, not mild RSV could damage the lungs and trigger asthma. Alternatively RSV symptoms may become severe because of sensitivity caused by the early stages of asthma.

As well as factors reported to cause asthma, a number are thought to exacerbate existing disease, including the presence and quantity of relevant allergens, cold air and exercise. Colds and respiratory viruses are thought to be responsible for 80%–85% of asthma attacks (Johnston et al 1995). Passive smoking has been correlated with asthma severity and a higher risk of hospital admission for asthma (Eisner et al 2005). Air pollution has long been thought of as a cause of asthma but studies before German reunification showed a lower prevalence of asthma in the more polluted East Germany (Nowak et al 1996), and New Zealand, with typically clean air has high levels of asthma (Beasley et al 1998).

It is therefore clear that there are both genetic and environmental influences on asthma development. Genetic polymorphisms may not cause asthma per se but predispose towards sensitization in particular environments. The critical events that determine whether allergy and asthma develop appear to occur in early childhood, up to around 3 years of age (Upham and Holt 2005), and may even be influenced in-utero (Skorge et al 2005). This provides a specific window of opportunity for asthma prevention.

The mechanisms of asthma

Asthma is an allergic response to an innocuous antigen, but how does that allergen trigger the initial immune response? The first cells in the lung met by inhaled antigen are alveolar macrophages; in non-atopic individuals these cells are highly phagocytic, express strong microbicidal activity, are poor antigen presenting cells (APC) and are immunosuppressive. In asthmatics, these cells have reduced immunosuppressive function (Aubas et al 1984), increased APC function (Larche et al 1998), increased production of the Th2 cytokines IL-4 and IL-5 (Tang et al 1998) and decreased production of IL-10 (Borish et al 1996). Antigen missed by the alveolar macrophages is picked up by dendritic cells (DCs), and presented to naïve $CD4^+$ T-cells. In healthy lungs mature pulmonary DCs produce IL-10 which induces T-cell tolerance (Akbari et al 2001), so diminished IL-10, as seen in asthmatics, may reduce antigenic tolerance. Activation of CD4 T-cells in the presence of certain cytokines polarizes the response towards Th1 or Th2. In the presence of IL-12 and IFN-γ Th1 cells are produced, whereas IL-4 induces cells to secrete Th2-type cytokines including IL-4, IL-5 and IL-13 (Del Prete et al 1991). In the asthmatic, there is a lack of IL-12 production from DC's and allergen is thus presented to T-cells by DC's and macrophages in a cytokine milieu that favors Th2 development (van der Pouw Kraan et al 1997). Since respiratory DC's are immature in phenotype and poor producers of IL-12 (Stumbles et al 1998), and allergens lack pathogen associated molecules such as TLR ligands, which trigger IL-12 from DC's (Schulz et al 2000), the immune response to inhaled allergens becomes abnormally skewed towards Th2 in the asthmatic (Robinson et al 1992).

B-cell activation by IL-4 directs isotype switching towards IgE. When produced by plasma cells, IgE binds to the high-affinity receptor FcεR1 on mast cells and basophils. When bound IgE is cross linked by antigen it causes rapid mast cell and basophil degranulation. The granules released contain histamine which promotes vascular permeability, smooth muscle contraction and mucus production, all symptoms of asthma. Cytokines released include IL-4, which feeds back onto B-cells, and IL-5, critical for eosinophil production and activation. Activated eosinophils release pro-inflammatory

mediators and cytokines including more IL-5 and IL-13. IL-13 induces B-cell class switching to IgE and mast cell activation to give an inflammatory response. Hence the production of Th2 cytokines acts in a positive feedback loop promoting IgE production and mast cell degranulation, increasing asthma symptom severity. Asthmatics have more activated Th2 cells in their bronchoalveolar lavage fluid (Robinson et al 1992), and in murine models Th2 cells alone can recapitulate most key features of human asthma (Cohn et al 1998). However IgE levels are important as "asthma is almost always associated with some type of IgE-related reaction" (Burrows et al 1989) and severity is related to total serum IgE levels (Carroll et al 2006). Not only are there higher levels of IgE, but also increased numbers of mast cells in the airway smooth muscle of asthmatics (Brightling et al 2002). In addition to immediate hypersensitivity reactions, this is likely to contribute to the airway remodeling events that are the consequence of chronic inflammation (Yu et al 2006).

As well as $CD4^+$ Th2 cells, $CD8^+$ Tc2 cells are thought to be involved in asthma, although less is known about their role. Respiratory DC's cross-present allergen into the MHC Class I pathway, resulting in an allergen-specific CD8 T cell response (Wells et al 2007). Although CD8 T cells generally suppress allergy and are strongly biased towards Tc1 development (Carter and Murphy 1999; Noble et al 2001), Tc2 cells with a cytokine profile similar to that of Th2 cells can be induced in mice and humans. Allergen-specific Tc2 cells infiltrate the lung and recruit an eosinophilic inflammatory response with airway hyperreactivity (Sawicka et al 2004). This may exacerbate the Th2 response and is likely to contribute to human asthma (Ying et al 1997). $CD8^+$ T-cells inhibit IgE responses by favouring Th1 development and IL-12 and IL-18 secretion from DC's (Thomas et al 2002; Salagianni et al 2007). Such cross-regulation between CD4 and CD8 T cell populations may play a key role in immune regulation and development of respiratory tolerance.

Despite the dominant role of Th2 cells and IgE-mediated responses in the disease, other mechanisms have been implicated, particularly in chronic asthma. Tumor necrosis factor-α (TNF-α, a Th1-associated cytokine, is present at high levels in the airways of chronic asthmatics, and its blockade can improve symptoms (Howarth et al 2005; Holgate and Polosa 2006). Transforming growth factor-β (TGF-β) produced from a number of cell types contributes to the airway remodeling process through its pro-fibrotic functions (McMillan et al 2005), as may the eosinophils present in the airway (Humbles et al 2004). IL-17 is also strongly upregulated in asthma (Hashimoto et al 2005), and may be involved in the neutrophil recruitment to the airways (Hellings et al 2003) which is more prominent in chronic disease. IL-17 is the hallmark cytokine of the newly described Th17 subset of T cells (Harrington et al 2005), but it is unknown whether these cells play an important role in airway inflammation.

Asthma prevalence and importance

Asthma is one of the commonest chronic diseases of affluent societies. It is estimated that around 300 million people in the world currently have asthma (Masoli et al 2004). The two largest international surveys of asthma prevalence and severity are the European Community Respiratory Health Survey (ECRHS) and the International Study of Asthma and Allergy in Childhood (ISAAC). ISAAC studied children aged between 13 and 14 years in 56 countries worldwide (Beasley et al 1998). They found the lowest asthma prevalence in Indonesia with 1.6%, and the highest in the UK, with 36.8% of children in the study reporting symptoms of asthma in the previous 12 months. They also found that countries reporting high asthma rates also reported high rates of allergic rhinoconjunctivitis and atopic eczema. This includes the UK, New Zealand, Australia, and the Republic of Ireland. Those with the lowest rates included Indonesia, several Eastern European countries, Greece, China and Taiwan. The ECHRS looked at asthma prevalence in 22 different countries and found similar results with the highest asthma rates in English speaking countries, and low rates in Mediterranean and Eastern European countries (Janson et al 2001). The results were similar for physician diagnosed asthma (Janson et al 1997); New Zealand and Australia had the highest rates with 11%–13% of the population sampled affected, with rates of anti-asthma medication use at 12%–16%. In the UK, 8.5% of the population are receiving treatment for asthma and 50% of these are experiencing severe asthma symptoms. One in six of these reported weekly attacks so severe they could not speak (Asthma UK 2003). In terms of healthcare costs the total US costs for asthma were estimated at $10.7 billion in 1994 (Weiss et al 2000). The UK cost was £889 million in 2003 (Asthma UK).

Asthma mortality rates also show large variations between countries, not only due to numbers and severity of cases but also access to prompt, specialist medical care. Countries with the highest asthma rates do not necessarily have the highest mortality rates, for example the UK mortality rate is 2.9–3.2 per 100,000 asthmatics, the New Zealand rate is 4.6. Whereas Russia and China have low asthma prevalence, mortality rates are very high (28.6 and

36.7 fatalities per 100,000 asthmatics respectively (Masoli et al 2004)), underlining the fact that most asthma deaths are preventable. Nevertheless, even in the UK, asthma kills around 1400 people every year (Asthma UK 2003).

In addition to worldwide variation, it is clear that in the past 50 years the worldwide prevalence of not only asthma but other allergic diseases such as allergic rhinitis and eczema has increased dramatically. Hay fever was regarded as extremely rare until the 19th century (Emanuel 1988), when it was associated with the upper classes of society. Later increases in atopy and asthma have been well documented in the UK (Burr et al 1989; Kuehni et al 2001). In 8–13 year old children the incidence of asthma, hay fever and eczema more than doubled over a 25 year period (Ninan and Russell 1992), and there was a six-fold increase in asthma indicidence between 1973 and 2003 (Burr et al 2006). In Scandinavian countries, similar increases have been observed. In Sweden, asthma, allergic rhinitis and eczema incidence roughly doubled between 1979 and 1991 (Aberg et al 1995), increases were also observed in Norway (Skjonsberg et al 1995), and in Finnish young men a 20 fold increase was reported between 1961 and 1989 (Haahtela et al 1990). In more recent years this trend may be slowing down or even reversing. In Italy, Ronchetti et al (2001) found increases in asthma between 1974 and 1992 but no further increases up to 1998. Similar results were seen in Switzerland and Germany (Braun-Fahrlander et al 2004; Zollner et al 2005). UK results are more confusing. Increases in wheeze and asthma attacks but a decrease in hospital admissions have been reported, although this is probably a result of better asthma drugs and management (Butland et al 2006). Although the incidence of asthma might now be falling in Europe, elsewhere, as in Taiwan (Yan et al 2005), the trend still appears to be upwards. In less affluent countries adopting an increasingly Westernized lifestyle, it appears that increasing asthma prevalence is an inevitable consequence, accompanied by much higher mortality rates.

Clearly the increase in asthma prevalence could not be caused by genetic factors as a change in the genetic pool would not occur in such a short period of time. Therefore, it must be caused by environmental factors. What are the changes in lifestyle that have been occurring in the last 50 years since the second world war which could cause such an impact on disease?

The hygiene hypothesis

The hygiene hypothesis was first postulated by Strachan in 1989 (Strachan 1989). In his paper he notes that the increase in prevalence of asthma and childhood eczema reported by Emanuel (Emanuel 1988) and Fleming (Fleming and Crombie 1987) could be due to several factors. He observed that the incidence of hay fever was inversely related to the number of children in the household when a child was aged 11, and that the trend was steeper when the siblings were older than the child. The incidence of eczema was also inversely related to the number of older, but not younger siblings. From this, Strachan concluded that in the past, allergic diseases could have been "prevented by infection in early childhood transmitted by unhygienic contact with older siblings" but this protection has been diminished over the past century by "declining family size, improvements in household amenities and higher standards of personal cleanliness". A more recent study of individuals born pre 1980 concluded that low socio economic status at birth as well as increasing birth order was associated with a lower risk of allergy (Kinra et al 2006). Over the years, this hypothesis has been revisited and expanded to include more atopic disorders and other features of modern living in an effort to elucidate the major cause or causes of the current asthma epidemic.

Some studies focused on the original issue of hygiene such as that by Zhang et al (2005) looking at the incidence of rhino-conjunctivitis, wheezing and asthma among school children in Western Australia, comparing asthma symptoms to household cleanliness. As with similar studies, this is based upon questionnaires filled in by families and relies on parental diagnosis of children's symptoms and the family's hygiene habits, both of which can be subjective. Despite these drawbacks, this and similar papers show an inverse relationship between asthma and house cleanliness, although parents of a child with asthma are more likely to keep a clean house in order to reduce their child's symptoms.

Other research has built on the idea that children born to large families are less likely to develop asthma, and suggested that interactions with other children at day-care centers can transmit infections in the same way as interactions with siblings. Some researchers found no association between day-care attendance and development of atopic diseases (Backman et al 1984) whereas others found that day-care attendance did protect against allergies, but only for children from smaller families, or children who attended from a younger age (6–11 months) (Kramer et al 1999; Ball et al 2000). Another theory is that children in rural communities are protected from asthma because they interact with livestock from an early age. Yemaneberhan et al (1997) compared asthma rates in urban and rural Ethiopia. They found wheezing and asthma were more prevalent in urban

areas, this increase was related to housing style, bedding materials and use of certain insecticides. Comparisons have also been made in the USA (Adler et al 2005) with similar results; farm-reared children (up to the age of 5) were less likely to have a history of wheezing or develop asthma than their non farm-reared counterparts. Interestingly they report no difference in incidence between farm and non farm-reared children for non-asthma allergy, again suggesting subtle but important differences in the causes of asthma compared to other allergies. In rural communities in Europe, similar results were obtained. Allergic disease correlates negatively with the amount of exposure to livestock (Von Ehrenstein et al 2000). Since fewer families in Europe are involved in farming compared to 50 years ago, this may have contributed significantly to the asthma increase.

Is exposure to domestic pets also a protective factor? Children reared in houses with high levels of pet allergen have sensitization to these allergens, which is strongly associated with asthma (Ingram et al 1995). On the other hand children exposed to pets in the first year of life have a lower frequency of allergic rhinitis at 7–9 years and of asthma at 12–13 years (Hesselmar et al 1999). Thus exposure to high doses of allergens early in life can induce operational tolerance which is long-lasting.

One common factor in these studies is the importance of childhood infections, whether from siblings, other children in day-care or farm livestock. Children are less likely to develop wheeze or asthma at age 7 if they have developed ≥2 episodes of runny nose before the age of 1, or ≥1 viral infection of the herpes type in the first 3 years of life (Illi et al 2001). Matricardi et al (1997) looked at hepatitis A seropositive and negative Italian men, prevalence of atopy and atopic respiratory diseases. They concluded that prevalence of atopy was lower amongst seropositive individuals; however, incidence of atopy amongst seronegative individuals was as low as that of seropositive individuals when they had three or more older siblings. Thus increases in asthma could be due to fewer childhood infections passed on by older siblings. Similar results show a lower prevalence of atopy amongst children who have had measles infection compared to those who have been vaccinated. Interestingly, the widely-used aluminium based adjuvants used in subunit vaccines have been shown to induce higher levels of IgE antibodies (Cogne et al 1985). Use of such vaccines rather than live/attenuated organisms may help skew the immune system towards a Th2 phenotype.

Another area of interest is that of mycobacterial infection, Shirakawa et al (1997) suggested that amongst Japanese schoolchildren there was a strong inverse association between delayed hypersensitivity to *Mycobacterium tuberculosis* and atopy, including asthma. Later studies in the Gambia (Ota et al 2003), and Australia (Marks et al 2003), largely failed to reproduce these findings. In areas where there is a high prevalence of geohelminth infections such as Africa, the Eastern Mediterranean and South East Asia, there is a lower prevalence of asthma, despite the Th2-type response elicited by many of such infections. Indeed it was shown that there is a lower prevalence of allergic disease in patients infected by geohelminths (Catapani et al 1997) again indicating the protective effect of infectious organisms. Conversely, use of antibiotics in infants may be associated with asthma (Wickens et al 1999), alternatively antibiotic use may be more common in asthmatic children (Celedon et al 2004). Antibiotics disturb the composition of the gut microflora, which is likely to be important for development of a robust immunoregulatory network.

These studies provide an increasing body of evidence demonstrating that the way we live today has a major impact on the health of our children. This increasingly "westernized" way of life, including smaller family size, lower exposure to farm animals, more urban living, and lower exposure to childhood diseases through increased hygiene awareness and vaccination, is apparently leading to increased incidence of not only asthma but other atopic disease including hay fever and eczema. So how important is allergen exposure versus hygiene or infection, and what are the immunological mechanisms that might explain why this is happening?

Mechanisms of the hygiene hypothesis

Th1/Th2 dysregulation

Individuals who develop asthma have an imbalance in the Th1/Th2 cytokine response with a bias towards Th2 cytokine production (IL-4, IL-5 and IL-13). The hygiene hypothesis suggests that a reduction in childhood infections due to improved hygiene and smaller family size may alter the Th1/Th2 cytokine balance, with insufficient stimulation of Th1 cytokine producing cells, giving an increasingly polarized Th2 response. Higher endotoxin concentrations have been found in farming rather than non farming households (von Mutius et al 2000). Endotoxin consists of lipopolysaccharide (LPS) which elicits Th1 responses through increased IL-12 production (Macatonia et al 1995), although the protective effects of LPS are seen only at high doses (Piggott et al 2005). Altering the cytokine balance in asthmatics towards a more Th1 like profile is almost certainly beneficial. In a murine model of asthma, IFN-γ administered during

either the primary sensitization or the secondary immune response decreased IgE production, prevented airway hyperresponsiveness and up-regulated Th1 cytokines (Lack et al 1996; Yoshida et al 2002). Unfortunately results of trials in human severe asthma were less impressive (Boguniewicz et al 1993). Transferring IFN-γ secreting Th1 cells into mice gives neutrophilic but not eosinophilic inflammation characteristic of an allergic response (Cohn et al 1998). Airway eosinophilia and hyperresponsiveness were abolished in mice by using recombinant IL-12 to enhance the Th1 response (Kips et al 1996). In humans, however, there was a decrease in eosinophil levels but no change in airway hyperresponsiveness (Bryan et al 2000); hence eosinophil levels are not necessarily an indicator of asthma.

If bacterial infections skew the cytokine response towards Th1, perhaps immunization with bacterial components can elicit a similar response. Eisenbarth et al (2002) showed that low levels of inhaled LPS, signaling through TLR-4, induced Th2 responses in a mouse model of allergic sensitization but high LPS levels induced Th1 like responses. Absence of LPS gave no significant lung response, as there is no "danger signal" received by the DCs. In humans, high dose of exposure to endotoxins present in house dust mite is protective for allergy but this is dependent on genetic influences (Simpson et al 2006). CpG DNA, a potent TLR9 ligand that induces IL-12 secretion and Th1 responses, prevents allergic airway inflammation (Shirota et al 2000). Furthermore a number of studies (Zuany-Amorim et al 2002; Smit et al 2003) have shown that immunizing mice with *Mycobacteria* prevented allergic manifestations and suppressed features of asthma. Unfortunately human trials using *M. vaccae* (Shirtcliffe et al 2003) have not been successful.

Analogous to the Th1/Th2 cytokine imbalance in asthmatics there may also be a $CD8^+$ Tc1/Tc2 imbalance. Indeed, Tc2 cells have been found in the lungs of asthmatics (Ying et al 1997), and as with $CD4^+$ T cells, low antigen concentrations in vitro stimulate murine Tc2 development, but higher doses induce Tc1 cells (Sawicka et al 2004). Furthermore, Tc2 cells have been demonstrated to induce airway hyperresponsiveness and recruit eosinophils to the lungs (Sawicka et al 2004). These responses may amplify those of $CD4^+$ T-cells. By contrast, Tc1 CD8 cells down-regulate IgE by stimulating DCs to produce IL-12 and IL-18 (Salagianni et al 2007), activate $CD4^+$ Th1 cells to produce IFN-γ which inhibits IgE producing B-cells (Thomas et al 2002), and inhibit allergic airway inflammation (Wells et al 2007).

Teffector/Treg dysregulation

The theory of increased immune bias towards Th2 causing increased asthma has two major drawbacks. Firstly, areas with high levels of helminth infection are areas where asthma is less prevalent, so helminth infection appears to confer protection against asthma. However, worm infections such as schistosomiasis elicit a strong Th2 response (Araujo et al 1996). Secondly, autoimmune diseases such as Type-1 diabetes and multiple sclerosis are also increasing in prevalence (Group 2000; Grytten et al 2006). These conditions are both characterized by Th1 or CD8 T cell inflammation, indeed work has shown that individuals with type-1 diabetes (Douek et al 1999) and multiple sclerosis (Oro et al 1996) are much less likely to develop asthma. How can conditions that exhibit a Th1 bias be increasing in prevalence at the same time and in the same countries as those with a Th2 bias? A likely explanation is that immune dysregulation is apparent in regulatory T cell populations, so instead of a balanced response to immune challenge leading to tolerance, the individual generates skewed Th1 or Th2 responses to autoantigens or allergens. Another possibility is that multiple sclerosis is mediated predominantly by Th17-type cells, rather than Th1 cells as previously thought (Weaver et al 2006). Th17 cells might also be involved in asthma, since IL-17-producing cells are present in the airways of asthmatics (Molet et al 2001; Hashimoto et al 2005). Notably, both Th17 and Th2 cell development are strongly inhibited by IFN-γ (Afkarian et al 2002; Harrington et al 2005). Thus, a lack of IFN-γ production early in an immune response to allergen might lead to dysregulated Th2 or Th17 responses.

There is growing interest in the function of regulatory T-cells (Tregs) in relation to asthma (Figure 1). There are several types of Treg, broadly characterized into naturally occurring (thymically derived), which are $CD4^+CD25^+FoxP3^+$ (Sakaguchi 2004), and inducible, $CD4^+$ (Hawrylowicz and O'Garra 2005) or $CD8^+$ (Noble et al 2006) Treg. Naïve T-cells stimulated in the presence of IL-10, or treated with glucocorticoids and vitamin D3, mature into IL-10 producing regulatory Tr1 cells (Groux et al 1997; Barrat et al 2002) that suppress $CD4^+$ T-cell proliferation in response to antigen. IL-10 inhibits dendritic cell function and release of histamine from activated mast cells (De Smedt et al 1997; Royer et al 2001). In allergic individuals, allergen-specific CD4 cells secrete less IL-10 and more IL-4 than normal individuals, which produce Tr1-like cells in response to allergens (Akdis et al 2004). Additional subsets of induced Treg include those that express FoxP3 after exposure to TGF-β (Chen et al 2003), and $CD8^+$, cytokine-induced Treg (Noble et al 2006). These

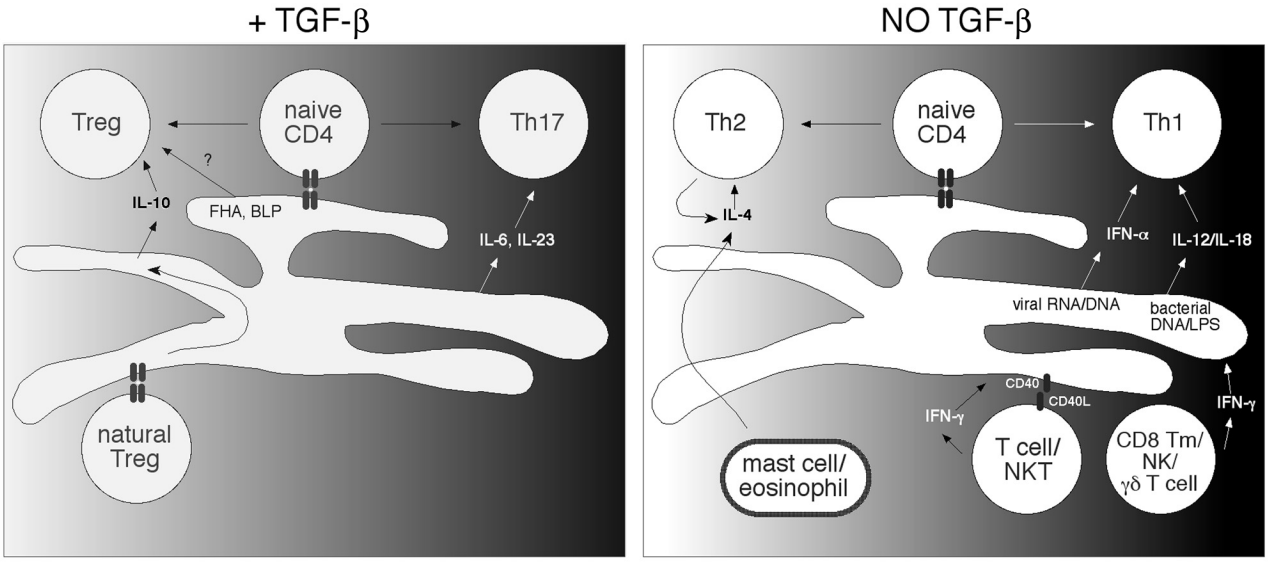

Figure 1 Regulatory networks that could be dysregulated in a hygienic environment. T cell development in the presence (left) or absence (right) of TGF-β is shown separately for clarity. The degree of shading represents the level of danger in the environment. FHA, filamentous hemagglutinin of Bordetella pertussis (McGuirk et al 2002); BLP, bacterial lipoprotein (Liu et al 2006).

can elaborate additional suppressive pathways dependent on cell contact and/or TGF-β (Zheng et al 2004).

How increased hygiene might modify Treg function is unclear. Suppression by Tregs is blocked by microbial activation of Toll-like receptor pathways in DCs (Pasare and Medzhitov 2003). Therefore, induction and maintenance of Treg populations is most likely to be at fault during the earlier stages of the disease. Ling et al found a significant reduction in the ability of naturally occurring $CD4^+CD25^+$ Tregs from atopic compared to non-atopic donors, to suppress allergen-stimulated $CD4^+CD25^-$ T-cell proliferation and IL-5 production in vitro (Ling et al 2004). The ability to suppress was even more reduced in individuals with active hay fever. Treg function was also found to be reduced in a number of other autoimmune conditions such as diabetes (Kukreja et al 2002). This is evidence of a role for these cells in disease etiology, but could simply reflect the fact that activated effector T cells are less susceptible to suppression (Liu et al 2006). However, recent data demonstrating that $Foxp3^+$ cells are induced by infection and are present at inflammatory sites (Wilson et al 2005; Baumgart et al 2006), support the idea that a lack of microbial exposure could prevent the development of a robust regulatory network of Treg populations. In addition, certain infectious agents appear capable of inducing a Tr1-type response through altering the balance of IL-10 and IL-12 secretion from DC (McGuirk et al 2002).

It seems likely that the natural gut microflora have a major influence over immunoregulatory functions, in addition to the impact of pathogenic infection. The gut contains over 10^{13} microbes which collectively express 100 times as many genes as the human genome (Gill et al 2006). This provides, along with food, an enormous range of foreign antigens with which the innate and acquired mucosal immune system interact. Diet, smoking and antibiotics all alter the microflora composition (Adamsson et al 1997). Differences have been observed between the microflora in infants who do and do not go on to develop asthma. Infants who develop asthma are more likely to have fewer aerobic bacteria (eg, Lactobacilli) and Bifidobacteria but more Clostridia and Staphylococci (Bjorksten et al 2001; Kalliomaki et al 2001), although this may be a symptom rather than a cause. Sudo et al showed that without Bifidobacterium in the microflora, germ-free mice showed increased IgE and IL-4 production and no oral tolerance (Sudo et al 1997). Oral tolerance, in addition to preventing food allergy, can suppress asthmatic responses in the lung (Russo et al 2001). Other than antibiotic use or more sterile food production, it is not clear how improved hygiene affects the microflora balance. Breastfeeding may influence the early microflora composition as well as inducing oral tolerance in the child to antigens taken up by the mother.

Interplay of innate responses with acquired immunity and memory populations

The immune response to a newly encountered allergen can be influenced by a diverse array of innate immune mechanisms. Natural killer (NK) cells, NKT cells and γδ T cells are potent

sources of IFN-γ early in the immune response (Hiromatsu et al 1992; Cui et al 1999; Martin-Fontecha et al 2004). This is critical for Th1 development, and thus suppression of Th2 responses, through activation of STAT-1 signaling, which upregulates Tbet expression (Afkarian et al 2002). The presence of microbes and associated Toll-like receptor (TLR) ligands during exposure to allergens is therefore likely to increase the availability of early IFN-γ, and prevent Th2 skewing. Likewise, the presence of viral RNA triggers strong IFN-α production in DCs which enhances IL-12 production and Th1 development (Farrar et al 2000; Van Uden et al 2001). Conversely, innate cells including mast cells, NKT cells and eosinophils can secrete IL-4 which would favour Th2 development. In several experimental models the presence of an infection suppresses allergic airway inflammation (Hansen et al 2000; Koh et al 2001; Wohlleben et al 2003). While these pathways might explain short term effects of microbial load on allergic sensitization, it cannot explain the long-term effects that are an essential component of the hygiene hypothesis. For example, Asians migrating to the UK from India up to the age of 4 are protected from asthma in adulthood, while those migrating later are not (Kuehni et al 2007). How can such early infection have such a lasting impact on the immune response to allergens not encountered until decades later? While innate immune cells may be involved, these are relatively short lived and innate cells, by definition, do not mediate immunological memory. It is therefore reasonable to predict that there is a link between the mechanisms behind the hygiene hypothesis and those of immunological memory.

The T cell compartment is thought to mediate the most long-term components of immune memory. In experimental models, it has been shown that CD8 memory T cell populations are extremely long-lived (Murali-Krishna et al 1999). Although individual memory T cells are not immortalized, antigen-specific CD8 populations are maintained through homeostatic proliferation in response to IL-15 and IL-7 (Zhang et al 1998). Crucially, this mechanism ensures that no antigen is required, so these populations can be retained indefinitely even if the infection is not present (Murali-Krishna et al 1999). Alternatively, the actions of regulatory T cells may prevent the total elimination of certain infections (Belkaid et al 2002) allowing subclinical, chronic infections to be established. This would provide continual, low level immune stimulation. Thus one can imagine that each infection encountered early in childhood might have a long-lasting impact on the immune system, resulting in an accumulation of mature memory cells. Although the proportion of circulating T cells with a memory phenotype does not increase dramatically with age due to separate homeostatic control of the naïve and memory pools (Lanzavecchia and Sallusto 2005), the nature of the memory pool could alter with age in response to environmental stimuli. This is probably also true of the Treg population (Akbar et al 2007). The production of early cytokine such as IFN-γ from memory CD8 T cells could thus increase in early life without changes in the proportion of memory cells. But how could such alterations prevent allergic sensitization?

Since large clonal expansions of CD8 Tc1 cells are rapidly induced by viral and other intracellular infections, is it easy to speculate that CD8 T cell populations are involved in transducing the signals induced by infection into those that might prevent allergic disease. CD8 memory cells are likely to prevent allergic sensitization through their ability to rapidly secrete IFN-γ and activate DCs (Das et al 2001; Kambayashi et al 2003; Noble et al 2003). Mature CD8 memory populations, induced by infection might therefore prevent Th2 responses to allergen through cross-reactivity, amplifying the Th1 arm of the response.

Alternatively, Treg populations primed by infection, rather than being derived from thymic selection as at birth, may accumulate in a "dirty" environment. These Treg may be more potently suppressive and act in a cross-reactive or non-specific fashion to inhibit responses to allergens. It is interesting to note that induction of IL-10-producing Tregs, which are more likely to be peripherally induced, is deficient in severe asthmatics (Xystrakis et al 2006). The IL-10 secretion can be improved by oral supplementation with vitamin D (Xystrakis et al 2006), the active form of which synergizes with glucocorticoids to induce Treg (Barrat et al 2002). Another reason to implicate involvement of IL-10 in the hygiene hypothesis is that it can be produced by both Th1 and Th2 cells, in addition to Treg and DCs (Trinchieri 2001; Shoemaker et al 2006). Therefore the Th2 responses induced by helminths would invoke potent IL-10 secretion, perhaps leading to more robust Treg populations. IL-12 induces IL-10 Treg in both CD4 and CD8 T cells (Gerosa et al 1996; Stock et al 2004; Noble et al 2006). Thus, Th1 and Treg development may be linked, and the concurrent rise in allergy and autoimmunity could be explained by a lack of the negative feedback loop that involves IL-10-mediated suppression.

Are Treg induced by cytokines alone, or could they respond directly to pathogen associated molecules? Foxp3 Treg express distinct patterns of TLRs compared to effector T cells (Sutmuller et al 2006), and their response to infectious

agents and cytokines is far from clear. A recent study showed that a TLR2 agonist could expand Foxp3$^+$ Treg in vivo, and these retained their suppressive capacity after proliferation (Liu et al 2006). Strategies are therefore starting to emerge for therapeutic enhancement of Treg in vivo, which unlike Treg cellular therapies will be applicable to asthma treatment.

Conclusions and therapeutic implications

Clearly the immunological mechanisms involved in the hygiene hypothesis are far from clear and a lot more research is needed. This is currently an interesting time in which research on the hygiene hypothesis is moving from epidemiological observations that support (or not) the theory, to more mechanistic studies and hypotheses. Will this emerging knowledge result in a reversal in the upward trend in asthma, or widespread use of novel therapeutics? Attempts to use Th1 cytokine inducing microbes to bias the response back to Th1 have thus far failed in humans, probably because such exposure is required in early life. The arguments against the theory of cytokine bias include the lower prevalence of asthma in areas with a high geohelminth burden and the increase in Th1 type autoimmune diseases in areas experiencing increases in Th2-mediated asthma. In the past there would have been exposure to higher antigen levels during gestation and the first year of life due to more time being spent outdoors with a more rural lifestyle. Early infections and endotoxin exposure in such an environment are also protective. Could we mimick these conditions to prevent or treat asthma in children?

It could be that small changes to our environment in early life to maintain a healthy gut microflora would reverse the trend of increasing asthma prevalence. However given the complexity of our environment and the scale of the problem, it is more likely that one factor alone will not reduce asthma. Instead, it may be necessary to introduce modifications to existing vaccines which ensure more robust development of childhood immunity, not just to the specific infection but in a non-specific fashion in order to prevent allergy. More understanding of the critical mechanisms is needed for this, but clearly TLR agonists are a likely component of such approaches – these include CpG DNA (TLR9), Pam3Cys (TLR1/2), monophosphoryl lipid A (TLR4) and poly I:C (TLR3), agonists, which activate DC and enhance IL-12 secretion. Some of these have been tested in humans, and do not induce the side-effects associated with traditional adjuvants such as complete Freund's adjuvant. Inclusion of this new generation of molecular adjuvants in vaccines might not only enhance the effectiveness of the vaccines themselves, but have a non-specific suppressive effect on allergic sensitization.

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