

T Cell Cytokine Production in Childhood Asthma

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Abstract: A recent asthma audit demonstrated that, 1.4 million children (one in eight) in the UK today are receiving treatment for their asthma, and that this figure has increased six-fold in the last 25 years. The chronicity of asthma has been associated with cytokine-mediated inflammation, in particular from T helper 1 (Th1) and T helper 2 (Th2) cells. Over the past 10 years, a number of studies have tried to unravel the role of T cell cytokines in childhood asthma, as there may well be differences between childhood and adult asthma. Research in this area has been hampered by ethical and practical difficulties. Although a number of studies use whole or separated blood for investigative purposes, the use of T cells obtained from the airways, obtained by bronchoalveolar lavage (BAL), or sputum may be more representative of *in vivo* cytokine expression. A large body of evidence suggests, that Th2 cytokines are upregulated in paediatric asthma. However, a number of more recent publications propose that Th1 cytokines may also have inflammatory effects in childhood asthma. In particular, IFN γ 's role in childhood asthma has been clearly documented in studies, from both blood and BAL. Such reports have questioned the concept of the Th1/Th2 imbalance in such childhood asthma. This review will discuss the current findings on cytokine production from T cells in children with atopic asthma, and attempt to unravel the cytokine complexities in childhood asthma.

Keywords: Childhood asthma, cytokines, T lymphocytes, T-helper 1 cells, T-helper 2 cells.

INTRODUCTION

Airway inflammation in asthma is a consequence of the infiltration of T lymphocytes, eosinophils, macrophages and mast cells. The finding of large numbers of infiltrating lymphocytes in the airways of asthmatic patients [1], led to a plethora of research into the role of T cells in the pathogenesis of asthma, including the work that provided evidence, that lymphocyte activation contributes to the development of eosinophil recruitment, hyperresponsiveness and symptoms of asthma [2]. Similar observations were noted in childhood asthma, where activated T cells correlated with eosinophilia and disease severity [3]. In 1986, Mosmann *et al.* described two types of T helper (Th) clones in mice, Th1 and Th2 cells [4], which were distinguishable by the profile of cytokine production. Effector Th1 cells are involved in delayed-type hypersensitivity through their production of IFN γ and IL-2, whereas Th2 cells secrete IL-4, IL-9, IL-10 and IL-13, and promote antibody-mediated humoral immune responses. Several studies suggest, that polarized human Th1 and Th2 cells produce a relatively similar pattern of cytokines, compared to their mouse analogs (Table I) [5, 6], and numerous immunological diseases in humans have been associated with a Th1/Th2 cytokine imbalance (reviewed in [7]). Originally most investigations on the activity of T cell cytokines in asthma were conducted in adults, due to the ethical limitations surrounding research in children, and it was believed that the pathophysiology of adult asthma was similar to that in paediatric asthma [8].

Table I. Cytokine Profiles of Human CD4 T Cell Subsets

Th0	Th1	Th2
IL-2	IL-2	<i>IL-2</i>
IL-3	IL-3	IL-3
IL-4		IL-4
IL-5		IL-5
IL-6		IL-6
IL-9		IL-9
IL-10		IL-10
IL-13		IL-13
IFN γ	IFN γ	
TNF	TNF	TNF
TNF	TNF	
GM-CSF	GM-CSF	<i>GM-CSF</i>

Upon activation, naïve T helper cells become an uncommitted cell termed Th0. These Th0 cells secrete multiple varieties of cytokines, and in response to stimulation, differentiate into either Th1 or Th2 cells, distinguishable by their cytokine repertoire.

*Cytokines highlighted in italics represent very low levels (if present at all)

METHODS USED TO INVESTIGATE CYTOKINE EXPRESSION IN CHILDREN WITH ASTHMA

Early evidence of T cell cytokine involvement in children with asthma came largely from indirect studies, such as measurements of cytokine concentrations in peripheral blood, rather than identifying specific, cytokine secreting cells. This led to the isolation of peripheral blood

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mononuclear cells and measurement of T cell cytokines in the supernatant from stimulated cultures. However, disadvantages of the latter method include the inability to simultaneously identify the T cell subset from which the cytokine(s), are secreted and the uncertainty of any contaminating cell types, which may also secrete the same cytokine. Some studies have used a bronchoscopic procedure to measure T helper cytokines in BAL supernatant from children with varying asthma phenotypes [9-11]. However, flexible bronchoscopy and bronchoalveolar lavage (BAL) is normally only possible in children when there is a clear medical indication to undertake such a procedure. The advent of a non-bronchoscopic lavage method for children [12], has provided important information on lung inflammation in children, including T cell cytokine production at the site of inflammation [13]. The establishment of a specific intracellular flow cytometric method [14], employing either whole/separated and/or cloned blood or BAL T cells, has greatly furthered our understanding of the regulation of T cell-derived cytokines in asthma. This method allows the identification of different T cell subtypes, based on the cytokines secreted from a heterogeneous population of cells following activation. Evaluation of cytokine production has been based predominantly on activation of cells with non-physiological polyclonal stimuli, such as phorbol 12-myristate 13-acetate (PMA) and ionophore, phytohemagglutinin (PHA), or anti-CD3 and anti-CD28, and therefore may not be as relevant as stimulation with antigen-bearing antigen presenting cells (APCs). Although the use of induced sputum, using both normal and hypertonic saline, in children with asthma has been documented as being a noninvasive, safe and successful method [15, 16], to date there are only two published reports using induced sputum as a tool for examining cytokine expression in children with asthma [17, 18]. In general, it has been reported that children should be 6 years or older for successful performance of induced sputum techniques [19]. However, a recent study used a two-way tube with a trap for obtaining sputum from babies as young as 1 month old [20]. It seems likely, that induced sputum may become a particularly useful tool for the analysis of cellular and biochemical markers in young children with asthma. Because direct access to the airways is not always possible, an alternative and relatively safe and easy method such as nasal lavage may be employed to investigate cytokine expression in children with asthma [21]. Finally, the use of exhaled breath condensate has recently emerged as a possible new and non-invasive method in paediatric respiratory studies [22], and has been used to study the levels of IL-4 and IFN γ in normal children, and those with asthma [23]. However, the technique still awaits standardisation before its usefulness in measuring airway inflammation can be fully validated.

Recent advances in methods to study T cells in airways diseases, have greatly aided the investigation into how the production of T cell cytokines, chemokines and other mediators regulate airway inflammation in children. This review will focus on reports that have specifically investigated cytokine production from T cells in blood, BAL or sputum from children with asthma, excluding reports on other atopic diseases such as dermatitis. It is important to keep in mind, that inflammatory cytokines may also derive from non-T cell sources, such as mast cells,

eosinophils, basophils, macrophages/monocytes and epithelial cells [24-27].

Th1/Th2 Cells in Childhood Asthma

Over the past decade, a series of *in vivo* and *in vitro* studies in adults showing an increase in Th2 cytokine expression, and a concomitant downregulation of secreted Th1 cytokines in allergic diseases, established the concept behind the so-called 'Th2 hypothesis' for asthma. It was therefore believed, that this held true for paediatric asthma. One of the first studies measuring cytokine concentrations in children with allergic disease, revealed a significant increase in the level of IL-4 in serum from atopic asthmatics compared to controls, which correlated with IgE [28]. Other subsequent studies in serum and blood supported the importance of IL-4 in childhood asthma [29, 30]. The differences in IL-4 levels are likely to be dependent on disease severity, since analysis of children with mild/moderate asthma revealed no differences in IL-4 concentrations compared to normal controls [31]. Low levels of IL-4 in BAL from children means it is particularly difficult to measure, despite 20-fold concentration of BAL fluid [10, 32]. Although these and other reports established IL-4's involvement in atopic asthma, interest soon focussed on whether the Th1/Th2 imbalance associated with asthma was a function of atopy per se. Using phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMCs) from atopic and non-atopic children with asthma, including normal controls, Tang *et al.* were the first to report, that an increase in IL-4 and a decrease in IFN γ expression was associated with atopy, rather than the presence of asthma [33]. Although this study involved relatively small numbers of subjects, and technically the method of cell separation may have an effect on cellular function, it was clear from this study that an imbalance in T cell cytokines is associated with atopic asthma in children. A similar, but more elaborate study using asymptomatic children and including all phenotypes of atopic and asthmatic children was recently undertaken, to further investigate the relationship between cytokine imbalance and atopy/asthma [34]. This study demonstrated that an increased IL-4 was associated with both atopy and asthma, and showed an increase in IFN γ in non-atopic asthma, with a corresponding decrease in atopic asthma, suggesting both a Th1 and Th2 cytokine increase in non-atopic asthma and a Th1/Th2 imbalance in children with atopic asthma.

It is clear from a number of studies, that both disease severity and age are among the most important variables related to cytokine expression [35, 36]. Using PHA-stimulated PMBCs, Nurse *et al.* showed that severe asthmatic children had significantly lower IFN γ concentrations, whereas moderate asthmatics had lower but not significantly different IFN γ levels compared to normal controls, suggesting a relationship between IFN γ levels and asthma severity. Surprisingly, this study failed to demonstrate any changes in IL-4, IL-5, TNF- α and GM-CSF levels between any of the patient groups and controls. This could be due to the fact, that the time of maximum cytokine release varies between cytokines, and this study only measured cytokines at 48 h [37]. A similar relationship for IFN γ and lack of associations for IL-4 and IL-5 have

been reported in paucisymptomatic and symptomatic children with difficult asthma [11]. *In situ* hybridisation experiments demonstrated a correlation of IL-5 mRNA expression from PBMCs with asthma severity, with no such correlations in mRNA for IL-2, IL-4, or GM-CSF, despite the increase in all cytokines in atopic asthmatics compared to control subjects [38].

The only study that has investigated T cell cytokines in sputum from children, found that IL-5 and GM-CSF concentrations are similar in induced sputum supernatants from children with current or stable asthma compared to control subjects, and that treatment with inhaled glucocorticoids had no effect on the concentration of IL-5 or GM-CSF [17]. In support of IL-5's relationship with asthma severity, serum studies showed that there was a greater proportion of detectable IL-5 levels in acute asthma, than in mild asthma or in controls, and that levels may be associated with atopic status [39]. However, conflicting findings suggest no correlation between IL-5 and asthma severity [40], although this study failed to exclude those subjects taking inhaled corticosteroid, despite confirmation both from the study itself and from other authors, that steroids have an inhibitory effect on cytokine production [13, 41, 42]. Increased levels of IL-5 have also been reported in BAL supernatant from children with acute asthma, and in PMBCs from atopic asthmatic children following stimulation with cat and dog allergen [10, 43]. Whether IL-5 is a useful marker in monitoring disease activity in children with asthma has yet to be fully established, however, elevated production of this cytokine appears to be related to asthma.

It is also worth noting, that T cell cytokine production is stimulus dependent, whereby T cells can make both IL-4 and IFN γ [44]. Although there are many reports on cytokine expression in blood using the non-specific mitogen, PHA as a stimulant, other studies have used the T cell mitogen, Concanavalin A (Con A), and superantigens, such as Staphylococcal enterotoxin B (SEB) or a number of allergens. Con A-stimulated PBMCs showed a decrease in IFN γ in atopic asthmatics compared to controls, although this study did not include asthmatic subjects without atopy [31]. The polyclonal stimulus, SEB, has been shown to cause a reduction in IFN γ , and an increase in IL-4 and IL-5 expression in atopic children compared with non-atopics [45]. Several studies confirmed the allergen-specific regulation of cytokine production in PBMCs. House dust mite (HDM) appears to cause upregulation of Th2 cytokines (IL-4, IL-5, IL-9, IL-10 and IL-13) in atopic children including atopic asthmatics, and conflicting results have been reported on HDM's effect on IFN γ expression [45-48]. *Candida albicans* (CA) has been shown to activate the secretion of Th1 cells in atopic subjects [47]. Therefore, it may be important to examine whether a non-specific stimulus is equivalent to physiological activation for the purposes of the study.

Methodological factors greatly influence results from studies on cytokine expression, and studies not investigating the expression of cytokines from single cells possess many flaws. One well established flow cytometric technique for studying intracellular cytokine production at the single cell level [14] has now been widely adopted for investigations

into childhood asthma. The first study to use such a technique in children with asthma was carried out in 1998 by Krug *et al.* who confirmed the idea that IL-4 and IL-5 play an important role in mild asthma and suggested that IL-4 from CD3⁺ cells is associated with atopy rather than asthma [35]. The association between IL-5 with the degree of hyperresponsiveness in this study, supported previous findings regarding the influence of disease severity on IL-5 expression. Although this study did not investigate the frequencies of IFN γ cells, results from 2 studies, using the same method with PBMCs, suggest a decrease in IFN γ cells, associated with atopic asthmatics compared to normals, which is confined to the CD4 population of cells [49, 50]. The intracellular study by Fu and colleagues showed an increase in IL-4⁺/CD4⁺ and /CD8⁺ cells, with a corresponding decrease in IFN γ subsets in asthmatic subjects compared to control subjects, although CD8⁺ cells did not show a significant decrease in IFN γ [51]. However, in this study a broad age range of subjects (5 to 22 y) were studied, where previous work has highlighted the importance of age-matched controls when measuring cytokine expression in children [36]. Also, the weakness in the power of this study can be partly explained by the extremely low numbers of controls (n=4), and wide variation in results.

Although the importance of certain Th2 cytokines, in particular IL-4 and IL-5, in children with atopic asthma have been well documented, recent data in both adults and children has challenged the concept of a Th1/Th2 imbalance, and has put forth some evidence to suggest a Th1 profile in asthma. Marguet *et al.* showed in a study investigating the role of sICAM-1 and IFN γ in children with airway disease, that levels of IFN γ in BAL supernatants from children with established asthma were significantly elevated, compared to those with chronic cough and a trend for an increase in those with asthma compared to recurrent wheeze. Although the median concentration of IFN γ in control subjects was lower compared to asthmatic subjects, statistical significance was not reached [9]. More solid evidence for IFN γ 's possible proinflammatory activities in childhood asthma came from findings, using an intracellular flow cytometric assay employing BAL T cells [13]. The percentages of IFN γ /CD3⁺ cells were significantly increased in atopic asthmatics compared with aged-matched atopic non-asthmatic subjects, or non-asthmatic non-atopic control subjects, with a predominance of IFN γ compared to IL-2 or IL-4-producing T cells. Both LPS-stimulated whole blood and purified blood T cells stimulated with Ca-ionophore and 12-O-tetradecanoylphorbol 13-acetate (TPA), showed reduced expression of IL-10 mRNA and protein production in allergic asthmatics compared to controls [52, 53]. Since IL-10 has an inhibitory effect on the production of IFN γ [54], this could explain the observed increase in IFN γ expression in the aforementioned studies.

Little work has been documented on the role of GM-CSF in childhood asthma, possibly due to its expression by both Th1 and Th2 subsets. However, it appears to be more strongly produced by Th1 cells, compared to Th2 cells. One study reports an increase in GM-CSF protein production from PBMCs, in subjects with intermittent asthma and those with moderate persistent asthma compared to control subjects, which correlated with the number of exacerbations [55]. Consistent with these findings, Gemou-Engsaeth and

colleagues provided evidence for an increase in PBMCs, expressing GM-CSF mRNA in asthmatics compared to controls [38].

Is it possible that similar to findings in adults, IFN levels are related to features of asthma, including time of last exacerbation? Although 2 reports suggest a decrease in IFN with severity of disease, neither investigates which population of T cells are responsible for such a decrease [11, 37]. To date, no studies in children have directly identified different subtypes of T cells, with respect to intracellular cytokine production. This however, may provide information on the specific cells responsible for the observed changes in cytokine production. Indeed, a recent adult study suggests that asthma is associated with an increase in IFN from CD8⁺ cells, whereas the atopic status of a subject is related to a decrease in the ability of CD4⁺ cell to produce IFN γ [56]. This notion may also apply to childhood asthma, since Akpinarli *et al.* showed, although not significant, that an increase in IFN γ may be a function of asthma alone [34], and studies mentioned above support a decreased IFN γ in atopic asthmatics, secreted from CD4 cells [49, 50]. Furthermore in the study by Brown *et al.* [13], the group of non-asthmatic atopic subjects had a significantly lower frequency of IFN γ + T cells than the atopic asthmatic group of children, and therefore, it may well be the case that decreased IFN γ and increased IL-4 levels in children are associated with atopic disease.

CONCLUSION

It must be remembered that, although all of the studies discussed in this review investigated cytokines secreted mainly from T cells, as mentioned previously, it is possible that some may be produced from other cell types. Therefore, in order to correctly investigate those cytokines solely secreted by T cells, either separated and/or cloned cells should be used, or else the cytokine and T cell surface markers should be identified simultaneously. Although intracellular cytokine analyses have many advantages over other methods, they do not provide exact quantification of cytokines. It, therefore, may be useful to combine the use of a single cell assay with a quantitative measurement of secreted protein. Although recent European Respiratory Society Task Force concluded, that bronchoalveolar lavage from children "is likely to represent a useful tool in wheezing infants by documenting the patterns of inflammatory marker expressions at various stages of the disease." [57], no studies have directly investigated whether T cells from sputum, BAL, or peripheral blood directly represent infiltrating T cells into the submucosa, since safety and ethical concerns have limited the use of biopsies in children with asthma. Future studies involving biopsies from children undergoing surgery may further our understanding of the relevance of indirect methods such as BAL, sputum or blood to reflect the *in vivo* status.

Although asthma in both children and adults may have similar underlying mechanisms, it is evident that the onset, age, disease activity/severity, medication, viral infections and other methodological factors, such as type of stimulant and/or allergen and period of stimulus may also account for different outcomes in studies investigating T cell cytokine

involvement in allergic diseases in childhood. Furthermore, evidence suggests that a shift from a Th2 to a Th1 response occurs after both allergen and PHA-stimulated T cell clones [58]. Although infants and young children demonstrate a reduced capacity for T cell responses compared to older children and adults [59, 60], little is known about specific cytokine production, during different stages of immune development in early childhood, and is an important area for future work.

While the Th1/Th2 paradigm provided a simplistic model for initially describing T cell involvement in asthma, it certainly does not fully support the complexities of this disease. Despite studies showing indirectly, that a Th1 cytokine shift in patients with *Mycobacterium tuberculosis* may have a protective effect against the incidence of asthma [61], and that the production of Th1 cytokines in infancy may protect from development of atopy in childhood [62], evidence suggests that pure Th1 and Th2 subpopulations may not exist in humans [58]. Also IFN γ possesses a number of proinflammatory activities, including the upregulation of ICAM-1 [9] and the receptor for TNF α [63], and therefore it is likely, that under certain circumstances IFN γ may exert its proinflammatory activities and potentiate the inflammatory response in children with asthma. It seems that certain Th1 and Th2 cytokines are indeed elevated in asthma phenotypes of children, however, their effects and the mechanisms by which they exert such effects in childhood asthma are largely unknown. There is by no means a complete understanding of T cell cytokine responses in childhood asthma, and unravelling such complexities is a crucial part of understanding the disease process.

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