

T cells in Allergy and Asthma

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The dependence of allergic immune responses on thymus-derived lymphocytes has been known since the late 1960s, shortly after the original descriptions of IgE. The detailed mechanisms by which T cells regulate B cell isotype switching have become clearer over the last ten years and it is now accepted that this involves a two signal process (Figure 1). IgE is not made as part of the primary immune response. The primary immune response has two distinct roles: the production of antibodies (primarily IgM but also some IgG), and the induction of memory B cells. In order to make IgE, a memory B cell that recognises part of an allergenic molecule, will pick up the molecule by means of its surface immunoglobulin (IgD). The allergen is then internalised and processed by the B cell so that short peptide fragments can then be displayed on the B cell surface in the context of MHC class II molecules. In the regional lymph node, the B cell offers this allergen-MHC complex to adjacent T cells and, if it finds a T cell that recognises the peptide fragment, a precisely controlled sequence of events will follow. T cells which are predisposed to assist the production of IgE preferentially produce IL-4 rather than interferon gamma when they are stimulated through their antigen receptors. The T cell will thus produce IL-4 which feeds back positively on the T cell as an autocrine growth factor. This IL-4 also induces germline transcription of the C epsilon portion of IgE and stimulates the B cell to display the B7 molecule on its surface. B7 is the counter ligand for the T cell CD28 molecule. When the CD28 is triggered, the T cell displays the CD40 ligand which provides a contact signal to the B cell facilitating isotype switching. With the extra

IL-4 released from the T cell and the contact signal delivered through CD40, the B cell then undergoes an irreversible switch that brings the gene fragment encoding the antigen combining site up next to the C epsilon gene and allows transcription of the mature IgE transcript¹.

Recognising the importance of T cells in the regulation of allergy, and the inflammatory nature of allergic asthma, investigators started to address the possible role of T cells in asthma during the early 1980s. To start with, the focus was on a possible defect in T cell suppression which might allow the allergic inflammatory process to proceed. Thus following allergen inhalation, subjects who showed single early responses had more CD8⁺ cells in their airways than those who showed dual (early and late phase) responses². It rapidly became clear that T cell infiltration and activation were features of all grades of asthma and that activation (as shown by CD25 expression) was associated with symptomatic asthma³. Activated T cells were identified in the peripheral blood during exacerbations of asthma⁴ but under stable conditions the T cell changes were largely confined to the airways. Treatment with inhaled corticosteroids reduced both the numbers and activation status of the airway T cells⁵.

An appreciation of the possible function of T cells has followed from the rapid expansion of our understanding of cytokines and chemokines. In the context of allergy and asthma we have focused on IL-4 because of its role in IgE switching; IL-5 plays a critical role in the maturation, survival and activation of eosinophils; IL-3 and IL-10 have been implicated in mast cell genesis; IL-13 has many of the same properties as IL-4; and finally

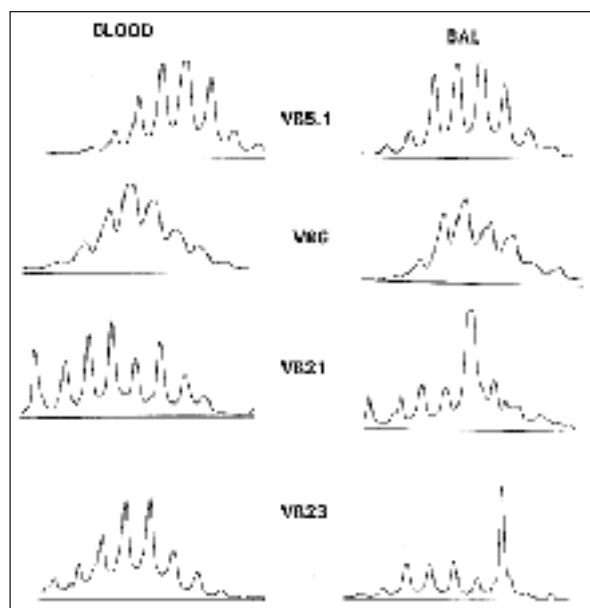


Fig. 1. Two signal model of B-cell isotype switching. On first exposure to any antigen, B-cells can make IgM without requiring assistance from T-cells. Some cells with the same specificity will be maintained as memory B cells, to provide a more rapid response in the event of re-exposure. Memory T-cells recognising fragments of the antigen may also develop. To make IgE, these two cell types must cooperate: in this model, a memory B cell recognises the allergen via its surface immunoglobulin, thereby trapping it. The antigen is ingested and then digested into short fragments, which are then presented on the B-cell surface in the groove of MHC class II molecules. These antigen-MHC complexes are shown to passing T cells within the regional lymph node or spleen. When a CD4⁺ T cell that recognises the MHC-antigen fragment complex meets the antigen-presenting B cell, the T-cell antigen receptor is activated, sending internal signals to the cell to produce autocrine growth factors (e.g. IL-2) as well as activating adhesion mechanisms to lock the two cells together. If the T cells is pre-programmed to facilitate IgE switching (i.e. a Th2 cell), then IL-4 is also produced: this has autocrine effects as well as inducing transcription of the C ϵ immunoglobulin gene in the B-cell (germ-line transcripts). Upon activation, the T-cell expresses CD40 ligand, which binds to CD40 on the B-cell surface and provides the second signal needed to switch the B-cell over to IgE production. Mean while, the B-cell displays the B7 counter-receptor which signals via CD28 on the T-cell to augment IL-4 production. Under these twin influences the B-cell germline DNA is rearranged, with excision of the intervening immunoglobulin DNA and the production of mature IgE transcripts.

interferon gamma is an important pro-inflammatory cytokine involved in classical delayed type hypersensitivity but also opposes the actions of IL-4⁶⁻¹⁰. Attempts to demonstrate the presence of

cytokine protein in human tissue samples have provided some unexpected insights. The overwhelming majority of cell staining for IL-4 protein turn out to be mast cells rather than T cells¹¹. Mast cells also contain significant amounts of IL-5 and IL-6 protein and it has now been shown that these proteins are synthesised by the mast cell and released upon activations¹².

These observations have encouraged the view that mast-cell-derived cytokines may create a pro-allergic milieu in which T-cell-allergen interaction is skewed towards the Th2 phenotype. In contrast, T cells do not seem to store detectable amounts of cytokine protein but manufacture cytokines de novo and release them rapidly. To assess the cytokine activity of T lymphocytes it is therefore necessary to use methods which detect mRNA such as in situ hybridisation or RT-PCR. Although the genes for IL-4 and IL-5 are located very close together on chromosome 5q₃₁₋₃₃, recent evidence indicates that their expression is not coordinately regulated¹³. Expression of mRNA for IL-5 is a feature of both allergic and non-allergic asthma¹⁴, and there is a close correlation between T-cell activation and serum IL-5 concentrations in various forms of asthma¹⁵.

A number of interesting insights into the relative contributions of IL-4 and IL-5 to asthma have emerged in studies of occupational asthma to isocyanates. Isocyanate asthma is a particularly interesting model which bridges the gap between atopic asthma, where exposure to the inciting allergen is lifelong, and intrinsic asthma where as far as we can tell, extrinsic allergens and IgE are not involved. Typically, isocyanate asthma develops after a latent period in which exposure occurs without symptoms. IgE antibodies cannot be directly implicated but the histology of the bronchial mucosa is more or less identical to that seen in other forms of asthma^{16, 17}. In biopsies taken from patients with chronic isocyanate asthma, there was upregulation of mRNA for IL-5 but not for IL-4, while when such patients underwent bronchial challenge, there was upregulation of both cytokines¹⁸. To add further confusion, T-cell clones derived from patients challenged with toluene diisocyanate were found to be predominantly CD8⁺ and secreted IL-5 but little or no IL-4¹⁹.

Taken together, this cytokine evidence indicates that the expression of asthma is closely linked to

upregulation of IL-5, while upregulation of IL-4 is perhaps more closely related to IgE and allergic sensitisation, but not directly to the clinical syndrome of asthma²⁰.

Experimental studies of the role of IL-4 and IL-5 in allergic disease have been hampered by the lack of a really good animal or *in vitro* model of asthma. There are many models of allergic sensitisation, and it is relatively easy to demonstrate that sensitised animals will develop airways eosinophilia and increased airways resistance, but these are essentially models of acute allergen exposure rather than models of the disease we recognise as asthma. In particular, almost none of the models give any long-lasting airways inflammation or anything similar to the airways remodelling and collagen deposition that occur in chronic human asthma. With these caveats, animal models have shown that T-cells are the main source of IL-5, but not IL-4, in the lungs of antigen-challenged mice²¹. Detailed discussion of these studies can be found elsewhere²², but it has been consistently shown that monoclonal antibodies directed against IL-5 can prevent airways hyperresponsiveness in several species including mice, guinea pigs and monkeys²³⁻²⁵.

In bronchial biopsies, increased expression of IL-5 mRNA is one of the hallmarks of asthma²⁶, but IL-4 mRNA is less diagnostic. Over the past 5 years we have conducted a series of studies addressing cytokine expression by BAL T cells. In baseline BAL samples, we have shown expression of IL-13, GM-CSF, TNF α and interferon gamma in unstimulated cells. A minority of samples show small amounts of IL-4 and IL-5. When these BAL T cells are stimulated with the polyclonal mitogen PHA, expression of IL-3, IL-4 and IL-5 increases. Twenty-four hours after local endobronchial allergen challenge, mRNA for IL-13 and GMCSF is readily detectable in unstimulated samples, but the level of expression of interferon gamma mRNA is reduced compared to baseline samples²⁷. Following PHA stimulation, these samples show marked upregulation of IL-4 and IL-3 with lesser amounts of mRNA for TNF α and interferon gamma compared to the ten minute PHA stimulated samples. To address this in more detail, we cloned BAL T cells by limiting dilution. Peripheral blood mononuclear cells (PBMC) were prepared by gradient centrifugation and adjusted to 10⁶

cells/ml. These were then irradiated and used as feeder cells for the cloning experiments. BAL T cells were plated out at 100, 30, 10, 3, 1 and 0.3 T cells per well in 96 well flat bottom microtitre plates with PHA 2 μ g/ml and 5 x 10⁴ feeder cells per well. The clones were fed with 50 u/ml IL-2 on day five and examined for growth on day fourteen. Wells which showed positive growth were selected for subculture, based on poissonian analysis which indicated a greater than 85% chance of monoclonality. The clones were expanded with weekly feeds of PHA and feeder cells. IL-2 50 u/ml was given every 3-4 days. After 6-8 weeks the clones were phenotyped by flow cytometry and cytokine profiles obtained by RT-PCR and ELISA.

The proportion of BAL T cells that could be cloned with PHA was about 1/50 for both allergen-challenged and saline-challenged airways. From the initial 17 panels of T cell clones, 72 T cell clones survived in culture for 6-8 weeks. 14 of these were obtained from baseline lavages, 17 from a lavage 4 hours after saline, 18 from sites lavaged 24 hours after allergen challenge and 23 from sites lavaged 24 hours after saline challenge. The clones were all CD3⁺, 50 were CD4⁺ and 22 were CD8⁺.

Cytokine analysis was performed by culturing aliquots of 5x10⁵ cells overnight with PHA 2 μ g/ml. After 16 hours, the supernatants were collected and then analysed by ELISA. The cells were harvested and their RNA was extracted with RNazol B. The polyadenylated mRNA was converted to cDNA by reverse transcriptase and then amplified by PCR with appropriate primer pairs. PCR products were visualised with ethidium bromide after gel electrophoresis. The clones derived from baseline lavages and the lavage 4 hours after challenge with saline showed strong expression of mRNA for IL-13. In addition, mRNA for TNF α , interferon gamma, IL-2 and GMCSF was also present (Table I). There were weaker signals for IL-4 and IL-13, while IL-5 mRNA was detectable in 7 out of 11 clones from the baseline lavage. There was no expression of IL-6 or IL-10 mRNA. Consistent with these mRNA profiles, IL-4 protein concentrations in the clones supernatants ranged from 1-64 pg/ml while IFN gamma concentrations were between 4 and 49 pg/ml. The cytokine profiles of clones derived 24 hours after

Table I. Cytokine mRNA and protein from T-cell clones derived from baseline lavage

Clone	CD4/8	TNF α	IFN- γ	GM-CSF	IL-2	IL-3	IL-4	IL-5	IL-6	IL-13	IL-4 (pg/ml)	IFN- γ (pg/ml)	IL-2 (pg/ml)
01	8	+	++	++	-	++	+	-	-	++	92	50	12
02	8	++	++	++	-	++	+	+	-	++	691	21	9
03	8	++	++	+	-	-	-	-	-	++	279	18	ns
05	8	++	++	+++	-	++	++	-	-	+++	<10	35	ns
06	8	++	++	++	-	++	++	+	-	+++	301	<5	17
07	8	++	++	+++	-	++	+	+	-	+++	616	56	ns
09	8	++	++	+++	-	++	-	+	-	++	439	41	16
10	8	++	++	+++	-	++	++	+	-	+++	63	18	16
11	4	++	++	++	-	++	-	-	-	+++	<10	<5	4
12	4	++	++	++	-	++	++	+	-	+++	761	51	24
14	4	++	++	++	-	++	++	+	-	+++	469	42	8

cytokine PCR bands compared to APRT control band which was assigned value of ++

cytokine protein measured by ELISA (cut-off limit for IFN- γ 5 pg/ml; for IL-4 10 pg/ml; for IL-2 6 pg/ml)

ns = no sample available

allergen challenge showed strong expression of mRNA for IL-3, IL-4, IL-5, IL-13, GM-CSF with no apparent expression of IL-2 or IL-10. TNF α , IFN gamma and IL-6 were weakly expressed. Clones from the saline site showed similar cytokine profiles to those obtained from the allergen challenge site (Table II). The supernatants from the clones derived 24 hours after challenge contained much higher amounts of IL-4 protein than

those from the baseline lavages. Some of the clones showed 10 times more IL-4 protein than the equivalent number of cells obtained in the initial polyclonal BAL samples. Although most clones that had strong mRNA signals for IL-4 also had large amounts of IL-4 in their supernatants, there was wide variation in the amount of IL-4 produced by clones with apparently similar amounts of mRNA. Interestingly, several of the T cell clones

Table II. Cytokine mRNA and protein from supernatants of T-cell clones derived 24 hrs after challenge with allergen or saline

Clone	CD4/8	TNF α	IFN- γ	GM-CSF	IL-2	IL-3	IL-4	IL-5	IL-6	IL-13	IL-4 (pg/ml)	IFN- γ (pg/ml)	IL-2 (pg/ml)
Allergen site													
17	nd	+	+	+++	-	+++	+++	++	+	+++	nd	nd	nd
20	8	+	+	+++	-	+++	+++	-	-	+++	1099	11	5
21	8	++	-	++	-	+++	+++	-	+	+++	1701	5	3
23	4	+	+	+++	-	+++	+++	++	+	+++	976	28	13
24	4	+	+	+++	-	++	+++	++	-	+++	24	18	19
26	8	+	+	++	-	+++	+++	+	-	+++	95	5	2
18	8	++	+	++	-	++	-	+	+	+++	979	35	ns
19	8	++	++	++	-	++	++	++	+++	+++	14	49	ns
22	4	-	-	++	-	++	+++	++	+++	+++	1205	5	ns
25	4	+	-	++	-	+++	+++	++	++	+++	773	28	ns
27	8	++	+	++	-	++	++	+	-	+++	147	5	ns
29	8	+	+	++	-	++	++	+	-	++	<10	34	ns
37	4	+	-	+	-	++	++	-	-	++	1057	39	ns
38	8	++	-	+	-	++	++	++	+	++	130	34	ns
Saline site													
30	4	+	+	+++	-	++	++	++	++	+++	34	25	26
32	4	+	+	++	-	+	+	+	-	+	<10	29	10
35	8	++	-	++	-	++	++	++	-	++	1122	36	ns
36	8	++	+	++	-	+++	+++	++	-	+++	455	5	ns
33	8	+	+	+++	-	+++	+++	+++	-	+++	122	38	20

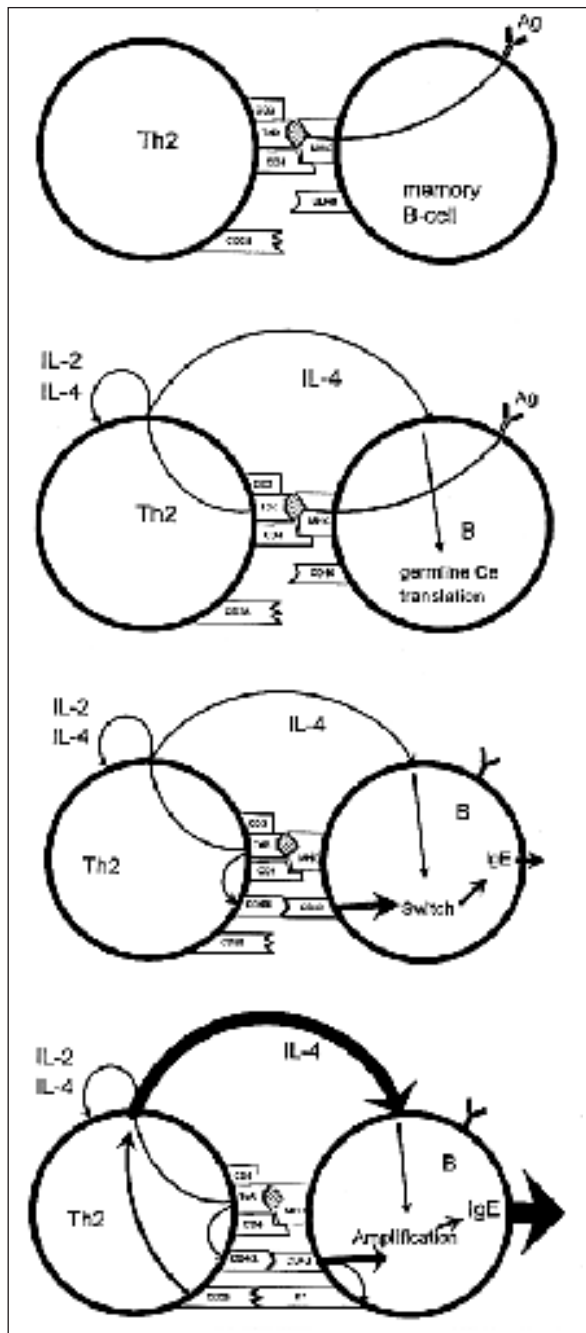


Fig. 2. BAL and peripheral blood T-cell receptor size profiles for individual TCR Vβ families. Blood and BAL T-cells were obtained from a patient with asthma. mRNA was extracted and a cDNA copy prepared by RT-PCR. Aliquots were then expanded with Vβ-family-specific primers and scanned by automated gene sequencer. Polyclonal profiles are shown for Vβ5.1 and Vβ6; clear clonal peaks can be seen in the BAL profiles for Vβ21 and Vβ23.

derived after allergen challenge were CD8⁺ and yet produced large amounts of IL-4 suggesting that these are cells of the “Tc2” subtype.

Recognising that T cell cloning can only assess those cells capable of proliferation (1 in 50) and also that the culture conditions may alter the functional phenotype, we have used intracytoplasmic cytokine staining techniques to look at cytokine profiles at the single cell level in fresh BAL samples²⁸. In this technique, T cells are stimulated by a combination of PMA and ionomycin to drive the translation of mRNA into protein, while at the same time, the Golgi apparatus is disrupted by the presence of monensin. This allows the cytokine protein to accumulate within the cell where it can be stained and demonstrated by flow cytometry. Somewhat unexpectedly, the principal difference between asthmatic and non-asthmatic BAL samples turned out to be an increased proportion of cells which produce IFN gamma in asthmatic as opposed to normal or atopic, non-asthmatic airways. Only a small proportion of T cells showed IL-4 protein by this method. A first sight this finding seems to conflict with the evidence that IL-4 and IL-5 are the main cytokines involved in asthmatic inflammation. However, it must be remembered that only a small proportion of T cells are likely to be involved in the response to allergens, and equally, IL-4 production is a relatively transient phenomenon so one would not expect to see a high proportion of cells producing IL-4 protein at any one time¹³. Moreover, production of interferon gamma could be seen as an appropriate response to allergic inflammation, helping to damp down the effects of IL-4. Equally, it has been shown that inhalation of interferon gamma is not helpful in trying to control asthma¹⁹ and it may be that the interferon gamma production is part of the pathogenesis of allergic inflammation.

One important consideration in interpreting the role of T cells in asthma has been to understand whether the expression of CD25 on airways T cells truly indicates that these cells are actively participating in the inflammatory response. In culture, CD25 expression is induced when T cells become activated and it was therefore assumed that the increased proportion of T cells expressing CD25 indicated active T cell involvement in asthma pathogenesis^{3,4}. However, more recently it has become clear that T cells can also express

CD25 if they are partially activated and then become "anergic" and unresponsive to further stimulation³⁰. When we compared the expression of CD25 with the production of IL-2 and IFN gamma in our BAL samples, we found that virtually none of the CD25+ cells were producing cytokines. This strongly suggests that the CD25+ T cell population in asthmatic airways is, in fact, anergic and is not participating actively in the inflammatory process.

This led us to start looking at T cell subpopulations in BAL in greater detail. We returned to our cDNA samples and looked at the clonality of T cell antigen receptor (TCR) V β gene usage. The TCR V β repertoire varies considerably between people and is dependant on the MHC type and on previous antigenic exposure³¹. The antigen combining site of the TCR is generated by random addition of nucleotides at the junction between the V, D and J segments of the TCR V β chain and the V-J junction of the TCR α chain. In order to be transcribed properly, the number of bases has to be divisible by 3 or else there will be a frame shift and a sterile transcript. It follows that if one looks at the length of TCR V β mRNA transcripts one would expect to see a normal distribution of TCR V β lengths within each family, assuming that the process that selects T cells is random. Where a single clone has been selected by antigen, there will be an excess of TCR V β transcripts of the length that corresponds to that particular T cell. We have analysed the TCR V β lengths for 25 different TCR V β families in a number of subjects and find that there are 3-5 clonal peaks in the BAL T cells from each subject (Fig. 2). Thus far, these peaks do not seem to be limited to certain families but increased numbers of subjects will be need to reach a definitive conclusion. Nevertheless, this approach does provide clear evidence of clonal expansion within the BAL compartment which is not mirrored in the peripheral blood. In further experiments, we have begun to show that these clonal populations have different cytokine profiles from the polyclonal background. As well as confirming the functional heterogeneity of T cells in asthma this approach has highlighted the importance of understanding the forces that drive the airway T cell repertoire and of avoiding over-interpretation of histological data.

CONCLUSIONS

The T lymphocyte which is uniquely placed to orchestrate the activities of the structural and mobile elements involved in the asthmatic inflammatory response. Its activities are focused by antigen recognition and mediated by the precise and coordinated release of cytokines which entrain other leukocytes and direct their functions. We still have much to learn about the role played by T cells in the airway and about the forces that shape the T cell repertoire. By understanding these better and identifying critical bottlenecks in the process, we may succeed in our ultimate goal of controlling and preventing asthma.

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