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Original article

Cytokine profiles of BAL T cells and T-cell clones obtained from human asthmatic airways after local allergen challenge

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Key words: asthma; cytokine; endobronchial challenge; T cell; T-cell cloning.

Background: This study assessed the heterogeneity of cytokine expression in asthma before and after local allergen challenge.

Methods: BAL T cells were obtained 10 min or 24 h after local endobronchial allergen challenge in atopic asthmatic subjects. T cells were cloned by direct limiting dilution. mRNA expression was assessed by RT-PCR, and cytokine protein production by ELISA.

Results: Unstimulated baseline BAL T cells expressed mRNA for IFN- γ , IL-13, and TNF- α . A minority of samples expressed IL-4 and IL-5, but no IL-3 mRNA was detected. PHA stimulation increased expression of IL-3, IL-4, and IL-5 mRNA in 4/6 samples. IL-13 and GM-CSF mRNA were found in BAL cells after allergen challenge, but expression of IFN- γ was reduced. Both IL-4 and IL-3 were strongly upregulated after PHA stimulation, while the expression of TNF- α and IFN- γ was reduced, compared to equivalent baseline samples. Seventeen panels of BAL T-cell clones were derived (average cloning efficiency 1/40 T cells). Seven panels survived to 8 weeks for analysis. Clones derived 4 h after saline challenge showed strong mRNA signals for IL-13, IL-4, and IFN- γ , whereas clones derived 24 h after allergen challenge expressed IL-13, GM-CSF, IL-3, IL-4, and often IL-5 (i.e., closer to the Th2 profile). There was considerable heterogeneity in the patterns of cytokine mRNA and protein production by different clones.

Conclusions: T cells from asthmatic airways produce IL-13, IFN- γ , and TNF- α , but after allergen challenge, type 2 cytokines are upregulated. mRNA and protein analysis provide complementary information on airways T-cell cytokine profiles.

Abbreviations: BAL: bronchoalveolar lavage; FEV₁: forced expiratory volume in 1 s; IFN: interferon; IL: interleukin; PBMC: peripheral blood mononuclear cells; PBS: phosphate-buffered saline; PC: cumulative provocative concentration producing 20%

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fall in FEV₁; PE: phycoerythrin; Per-CP: peridin chlorophyll-protein conjugate; PHA: phytohaemagglutinin; TNF: tumour necrosis factor.

It is now generally agreed that asthma is an inflammatory disorder in which a special form of chronic inflammation affects the airways. This inflammation is considered to be responsible for the physiologic disorder observed in clinical asthma. It is also now considered likely that T cells orchestrate the inflammatory response (14). In the bronchial mucosa of asthmatics, many T cells can be found both above and below the epithelial basement membrane. Most of these express CD4 and CD45RO; i.e., the memory T-helper phenotype (1, 2). T cells expressing markers of activation are increased in number in lavage (40) and in mucosal tissue biopsies (7), with a positive correlation between the extent of T-cell activation and the level of bronchial hyperresponsiveness (2). Increased numbers of activated T cells are found in the peripheral blood during acute episodes of asthma, and their numbers decrease as the acute episode resolves (5). Conversely, treatment with inhaled corticosteroids leads to a dramatic decrease in the mucosal content of mast cells and eosinophils in parallel with reduced expression of markers of T-cell activation (41). In asthmatic airways, local allergen challenge results within 4 h in the recruitment of T cells, neutrophils, and eosinophils into the bronchial epithelium and submucosa, in parallel with an upregulation of the endothelial adhesion molecules E-selectin and ICAM-1 (23). *In situ* hybridization studies have shown expression of mRNA encoding IL-5, IL-4, and GM-CSF in asthmatic mucosal biopsies and in BAL T cells, both in cross-sectional studies and after allergen challenge (10, 33). These observations suggest that the T cells present in asthmatic airways and those recruited during the late-phase reaction belong to the human homologue of the murine Th2 CD4⁺ T-cell subset (24, 33).

T cells are intimately involved in the generation of allergic responses: after the uptake and presentation of allergens by antigen-presenting cells, helper T (Th) cells become activated and produce an array of soluble messengers (cytokines) which stimulate surrounding cells. In the context of allergic inflammation, IL-4 and IL-13 promote the switching of B cells from IgM to IgE production (30) while IL-3, IL-5, and GM-CSF promote the development and activation of eosinophils (35). Moreover, IL-13 has recently been linked to several other pathologic features of asthma, including subepithelial fibrosis, mucus hypersecretion, and

eotaxin production (44). The importance of these cytokines has been emphasized by observations that genetic control of asthma may be linked to polymorphisms in the region of chromosome 5q31-q33, the region which includes the IL-3, IL-4, IL-5, IL-9, IL-13, and GM-CSF gene cluster (29). Further interpretation of this putative role has been hampered by difficulties in obtaining sufficient cells to allow accurate characterization of their specificity and function. Recent advances in T-cell culture techniques have made it possible to clone individual T cells and assess their functional phenotype. This approach has demonstrated increased proportions of Th2-like cells from the blood of atopic individuals (38) and from the conjunctiva of patients with vernal conjunctivitis (22). T cells have also been cloned from mucosal biopsies obtained after challenge with pollen or toluene diisocyanate (21, 28). In this study, we have studied the cytokine profiles of T cells from asthmatic airways before and after allergen challenge, and we have then cloned T cells from BAL samples in order to address functional heterogeneity among the T cells present in the lavage samples.

Material and methods

Asthmatic subjects were recruited by advertising for volunteers (Table 1). At the time of enrolment, all subjects had stable pulmonary function with a forced expiratory volume in 1 s (FEV₁) greater than 89% of that predicted for their age and height (mean $\pm$ SEM 110.1 $\pm$ 3.6% predicted). They all had asthma as defined by the American Thoracic Society criteria with hyperreactive airways to inhaled histamine, with a geometric mean provocative concentration of agonist required to reduce FEV₁ from baseline by 20% (PC₂₀) of 1.25 mg/ml (range 0.30–4.67 mg/ml). The subjects were all atopic as defined by a skin wheal response of $\geq$ 3 mm to *Dermatophagoides pteronyssinus* and/or mixed grass pollen extracts (Hollister Stier, Spokane, WA, USA). None of the subjects had experienced an upper respiratory tract infection within 6 weeks of investigation. None had been treated with oral corticosteroids, sodium cromoglycate, or theophylline for at least 6 weeks before their participation in this study. Four subjects were receiving inhaled steroids (beclomethasone dipropionate 200–1000 μ g/day). Inhaled steroids were stopped 48 h before challenge and bronchoscopy. The study was approved by the Southampton Hospitals and University Ethics Committee, and all subjects gave their written informed consent.

Table 1. Clinical details of subjects studied

Subject	code	Age (years)	Sex	FEV ₁ (% predicted)	PC ₂₀	Inhaled Steroid	Allergen concentration
JD	1	23	F	103.4	1.19	BC200	DP10 ⁻⁵
AH	2	35	M	111.7	3.08	BC1000	DP10 ⁻⁵
CL	3	20	F	122.7	–*	BC400	GP10 ⁻⁵
DT	4	29	F	99.0	0.3	BC400	DP10 ⁻⁴
LB	5	19	F	103.7	4.67	–	DP10 ⁻⁴
AL	6	21	F	131.4	2.21	–	DP10 ⁻⁵
HB	7	23	F	119.6	0.61	–	DP10 ⁻⁵
ST	8	27	M	108.1	3.73	–	DP10 ⁻⁴
DF	9	37	M	116	0.41	–	DP10 ⁻⁵
LH	10	27	M	89.0	3.64	–	DP10 ⁻⁴
IF	11	20	F	107.0	2.50	–	DP10 ⁻⁵
Mean		25.6		110.1			
SEM		1.8		3.6			
Geom. mean				1.25			

DP: *Dermatophagoides pteronyssinus* (diluted from stock solution 30 000 AU/ml); GP: mixed grass pollen (diluted from stock solution 1:20 w/v); BC: beclomethasone dipropionate (µg/day).
 *Histamine reactivity could not be measured in patient CL because she had used salmeterol inhaler before attending for assessment.

Study design

The allergen extract used for local bronchial challenge was that which produced the larger wheal response on skin prick testing. In each subject, a skin wheal dose response series was undertaken with 10-fold dilutions of the chosen allergen. The concentration selected for the local bronchial challenge was 10% of the highest dilution of allergen that evoked a wheal diameter of 3 mm. Bronchoscopy was performed by a standard technique, conforming to published guidelines (27). Briefly, subjects were premedicated with nebulized salbutamol (2.5 mg), intravenous atropine (0.6 mg), and midazolam (0–8 mg). Topical upper airway anaesthesia was achieved with 4% lignocaine spray. Supplementary oxygen was administered by nasal cannulae, while oxygen saturation and pulse rate were monitored throughout. The fiberoptic bronchoscope (Olympus BF-XT20 or BF-IT20) was introduced by the oral route, and additional lignocaine (2%) given as required to achieve lower airway anaesthesia. The bronchoscope was wedged in the anterior segment of the right upper lobe and 20 ml prewarmed, pyrogen-free isotonic saline was injected via the suction channel. The bronchoscope was resited in the medial segment of the middle lobe, and 20 ml prewarmed allergen solution was injected. The bronchoscope was then withdrawn to the right main bronchus and any excess fluid aspirated. The two challenged segments were observed for

10 min to confirm constriction of the allergen-challenged airway. BAL was performed with 6×20 ml aliquots of prewarmed isotonic saline, initially in the saline-challenged segment and then in the allergen-challenged segment. The bronchoscope was then withdrawn and the subject allowed to recover. Twenty-four hours later, bronchoscopy was repeated, with the same premedication. After achieving local anaesthesia, BAL was performed with 6×20 ml aliquots of prewarmed isotonic saline; again, initially in the saline-challenged segment and then in the allergen-challenged segment. In one subject (D.F.), a single lavage was performed 4 h after saline challenge (no allergen was used in this subject).

BAL T-cell cultures

The BAL cells were passed through a 100-µm mesh polypropylene filter (Cellstrainer, Falcon), washed, and enriched for T cells by adherence to plastic Petri dishes (60 min at 37°C). The nonadherent cells were enriched further by magnetic separation after allowing any remaining phagocytes to ingest sheep-anti-mouse-Ig-coated magnetic beads (Dynabeads, Dynal, Liverpool, UK) (incubation for 45 min at 37°C followed by immunomagnetic separation). The purity of T cells obtained was 75–95%. The main contaminating cells were small, nonadherent macrophages. Cell suspensions were prepared in RPMI-1640 medium

(Gibco, Paisley, UK), which was supplemented with L-glutamine 2 mM, sodium pyruvate 1 mM, penicillin 100 U/ml, streptomycin 100 µg/ml, amphotericin 0.5 µg/ml, and 5% normal human AB serum. Enriched BAL T cells were cultured overnight with either medium alone or PHA 2 µg/ml. The cells were collected and total RNA was extracted for RT-PCR. The supernatants were frozen for later estimation of IL-4 and IFN- γ by ELISA.

T-cell cloning

Peripheral blood mononuclear cells (PBMC) were prepared by Ficoll-Hypaque gradient centrifugation of heparinized venous blood. After washing, the interface cells were counted and adjusted to 10^6 cells/ml. To develop T-cell clones, enriched BAL T cells were plated out at 100, 30, 10, 3, 1, and 0.3 T cells per well in 96-well, flat-bottomed microtitre plates with PHA 2 µg/ml and irradiated autologous PBMC at 5×10^4 /well. Surplus PBMC to be used as feeder cells were resuspended in supplemented medium containing 7.5% dimethylsulfoxide and 10% foetal calf serum, and frozen down in liquid nitrogen until required. Feeder cells were irradiated (3000 rad) with a caesium source immediately before use. The clones were fed with 50 U/ml IL-2 on day 5 and examined for positive growth on day 14. Wells selected for subculture were those in which Poissonian statistics indicated a probability of >85% of monoclonality. These positive wells were expanded into 48- and 24-well plates with weekly feeds of PHA and irradiated PBMC. 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 were obtained by RT-PCR and ELISA.

FACS phenotyping of T-cell clones

T-cell clones were phenotyped with FITC-conjugated antibodies to CD4 and CD19, PE-conjugated antibodies to CD8 and CD16, and a PerCP-conjugated antibody to CD3 (Becton-Dickinson, Abingdon, UK). The stained cells were analysed on a FACScan cytometer (Becton-Dickinson) by a Boolean gate analysis technique which enables all markers to be stained in one sample (13). The antibodies were diluted in PBS, and 20 µl of each antibody was added to a polypropylene tube containing 1×10^5 cells in 100 µl. An aliquot of cells was also stained with FITC-, PE-, and PerCP-conjugated control antibodies. The cells were incubated for 30 min in the dark and then washed twice with PBS before being analysed.

Cytokine mRNA analysis

Aliquots of 5×10^5 BAL T cells or T-cell clones were cultured overnight with PHA 2 µg/ml in 500 µl complete medium in 48-well plates. After 16 h, supernatants were collected and frozen for further analysis by ELISA. The cells were harvested, and total RNA was extracted with RNazol B (AMS Biotechnology, Witney, UK). Polyadenylated mRNA was converted to cDNA by reverse transcription using AMV-reverse transcriptase and oligo-d(T)₁₈ as a primer. cDNA for the various cytokines was amplified by PCR with appropriate specific primer pairs. PCR products were visualized with ethidium bromide after gel electrophoresis. A total of 10 cytokines were examined (TNF- α , IFN- γ , IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, and GM-CSF), and two housekeeping genes: APRT, which is expressed in all cells, and the T-cell receptor β -chain gene, which is expressed only in T cells. Band intensity was assessed in relation to the APRT band and graded as follows: ++ (equal to APRT), +++ (more than APRT band), + (strong signal but less than APRT), or $\pm$ (faint band present only).

IL-4 and IFN- γ ELISAs

Immunoreactive IL-4 was detected in supernatants by an ELISA technique developed by Dr Janis Shute (University Medicine, Southampton, UK) using antibodies obtained through scientific collaborations with Ciba-Geigy (Basel, Switzerland) and Genzyme (Maidstone, Kent, UK). IFN- γ was detected by a sandwich ELISA with paired antibodies which were the gift of Dr Sefik Alkan, Ciba-Geigy, Basel, Switzerland. The sensitivities of the ELISAs were 10 pg/ml for IL-4 and 5 pg/ml for IFN- γ .

Results

BAL T-cell samples

Unstimulated bulk samples of BAL cells obtained 10 min after allergen or saline challenge expressed IFN- γ (5/6 cases), IL-13 (4/6), and TNF- α (5/6). IL-4 mRNA was detected in 1/6 and IL-5 mRNA in 2/6. IL-3 mRNA was not detected. Overnight stimulation with PHA increased the expression of IL-3, IL-4, and IL-5 in 4/6 samples (Table 2). Within individuals, the samples from the allergen and saline 10-min sites showed almost identical patterns of mRNA expression. The pattern of cytokine mRNA expression varied in BAL cells obtained 24 h after saline challenge, with IL-3 and IL-4

Table 2. Cytokine mRNA from BAL T cells (10 min after challenge)

Subject	Stimulus	TNF- α	IFN- γ	IL-3	IL-4	IL-5	IL-13
AH	Medium	–	+	–	+	–	–
DT	Medium	+++	+	–	–	–	++
HB	Medium	+	–	–	–	–	+
ST	Medium	+++	+++	–	–	–	++
LH	Medium	++	++	–	–	+	++
IF	Medium	++	++	–	–	+	–
AH	PHA	++	++	+	++	+	++
DT	PHA	++	+	–	–	–	–
HB	PHA	++	++	–	–	–	++
ST	PHA	+++	+++	++	$\pm$	–	++
LH	PHA	+++	++	++	++	+++	++
IF	PHA	++	++	+	+	+	+

Band intensity estimated relative to APRT control band, which was assigned arbitrary value ++.
Results from saline site and allergen site were assessed separately but showed almost identical band intensities ($\pm$ band very weak but present).

in 2/5 samples; IFN- γ , IL-5, and IL-13 in 3/5. After overnight PHA stimulation, IFN- γ and TNF- α were found in all five samples, IL-3 and IL-4 in 3/5, and IL-5 and IL-13 in 4/5. Although the cell numbers were equivalent, the BAL T-cell samples from the allergen-challenged sites gave weaker PCR signals than those from saline-challenged sites (Fig. 1; Table 3).

When stimulated *in vitro* by PHA, similar concentrations of immunoreactive IL-4 were generated by cells from the allergen and saline sites after 10 min (mean 13.5 vs 9.9 pg/ml, respectively). Slightly more IFN- γ protein was generated by the BAL cells obtained from the allergen site at 10 min (mean 439 vs 348.8 pg/ml). When BAL cells obtained 24 h after challenge were cultured with PHA, there was more IL-4 production by cells from the allergen site than the saline sites (mean 162.3 pg/ml vs 31.9 pg/ml; $P=NS$) but less IFN- γ (mean 221.1 vs 403.7 pg/ml).

T-cell clones

The proportion of BAL T cells that could be cloned with PHA was about 1/40 for both the allergen- and saline-challenged sites. There was no difference in the frequency of obtaining clones between the allergen and saline sites. Wells selected for subculture were those in which Poissonian statistics indicated a probability of >85% of monoclonality. A total of 72 T-cell clones survived in subculture for up to 6–8 weeks. These represent the progeny of seven of the initial 17 panels. Fourteen clones were obtained from a paired

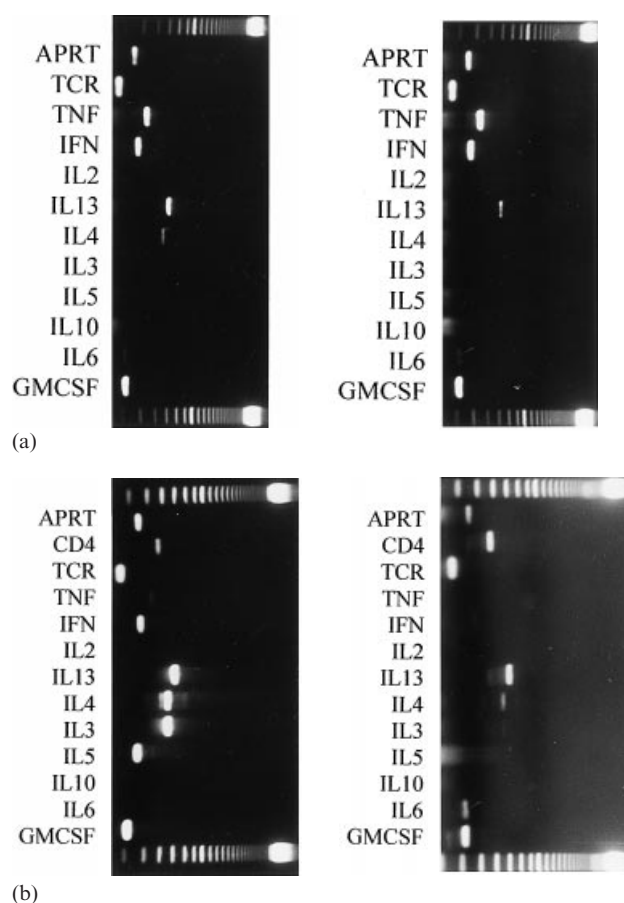


Figure 1. Cytokine mRNA detected by RT-PCR in BAL cells obtained (a) at baseline and (b) 24 h after allergen challenge. In each panel, left gel shows cytokine cDNA from unstimulated cells, and right panel shows cytokine cDNA from cells stimulated for 16 h with PHA.

Table 3. Cytokine mRNA from BAL T cells (24 h after saline or allergen challenge)

Subject	Stimulus	Saline-challenged sites						Allergen-challenged sites					
		TNF- α	IFN- γ	IL-3	IL-4	IL-5	IL-13	TNF- α	IFN- γ	IL-3	IL-4	IL-5	IL-13
AH	Medium	++	-	-	++	+	-	-	-	-	+	-	+
CL	Medium	++	-	-	-	-	-	++	-	-	-	-	-
ST	Medium	++	++	+	+	+	+++	++	+	+	++	+	++
LH	Medium	+	+	+	-	-	+	+	+	-	-	+	+
IF	Medium	++	++	-	-	+	-	+	+	-	+	-	++
AH	PHA	++	++	+	+	+	+	++	++	-	++	+	++
CL	PHA	++	++	++	-	-	-	++	-	++	-	-	-
ST	PHA	++	++	+++	+++	++	+++	++	++	+++	+++	++	+++
LH	PHA	++	+	-	-	+	+	-	-	-	-	-	-
IF	PHA	++	++	-	++	++	++	+	++	-	-	-	+

Band intensity estimated relative to APRT control band, which was assigned arbitrary value ++.

10-min lavage (three from the saline site and 11 from the allergen site) (ST). A total of 17 clones were obtained from a 4-h post-saline lavage (no allergen used in this subject – D.F.). Clones were obtained from two subjects 24 h after allergen and saline challenge. Eighteen clones were obtained from the first subject (C.L.), 15 from the saline site, and three from the allergen site. A total of 23 clones were obtained from the second subject (S.T.), eight from the saline site, and 15 from the allergen site. The T-cell clones were all CD3⁺ with no contaminating monocytes, NK cells, or B cells. Of the 72 clones phenotyped, 50 clones were CD4⁺, and 22 were CD8⁺.

Cytokine profiles of T-cell clones

Cytokine analysis was performed on T-cell clones from two subjects. These were the saline-challenged subject sampled at 4 h (D.F. – Fig. 2) and one of the subjects challenged and sampled at 24 h (S.T.). Clones derived from the baseline lavage (10 min after challenge) were also available for S.T. (Table 4). The clones derived from the baseline lavage and the lavage 4 h after challenge with saline showed strong expression of mRNA for IL-13. In addition, substantial mRNA signals for TNF- α , IFN- γ , IL-3, and GM-CSF were detected. IL-2 mRNA was found in the saline-challenged 4-h site (D.F. – Fig. 2), but not in the baseline clones (S.T. – Table 4). There were weaker mRNA signals for IL-4 and IL-5. IL-5 mRNA was weakly detectable in 7/11 clones from the baseline S.T. lavage, but IL-6 and IL-10 were not detected. Consistent with the RT-PCR profiles, the IL-4 concentrations in the D.F. clone supernatants ranged from 0 to 64 pg/ml, while IFN- γ concentrations ranged from 0 to

49 pg/ml. In keeping with the stronger IL-4 mRNA signal, the baseline S.T. clones produced more IL-4 protein (Table 4). The cytokine profiles of T-cell clones derived from the 24-h post-allergen-challenge segment showed strong expression of mRNA for IL-3, IL-4, IL-5, IL-13, and GM-CSF (Fig. 3). There was no expression of IL-2 or IL-10 in any of these clones, and TNF- α , IFN- γ , and IL-6 were only weakly expressed. The five clones from the saline site showed a similar cytokine profile to the clones from the allergen site (Table 5).

The supernatants from the clones derived 24 h after challenge contained larger amounts of IL-4 protein than those from the 4-h saline site. This was true for both the allergen-challenged site (range 14–1701 pg/ml) and the saline-challenged site (0–1122 pg/ml). Some of the clones showed a 10-fold increase in IL-4 production compared to

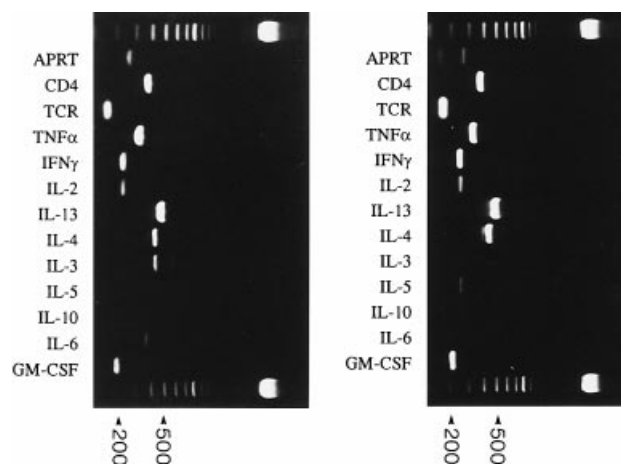


Figure 2. Cytokine mRNA profile of two T-cell clones derived from BAL performed 4 h after saline challenge.

Table 4. 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)
01	8	+	++	++	-	++	+	-	-	++	92	50
02	8	++	++	++	-	++	+	+	-	++	691	21
03	8	++	++	+	-	-	-	-	-	++	279	18
05	8	++	++	+++	-	++	++	-	-	+++	<10	35
06	8	++	++	++	-	++	++	+	-	+++	301	<5
07	8	++	++	+++	-	++	+	+	-	+++	616	56
09	8	++	++	+++	-	++	-	+	-	++	439	41
10	8	++	++	+++	-	++	++	+	-	+++	63	18
11	4	++	++	++	-	++	-	-	-	+++	<10	<5
12	4	++	++	++	-	++	++	+	-	+++	761	51
14	4	++	++	++	-	++	++	+	-	+++	469	42

Cytokine PCR bands compared to APRT control band, which was assigned value of ++.
Cytokine protein measured by ELISA (cutoff limit for IFN- γ 5 pg/ml; for IL-4, 10 pg/ml).

the bulk T-cell overnight cultures. Most clones that had strong IL-4 mRNA signals also had large amounts of IL-4 in their supernatants, but there was a wide scatter in the

amount of IL-4 produced from clones with apparently similar amounts of mRNA (Tables 4 and 5). Interestingly, several of the T-cell clones from subject S.T. were CD8⁺

Table 5. Cytokine mRNA and protein from supernatants of T-cell clones derived 24 h 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)
Allergen site												
17	nd	+	+	+++	-	+++	+++	++	+	+++	nd	nd
20	8	+	+	+++	-	+++	+++	-	-	+++	1099	11
21	8	++	-	++	-	+++	+++	-	+	+++	1701	5
23	4	+	+	+++	-	+++	+++	++	+	+++	976	28
24	4	+	+	+++	-	++	+++	++	-	+++	24	18
26	8	+	+	++	-	+++	+++	+	-	+++	95	5
18	8	++	+	++	-	++	-	+	+	+++	979	35
19	8	++	++	++	-	++	++	++	+++	+++	14	49
22	4	-	-	++	-	++	+++	++	+++	+++	1205	5
25	4	+	-	++	-	+++	+++	++	++	+++	773	28
27	8	++	+	++	-	++	++	+	-	+++	147	5
29	8	+	+	++	-	++	++	+	-	++	<10	34
37	4	+	-	+	-	++	++	-	-	++	1057	39
38	8	++	-	+	-	++	++	++	+	++	130	34
Saline site												
30	4	+	+	+++	-	++	++	++	++	+++	34	25
32	4	+	+	++	-	+	+	+	-	+	<10	29
35	8	++	-	++	-	++	++	++	-	++	1122	36
36	8	++	+	++	-	+++	+++	++	-	+++	455	5
33	8	+	+	+++	-	+++	+++	+++	-	+++	122	38

Cytokine PCR bands compared to APRT control band, which was assigned value of ++.
Cytokine protein measured by ELISA (cutoff limit for IFN- γ 5 pg/ml; for IL-4, 10 pg/ml).
nd: not done.

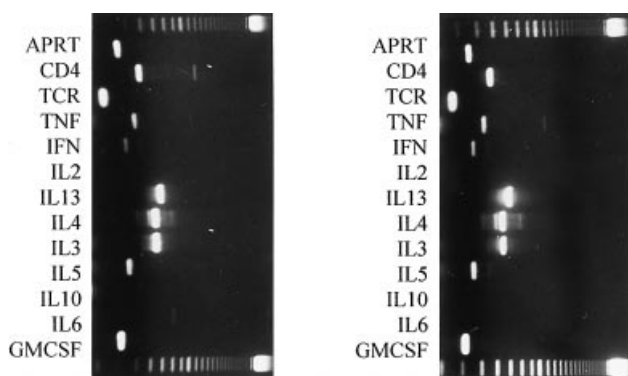


Figure 3. Cytokine mRNA profile of two T-cell clones derived from BAL performed 24 h after allergen challenge. Clones derived by primary limiting dilution and 8-week stimulation with PHA (weekly) and IL-2 (twice weekly).

rather than $CD4^+$, and yet produced large amounts of IL-4. IFN- γ production was more modest. Compared to clones derived from the baseline lavages in this subject (Table 4), there was an increase in the relative production of IL-4 (IL-4/IFN- γ ratio rising from a mean of 9.7 to 19.7). There was no correlation between the amounts of IL-4 and IFN- γ produced by these clones, even when the $CD4^+$ and $CD8^+$ subsets were considered in isolation (Fig. 4).

Discussion

The airways of patients with mild atopic asthma contained T cells which basally expressed mRNA for the cytokines IFN- γ , TNF- α , IL-13, and GM-CSF. mRNA for IL-3 and IL-4 was not basally expressed but could be induced by mitogen stimulation. At 24 h after local allergen challenge, there was a shift in cytokine repertoire toward a Th2-like pattern, with less mRNA for TNF- α or IFN- γ and increased expression of the type 2 cytokines IL-3, IL-4, and IL-5. This pattern was enhanced after PHA stimulation. Using T-cell cloning techniques, we were able to show that this general pattern reflects considerable heterogeneity among the airways T cells, with little evidence of "pure" Th1 or Th2-pattern T cells. Moreover, there was wide variation in the amount of IL-4 produced by cloned T cells, which appeared similar when judged by RT-PCR mRNA profiles. Among the BAL T-cell clones were a considerable number of $CD8^+$ clones which fit the recently described Tc2 phenotype (32). These results emphasize the importance of using several techniques to investigate the question of cytokine production by human airways cells and particularly the need to study protein and mRNA together to arrive at useful and valid conclusions.

Only a limited number of studies have addressed cytokine production by BAL cells before and after allergen challenge. Most have relied on *in situ* hybridization to assess cytokine expression rather than protein production. Robinson et al. found increased expression of mRNA for IL-2, IL-3, IL-4, IL-5, and GM-CSF in BAL T cells from asthmatics as compared with control subjects (33). Inhalation allergen challenge led to a further increase in the proportions of BAL cells expressing mRNA for IL-4, IL-5, and GM-CSF but no change in IL-3, IL-2, or IFN- γ mRNA expression (32). However, when cytokine proteins were measured in BAL from patients with asthma, significantly elevated concentrations were found for TNF, GM-CSF, and IL-6, but not IL-4 or IL-1 α , and when symptomatic airways were compared with asymptomatic asthmatic airways, only IL-1 β and IL-2 showed increases associated with symptoms (3). Eighteen hours after inhalation challenge, increased concentrations of IL-1, IL-2, IL-5, IL-6, IL-8, and TNF- α , but no increase in IL-4 or IFN- γ , were found in BAL fluid (37). Others have reported increased concentrations of IL-4 and IL-5 in concentrated BAL fluid 24 h after local allergen challenge, an observation which was also associated with eosinophil influx and the release of soluble VCAM-1 (43). Other cytokines were either not measured or not reported in that study. In contrast, Huang et al. found marked increases in IL-13, but relatively little IL-4 or IL-5 protein, 18–24 h after segmental allergen challenge, and identified the cellular source of the IL-13 as the mononuclear cell fraction (12). Another recent study has also measured IL-13 in human asthma (15), and, taken together, these studies suggest that IL-13 may be quantitatively more important than IL-4 in the local regulation of IgE switching in asthmatic airways. Moreover, while the actions of IL-4 and IL-13 on B cells are similar, it is now

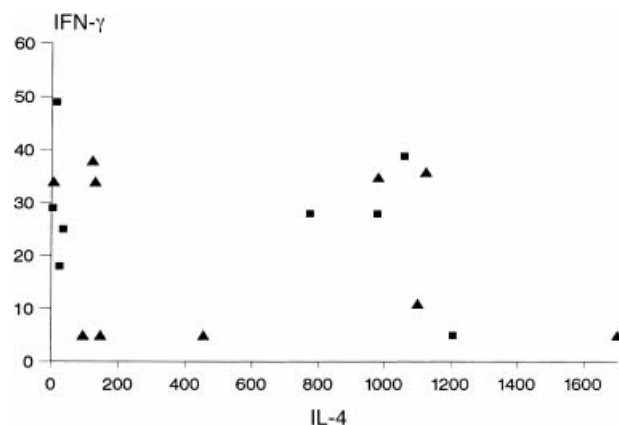


Figure 4. Comparison of IL-4 and IFN- γ protein production in supernatants of T-cell clones derived from BAL of site challenged 24 h previously with allergen.

clear that IL-13 has several additional properties that may be directly relevant to the pathophysiology of asthma (44).

This study has also demonstrated that T cells can be cloned directly from human airways (without an initial bulk culture), and that sufficient cell numbers can be derived for phenotypic and functional analysis. Both CD4⁺ and CD8⁺ clones were derived. However, several of the panels of clones could not be expanded, a fact which limits the general applicability of the technique and results. With this proviso, the majority of T cells which could be cloned expressed mRNA for cytokines of the Th2 T-cell subtype, consistent with previous reports using *in situ* hybridization (10, 33), but there was a considerable degree of functional heterogeneity between clones from the same site.

Unlike *in situ* hybridization, which assesses mRNA expressed in all cells present in the sample, the cloning data are informative about the subset which are capable of proliferation; in this case, about 2–3% of the BAL T cells. It has long been known that airways T cells are relatively reluctant to proliferate (39), possibly due to the effects of pulmonary surfactant on activation (26) or the ability of alveolar macrophages to induce activation-induced cell death (8, 11, 20, 42). In the present study, the T cells were extensively washed and depleted of macrophages, but still only about 1/40 grew in the primary limiting dilution analysis. This supports other suggestions that many airways T cells may be end-differentiated and intrinsically anergic (39).

Three other groups have recently reported data on T-cell clones derived from asthmatic airways. Comparison between the various studies is limited by methodological variations. In a study which used methods similar to ours, BAL was performed in four atopic asthmatic subjects 6 h after allergen inhalation (16). BAL T cells were sorted by FACS and then cloned directly by limiting dilution with PHA and irradiated feeder cells. No exogenous IL-2 was added and cloning efficiency was 3–40%. Both CD4⁺ and CD8⁺ clones were obtained, from BAL cells and PBMC. Cytokine production was assessed in 10 BAL clones (five from an asthmatic subject, five from a control subject). Both panels showed mixed production of both IL-4 and IFN- γ protein, with heterogeneous patterns and amounts of each cytokine. In a second study (28), T cells from bronchial biopsies were cultured in bulk in the presence of relevant allergens, IL-2, IL-4, and irradiated PBMC for 20 days before limiting dilution analysis with IL-2 and PHA. This method gave a cloning efficiency of 11–39% in three subjects, and a total of 120 clones were obtained, which varied in their cytokine profile, but in most of the clones there was a predominance of IFN- γ over IL-4. No attempt was made to

determine whether any of the clones were “sister clones” derived from a common parent T cell which had proliferated in the initial bulk culture. The higher cloning efficiency seen in that study reflects the death, during primary bulk culture, of T cells which are incapable of proliferation. In a third study (21), biopsies were obtained from two patients with occupational asthma due to toluene diisocyanate who had undergone allergen provocation. The biopsies were subjected to an initial bulk culture with IL-2, but no antigen or mitogen, before proceeding to limiting dilution analysis. In this instance, the clones were predominantly CD8⁺ (82% and 83%). All the CD8⁺ clones produced IFN- γ , with 43% also producing IL-5, but only 12% producing IL-4. Three months after cessation of exposure to isocyanates, no BAL T-cell clones could be obtained from the same patients with the same methodology.

A major concern about using T-cell cloning to assess functional phenotype is the possible influence of culture conditions on the eventual phenotype of the clone. Proliferation of particular T-cell subtypes may be favoured by the presence of various cytokines. Thus, Th2 cells proliferate better in the presence of IL-4 or IL-10, while the growth of Th1-type cells is enhanced in the presence of IL-2, IL-12, and IFN- γ (34, 36). In addition, the culture conditions may bias pluripotential precursor cells toward Th1 or Th2 phenotype; e.g., IL-4 encouraging differentiation to a Th2 pattern and TGF- β to a Th1 pattern (25). However, our data indicate that cloned airways T cells still show a considerable heterogeneity of cytokine expression after 6–8-week culture, and this presumably reflects their initial cytokine profile. Further confirmation comes from the bulk BAL T-cell cultures, which represent the integrated response of many T cells, but nevertheless indicate a Th2 pattern at the allergen sites from which the Th2-type T-cell clones were subsequently derived.

Because CD4⁺ T cells of the Th2 subtype elaborate IL-4, IL-5, IL-10, and IL-13 rather than TNF- β , IFN- γ , IL-2, and IL-12, the Th2 subset is associated with atopy and with tissue responses involving IgE, mast cells, and eosinophils (24, 38). It is interesting to note that several of the CD8⁺ T-cell clones secreted IL-4 and expressed IL-5. The ability of CD8⁺ T cells to produce type 2 cytokines has only recently been recognized (32), and the precise role of these “type 2” CD8⁺ cells remains unclear (32), but it seems likely that both CD4⁺ and CD8⁺ cells may contribute to the development of allergic inflammation.

From other data, only a small proportion of blood or airways T cells recognize an individual allergen such as house-dust mite (4, 6, 31); hence, when an initial bulk

culture with allergen is used, the functional phenotype of clones that are eventually obtained may not represent those that might have been derived by a primary limiting dilution, or by using a mitogen (9). While T-cell cloning can provide specific answers to a range of precise questions, its role in assessing overall cytokine profiles will probably be superseded by flow cytometric techniques in which cytokine protein production can be assessed at the single-cell level (17). Nevertheless, cytometric methods also have their limitations, including a limited range of suitable antibodies, paralysis of internal transport mechanisms, and the fact that the cell is rendered incapable of isolation and subculture.

Finally, it is interesting to note that there were substantial differences in cytokine protein production between BAL

T-cell clones with similar mRNA profiles. This may be due in part to post-translational regulation of cytokine production, which has been demonstrated for IL-2, IL-4, and IFN- γ , and which may also apply to other cytokines (18).

In conclusion, resting T cells in asthmatic airways express an intermediate cytokine profile, with IL-13 and IFN- γ prominent, but type 2 cytokines are markedly upregulated after local allergen challenge. These phenotypes appear stable when the T cells are cloned, but these mRNA profiles are not always reflected in cytokine protein production. Accurate analysis of BAL T-cell cytokine profiles requires a combination of techniques, which should be regarded as complementary rather than uniquely informative.

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