**Oral Diseases (2006) 12**, 455–462. doi:10.1111/j.1601-0825.2005.01220.x

http://www.blackwellmunksgaard.com

## ORIGINAL ARTICLE

# Differential regulation of IL-2 and IL-4 in patients with tobacco-related oral squamous cell carcinoma

**P Manchanda[1]*, SC Sharma[2], SN Das[1]**

*Departments of [1]Biotechnology and [2]Otorhinolaryngology, All India Institute of Medical Sciences, Ansari Nagar, New Delhi – 110 029, India*

**AIM: The aim of the study was to investigate the systemic immunity in terms of major lymphocyte subsets and the expression of IL-2 and IL-4 in T-cell subsets from peripheral blood of patients with tobacco-related intraoral squamous cell carcinoma.**

**METHODS: $CD3^+$, $CD4^+$ and $CD8^+$ T-cell subsets and $CD16^+$ $CD56^+$ natural killer cells, and intracellular cytokines in T-cell subsets were determined by two-colour flow cytometry and confocal microscopy.**

**RESULTS: Oral cancer patients showed a significantly reduced ($P < 0.001$) $CD3^+$ and $CD4^+$ T-cell subsets with a lower CD4/CD8 ratio when compared with the normal controls. The frequency of $CD3^+$ $IL\text{-}4^+$ and $CD8^+$ $IL\text{-}4^+$ T cells were significantly higher ($P < 0.001$) while $CD4^+$ $IL\text{-}2^+$ were significantly lower ($P < 0.02$) in patients when compared with the normal controls. Late stage of the tumour was associated with reduced expression of IL-2 in both $CD4^+$ ($P < 0.05$) and $CD8^+$ ($P < 0.03$) subsets.**

**CONCLUSIONS: The tobacco-related intraoral squamous cell carcinoma seems to be associated with multiple systemic immune defects particularly, an impaired $CD3^+$ and $CD4^+$ T cells in the peripheral blood as well as a differential regulation of IL-2 and IL-4 in $CD4^+$ and $CD8^+$ T-cell subsets. The cytokine response in these patients seems to be skewed from protective Th1 to immunosuppressive Th2 type. Thus these patients could be ideal candidate for immunomodulation therapy.**

*Oral Diseases* (2006) **12**, 455–462

**Keywords:** T-cell subsets; interleukin-2; interleukin-4; oral cancer

## Introduction

Most often intraoral squamous cell carcinoma is included under the group of head and neck carcinoma mostly due to its lower incidence rate, although it is a well-defined group of tumours with a distinct biological behaviour. The tobacco-related oral squamous cell tumours are slow growing, endophytic, mostly well differentiated, diploid and locally aggressive. It remains localized for a longer time and distant metastasis is rare, suggesting a role of immune functions in containment of these tumours (Das *et al*, 1986). Patients with head and neck squamous cell carcinoma manifest significant immunosuppression that is often profound in oral cancer when compared with that in patients with other malignancies (Schantz and Guillamondegui, 1988). Immunosuppression in head and neck squamous cell carcinoma has been postulated to occur in a hierarchical manner, where the primary tumour region is the most affected site, followed by draining lymph nodes (Wang *et al*, 1991). Earlier studies of oral cancer patients have shown a higher antibody response (Khanna *et al*, 1982) and impaired T-cell functions (Das *et al*, 1983) that were also related to the poor prognosis of the patient (Das *et al*, 1986). However, the exact mechanism of altered immunity in these patients is not known.

A number of cytokines have proved to be promising candidates for the induction of immune response against cancer in tissue culture system and in nude mice previously inoculated with malignant cells (Lotze, 1992). These are the small molecular weight regulatory molecules involved in the initiation and maintenance of non-specific (innate) and specific (acquired) immunity and appear to be involved in the host's immune response against cancer (Klein and Boon, 1993). Additionally, cytokines mediate cellular communications as well as many aspects of growth, differentiation and regulatory functions.

The role of Th-like cytokines in regulating T-cell mediated immune response was further clarified in recent studies. Th1 cytokines (IL-2 and IFN-$\gamma$) were shown to have an essential role in generating cytotoxic

Correspondence: Dr Satya N Das, Department of Biotechnology, All India Institute of Medical Sciences, Ansari Nagar, New Delhi – 110 029, India. Tel: + 91 11 26 59 35 48, Fax: + 91 11 26 86 26 63, E-mail: satya_n_das@hotmail.com; satyandas@gmail.com
*Present address: Department of Biology, Boston College, Boston, MA 02467, USA.
Received 30 September 2005; revised 27 October 2005, 5 November 2005; accepted 9 November 2005

T-lymphocytes and natural killer (NK) cells, the main effector cells in T-cell mediated immunity against viruses, parasites and tumours (Seder and Paul, 1994); while Th2 cytokines (IL-4, IL-5 and IL-6) were reported to promote immunoglobulin synthesis by B-cells (Coffman *et al*, 1991; Del Prete *et al*, 1991). A cross regulation of Th-like cytokine profile has been observed in several infection models as well as in patients with solid tumours. IL-4 has been shown to inhibit protective cell mediated immune response by down regulating IL-2 production and inhibition of IL-2 gene transcription (Goto *et al*, 1999).

Imbalances of Th1 and Th2 cytokines have been alleged to be responsible for some pathological conditions such as infections (Meyaard *et al*, 1996), allergic (Jung *et al*, 1995) and autoimmune disorders (Chang *et al*, 2002). Abnormal cytokine responses have been reported in a variety of solid tumours including head and neck squamous cell carcinoma (Knerer *et al*, 1996; Woods *et al*, 1998), ovarian carcinoma (Rabinowich *et al*, 1996), colorectal cancer (Pellegrini *et al*, 1996) and renal cell carcinoma (Wang *et al*, 1995). Recently, we have reported a deregulated expression of Th2 cytokine gene in patients with intraoral squamous cell carcinoma (Agarwal *et al*, 2003) that was related to progression of the disease. However, frequency of cytokine expressing T-cell subsets in peripheral blood of these patients remains speculative. Therefore, the present study has been carried out to investigate the systemic immunity in terms of major lymphocyte subsets and the expression of IL-2 and IL-4 in T-cell subsets from peripheral blood of patients with tobacco-related intraoral squamous cell carcinoma.

## Materials and methods

*Patients and healthy subjects*

Twenty-eight patients (26 males and two females) with a history of tobacco chewing for a period ranging from 5 to 25 years and a confirmed diagnosis of primary intraoral squamous cell carcinoma were randomly selected for this study. Mean age of the patients was 51.6 ± 12.7 years. Majority of patients (21 of 28) were suffering from late stage (III & IV) of the disease. None of the patients had distant metastasis of the tumour. The details of the patients and clinico-pathological features of the tumour have been shown in Table 1. None of the patients had received any kind of immunosuppressive therapy prior to the study, thus the results obtained are expected to reflect the changes associated with the tumour load. Twenty age and sex matched normal healthy individuals served as controls. The experiments were carried out with the written informed consent of each subjects and the competent authority of the All India Institute of Medical Sciences approved the study protocol.

*Immunophenotyping of T-cell subsets and NK-cells*

Heparinized blood from each individual was taken for immunophenotying and intracellular cytokine staining. One hundred microlitres of whole blood was taken in three Eppendorf vials containing 20 $\mu$l of fluorochrome labelled monoclonal antibody (BD- Pharmingen, San Jose, CA, USA) i.e. CD3 Fluoroisothiocyanate (FITC)/CD16.56 Phycoerythrin (PE); CD4 FITC/CD8 PE and antimouse isotype antibody (lgG2a, FITC/lgG1, PE). After vortexing at low speed for 1 min the vials were incubated at room temperature for 30 min in dark and washed twice with phosphate-buffered saline (PBS; pH 7.4) by centrifugation at 200 ***g*** for 5 min in a refrigerated centrifuge. The cell pallet was resuspended in FACS lysing solution and incubated for 5 min at 37°C to remove the erythrocytes. Finally the cells were washed twice with PBS, resuspended in 1% paraformaldehyde and stored at 4°C in dark until acquired in the Flow Cytometer.

**Table 1** Clinicopathological parameters of the patients with oral cancer

| *Sr. no.* | *Age* | *Gender* | *Tumor, node, metastasis* | *Stage* | *Grade* | *Site* |
|---|---|---|---|---|---|---|
| 1 | 60 | M | $T_3$, $N_2$, Mo | IV | 2 | RMT |
| 2 | 45 | M | $T_4$, $N_{2c}$, Mo | IV | 2 | Tongue |
| 3 | 37 | M | $T_4$, $N_2$, Mo | IV | 2 | BM |
| 4 | 65 | M | $T_1$, $N_2$, Mo | IV | 1 | Palate |
| 5 | 36 | M | $T_2$, $N_1$, Mo | III | 0 | Tongue |
| 6 | 86 | M | $T_3$, $N_0$, Mo | III | 2 | Tongue |
| 7 | 49 | M | $T_2$, $N_0$, Mo | II | 2 | Tonsil |
| 8 | 50 | F | $T_3$, $N_0$, Mo | III | 2 | Palate |
| 9 | 40 | M | $T_3$, $N_0$, Mo | III | 2 | Tongue |
| 10 | 60 | M | $T_3$, $N_0$, Mo | III | 0 | Tongue |
| 11 | 45 | M | $T_4$, $N_1$, Mo | IV | 0 | BM |
| 12 | 65 | F | $T_2$, $N_0$, Mo | II | 0 | Palate |
| 13 | 42 | M | $T_2$, $N_0$, Mo | II | 2 | Tongue |
| 14 | 65 | M | $T_3$, $N_0$, Mo | III | 0 | Lip |
| 15 | 32 | M | $T_1$, $N_0$, Mo | I | 0 | Tongue |
| 16 | 45 | M | $T_3$, $N_0$, Mo | III | 2 | Tongue |
| 17 | 61 | M | $T_3$, $N_1$, Mo | III | 0 | Palate |
| 18 | 50 | M | $T_3$, $N_2$, Mo | IV | 2 | Tongue |
| 19 | 30 | M | $T_1$, $N_0$, Mo | I | 0 | Tongue |
| 20 | 50 | M | $T_3$, $N_1$, Mo | III | 0 | BM |
| 21 | 68 | M | $T_4$, $N_2$, Mo | IV | 0 | Tongue |
| 22 | 67 | M | $T_1$, $N_0$, Mo | I | 0 | Tongue |
| 23 | 42 | M | $T_2$, $N_0$, Mo | II | 2 | Lip |
| 24 | 50 | M | $T_3$, $N_0$, Mo | III | 0 | Tongue |
| 25 | 55 | M | $T_3$, $N_1$, Mo | III | 0 | BM |
| 26 | 40 | M | $T_3$, $N_0$, Mo | III | 0 | Lip |
| 27 | 62 | M | $T_3$, $N_2$, Mo | IV | 2 | RMT |
| 28 | 45 | M | $T_3$, $N_1$, Mo | III | 0 | Tongue |

BM, buccal mucosa; RMT, retro-molar trigone.

*Lymphocyte culture, intracellular staining and two colour flow cytometry*

Intracellular cytokine expression (IL-2, IL-4) in T-cell subsets was studied on isolated and *in vitro*-stimulated peripheral blood mononuclear cells (PBMC). Briefly, PBMC were separated from 10 ml of heparinized blood by Ficoll hypaque gradient centrifugation and a suspension of $1 \times 10^6$,cells $ml^{-1}$ was made in RPMI-1640 with 10% foetal calf serum (Sigma-Aldrich, St Louis, MO, USA). The cells were stimulated with a combination of 1 ng $ml^{-1}$ of phorbol myristate acetate (Sigma-Aldrich), 2 $\mu$M ionomycin (Sigma-Aldrich) in the presence of 2 $\mu$m monnensin (Sigma-Aldrich) and cultured for 6 h at 37°C in a humidified chamber containing 5% $CO_2$.

The carboxylic ionophore, monnensin was used to increase the sensitivity of cytokine detection through its inhibitory effect on protein secretion by interfering with the function of golgi apparatus (Maino and Picker, 1998). After termination of the culture, cell pallet was obtained by centrifugation and it was re-suspended in a staining buffer containing optimal concentration of FITC labelled cell surface markers (CD3, CD4/CD8; BD Pharmingen, San Jose, CA, USA) and further incubated for 15 min at room temperature in dark. After two washings with PBS, cells were treated with 4% paraformaldehyde and kept in dark for 20 min. The cells were washed twice by centrifugation and pallets were resuspended in FACS permeabilization buffer (BD Biosciences, San Jose, CA, USA) containing PE labelled antihuman IL-2 and IL-4 mAbs (BD Pharmingen) respectively for intracellular staining and incubated for 30 min at room temperature in dark. The cells were washed twice with PBS and resuspended in 1% paraformaldehyde and acquired in flow cytometer (FACs Calibur; BD, San Jose, CA, USA) and results were analysed using CELL QUEST software (BD).

*Laser confocal microscopy*

Representative samples from the above preparation were used for confocal laser microscopy. Briefly, a cytospin preparation was made on cleaned slides, one drop of mounting medium (90% glycerol and antifade solution) was added, cover slip was placed and it was sealed with nail polish. The slides were viewed under Reiss Inverted Laser Confocal Microscope model Axiovert 200 equipped with 50 W Mercury arc lamp and Argon–Krypton laser. Oil objective (100×) was used throughout the study. Adobe Photoshop software (Adobe System Inc, CA, USA) was used to collect and format the confocal images.

*Statistical analysis*

Statistical analysis of the data was done using Epiinfo version 6.04d software (CDC, Atlanta, GA, USA). One-way analysis of variance (ANOVA) and Student's *t*-test was applied for evaluation of statistical significance of the data. Test statistics was considered significant if probability of a chance finding was $< 5\%$ ($P < 0.05$).

## Results

*T-cell subsets and NK cells*

Total T-cell ($CD3^+$), its subsets ($CD4^+/CD8^+$) and NK ($CD3^-$, $CD16^+$, $CD56^+$) cells were determined from the whole peripheral blood (before isolation and stimulation of the PBMC) by two-colour flow cytometry (Figure 1).

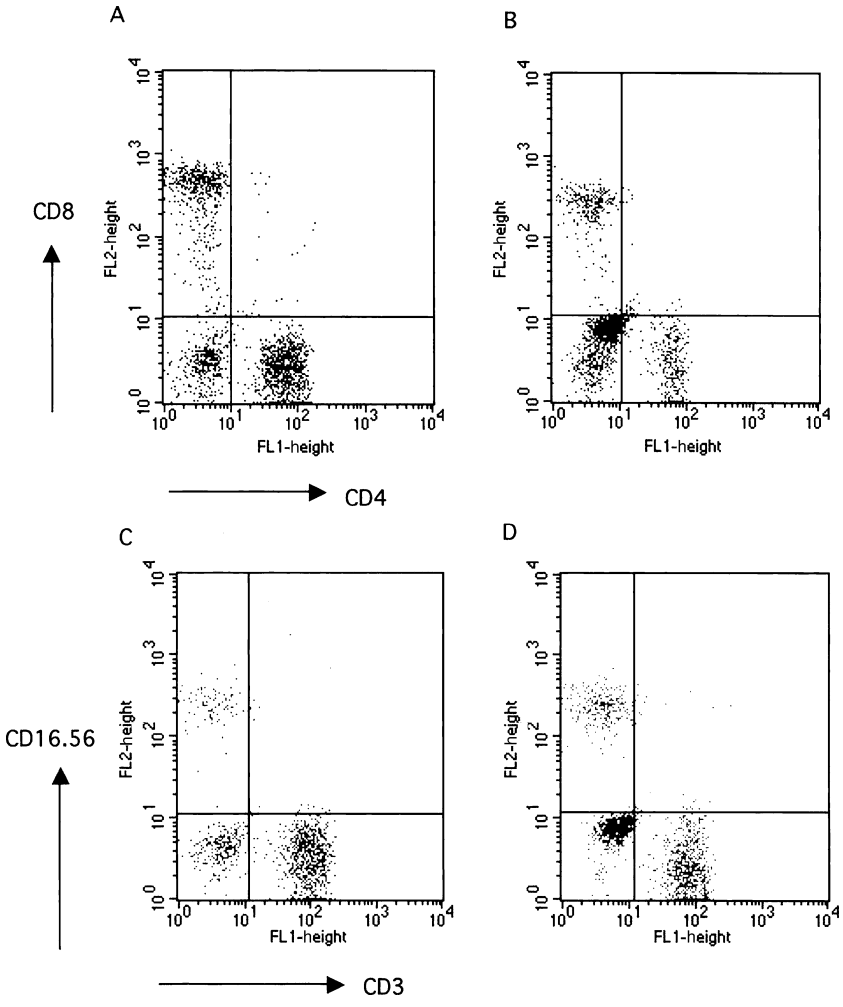


**Figure 1** Representative two-colour dot plot showing $CD3^+$, $CD4^+$, $CD8^+$ T-cell subsets and $CD16.56^+$ natural killer cells in peripheral blood of normal control (**a**, **c**) and oral cancer patients (**b**, **d**)

**Table 2** Distribution of lymphocyte subsets in normal controls and oral cancer patients

| *Subsets* | *Normal controls (n = 20)* | *Patients (n = 28)* | *P-value* |
|---|---|---|---|
| $CD3^+$ (%) | 70.1 ± 4.40 | 61.5 ± 3.8 | < 0.001 |
| $CD4^+$ (%) | 40.0 ± 5.0 | 31.5 ± 4.7 | < 0.001 |
| $CD8^+$ (%) | 27.5 ± 4.3 | 28.1 ± 4.5 | NS |
| $CD4^+/CD8^+$ ratio | 1.5 ± 0.2 | 1.1 ± 0.3 | < 0.05 |
| $CD3^- CD16^+ 56^+$ (%) | 15.4 ± 6.1 | 13.0 ± 6.8 | NS |

Values are given as mean ± s.d.
NS, not significant.

Oral cancer patients had significantly reduced level of $CD3^+$ and $CD4^+$ T cells subsets ($P < 0.001$) and $CD4^+/CD8^+$ ratio ($P < 0.05$) when compared with the normal controls. However, no significant difference was observed in the levels of $CD8^+$ T cells and $CD16.56^+$ NK cells between the two groups (Table 2).

*Intracellular cytokines (IL-2, IL-4) in T-cell subsets*
The results have been shown in Figure 2. The frequency of $CD3^+$ $IL\text{-}4^+$ T cells was significantly higher ($P < 0.001$) while $CD3^+$ $IL\text{-}2^+$ cells were less frequent in patients when compared with that of the normal controls. In case with $CD4^+$ T-cell subsets, the frequency of $CD4^+$ $IL\text{-}2^+$ subset was significantly lower

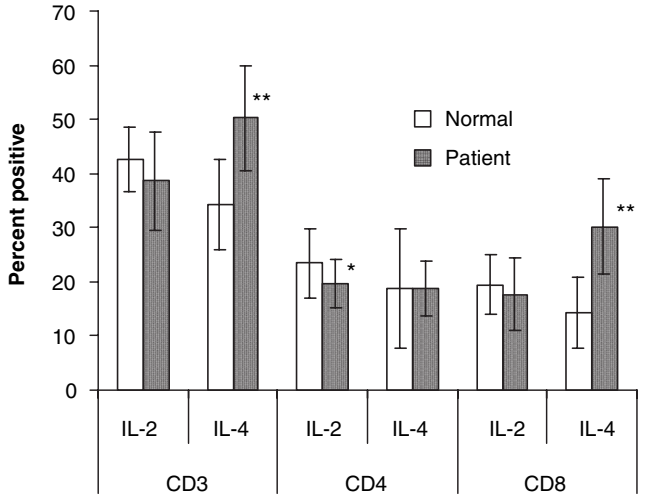


**Figure 2** Frequency of IL-2 and IL-4 positive T-cell subsets in normal subjects and oral cancer patients (*$P < 0.02$, **$P < 0.001$). The ratio of IL-2:IL-4 expressing $CD3^+$, $CD4^+$ and $CD8^+$ T-cell subsets were 1:0.8, 1:0.8, 1:0.7 in normal subjects and 1:1.3, 1:0.9, 1:1.7 in patients respectively

($P < 0.02$) in patients when compared with controls while no significant difference was observed in $CD4^+$ $IL\text{-}4^+$ subsets between the two groups. Contrarily, frequency of $CD8^+$ $IL\text{-}4^+$ subsets were significantly higher ($P < 0.001$) in patients when compared with that of the normal controls while no significant difference was found in $CD8^+$ $IL\text{-}2^+$ T cells between the two groups. The ratio of IL-2 and IL-4 expressing subsets was higher in $CD3^+$ and $CD8^+$ subsets in patients (1:1.3 and 1:1.7 respectively) when compared with the normal controls (1:0.8 and 1:0.7 respectively), suggesting a predominantly Th2 type of cytokine response contributed by $CD8^+$ T-cell subsets. Late clinical stage of the tumour was associated with a significantly lower frequency of $CD4^+$ $IL\text{-}2^+$ ($P < 0.03$) and $CD8^+$ $IL\text{-}2^+$ ($P < 0.03$) T-cell subsets. Furthermore, in the late stage tumours the ratio of IL2 and IL-4 expression both in $CD4^+$ and $CD8^+$ subsets was higher when compared with that in the early stage tumours suggesting a shift from Th1 to Th2 type of cytokine response (Table 3). No significant correlation was observed in cytokine (IL-2, IL-4) expressions in T-cell subsets in relation to histological differentiation of the tumour (data not shown).

The result of confocal microscopy has been shown in Figure 3. Surface markers (CD8 FITC) and intracellular cytokine (IL-2/IL-4 PE) fluorescence were seen in all the samples as shown in Figure 3a–d. Overall results of confocal microscopy were similar to that observed by flow cytometry. Frequency of $CD4^+$ $IL\text{-}4^+$ and $CD8^+$ $IL\text{-}4^+$ cells were more frequent in patients with intraoral squamous cell carcinoma when compared with the normal controls.

## Discussion

We investigated the $CD3^+$ T cells, their $CD4^+$, $CD8^+$ subsets, $CD16.56^+$ NK-cells and their contribution in Th-like (IL-2/IL-4) cytokine response from the peripheral blood of patients with tobacco-related intraoral squamous cell carcinoma, which is a distinct sub-group of the head and neck cancer. The rationale for this study was (i) that the circulating T-lymphocyte subsets constitute helper and effector populations responsible for tumour-targeted immune response and (ii) our earlier observations of impaired T-cell functions (Das *et al*, 1986) as well as abnormal expression of cytokine genes (Agarwal *et al*, 2003) in this sub-group of patients. The results showed a significantly reduced $CD3^+$ and $CD4^+$ T-cell subsets resulting to a lower CD4/CD8 ratio in

**Table 3** Interleukin (IL)-2 and IL-4 expression in T-cell subsets in oral cancer patients in relation to the clinical stage

| | | *CD4 Per cent (mean ± s.d.)* | | | *CD8 Per cent (mean ± s.d.)* | | |
|---|---|---|---|---|---|---|---|
| *Tumour stage* | *No.* | *IL-2* | *IL-4* | *IL-2:IL-4* | *IL-2* | *IL-4* | *IL-2:IL-4* |
| Early | 7 | 23.0 ± 7.2 | 25.7 ± 5.2 | 1:1.1 | 18.4 ± 8.0 | 28.5 ± 8.4 | 1:1.5 |
| Late | 21 | 15.4 ± 3.3** | 23.0 ± 5.3 | 1:1.5 | 14.0 ± 6.2* | 26.9 ± 5.6 | 1:1.9 |

*$P < 0.05$; **$P < 0.03$.

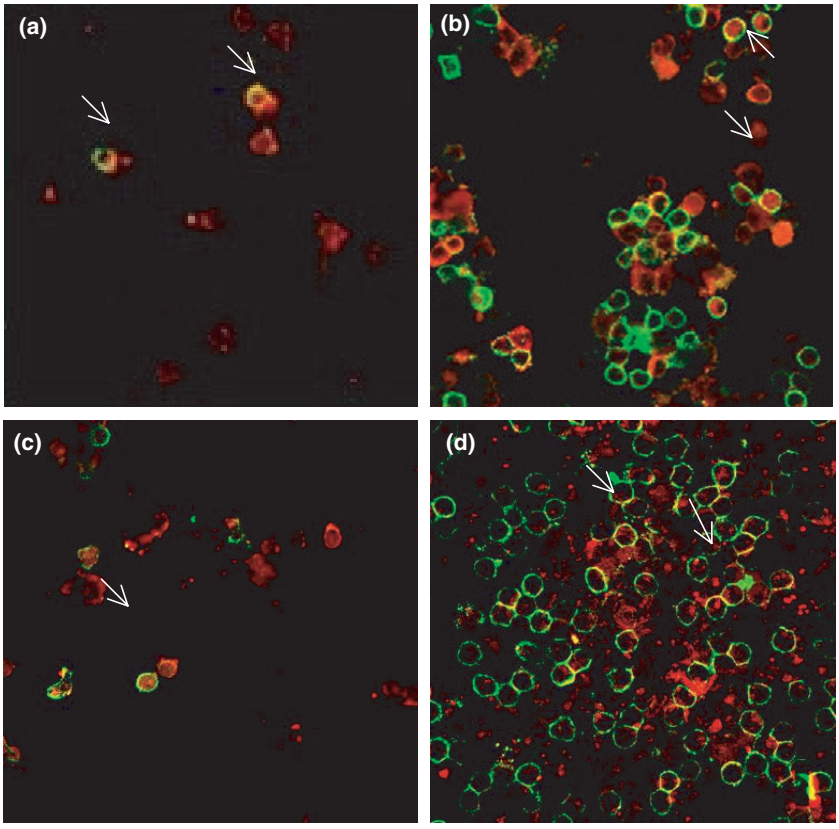


**Figure 3** Representative photograph showing intracellular cytokine expression (IL-2/IL-4 - PE) and cell surface markers (CD8 FITC) by two-colour confocal microscopy. Panel **a** and **b** shows IL-2 expression while panel **c** and **d** shows IL-4 expression in normal subjects and oral cancer patients respectively. Arrow indicates double positive cells

patients suggesting a systemic defect of adaptive immune response while innate immune response seems to be unaffected as reflected by the relatively normal levels of $CD16.56^+$ NK-cells in patients with intraoral squamous cell carcinoma. As $CD4^+$ T cells are mediator of delayed type of hypersensitivity response as well as amplification of cellular immune function, the reduced $CD4^+$ subset in oral cancer patients suggest an inherent defect of cellular immunity in these patients. The results are in agreement with our earlier observations where we have shown a greater impairment of DTH response, T-cell population as well as mitogenic response, in patients with oral squamous cell carcinoma (Das *et al*, 1983, 1986).

Immunological dysfunction plays an important role in the escape of tumour cells from effector immune functions, resulting in the development, establishment and progression of the cancer. The incidence of malignancy is 100 times more common in immunodeficient subjects (Kersey *et al*, 1973) when compared with the normal population. However, tobacco-related oral cancer has been reported in a very few patients with primary immunodeficienes (Scully, 1982). The effect of tobacco smoking on immune functions such as white blood cell counts, T cells and NK-cells is not well understood. Chronic exposure to tobacco smoke or nicotine has been related to impairment of antigen-mediated T-cell signalling, reduced antibody-forming cells (Kalra *et al*, 2000) and enhanced production of IFN-$\gamma$, IL-4 and IL-10 by memory T cells (Petro *et al*, 1999) in murine models. However, no significant effect was observed on IL-4 and IFN-$\gamma$ release by human PBMC or cloned T cells (Le Cam *et al*, 1996). In a study on 1027 normal volunteers we observed a significant increase in CD4/CD8 ratio and NK cells in habitual smokers (Saxena *et al*, 2004). Tobacco use has been related to a long-term chronic effect on many important aspects of the inflammatory immune response (Palmer *et al*, 2005). A greater impairment of cellular immunity has been reported in head and neck cancer patients than those with other malignancies (Olkowski and Wilkins, 1975; Wanebo *et al*, 1975). Impairment of immune function was further related to shorter diseases free survival after treatment (Das *et al*, 1986).

Recent studies have established that during ongoing immune response the cytokines produced by Th1 and Th2 subsets of $CD4^+$ T cells are critical to the effectiveness of that response. Th1 cytokines (IFN-$\gamma$ and IL-2) have been found to mediate DTH and cytotoxic T-cell response while Th2 cytokines (IL-4, IL5 and IL-10) mediates antibody secretion by B-lymphocytes (Del Prete *et al*, 1991). Type of environmental cytokines plays a determinant role in directing the development of T cells into IFN-$\gamma$ and IL-2 expressing Th-1 subset or IL-4, IL-5 and IL10 expressing Th-2 subsets (Coffman *et al*, 1991; Seder and Paul, 1994). In the present study the frequency of IL-4 expressing $CD3^+$ T cells were significantly higher in oral cancer patients when compared with the normal controls. When analysed in the T-cell subsets, $CD4^+$ T

cells showed a significantly lower frequency of IL-2 expressing cells, while $CD8^+$ T-cell subset expressed IL-4 more frequently when compared with the normal controls. These results suggest a differential regulation of IL-2 and IL-4 in $CD4^+$ and $CD8^+$ T-cell subsets in patients with intraoral squamous cell carcinoma. In majority of the studies related to cancer or in other pathological conditions cytokine expression has been studied mostly in context with $CD4^+$ T-cell subset to define Th1 and Th2 type of hierarchy. However, $CD8^+$ T cells seems to be an important contributing factor in modulating immune response through expression of cytokines particularly in cancer patients where there is increased loss or anergy of $CD4^+$ T cells. Majority of tumours of various origin including intraoral cancer are more frequently infiltrated with $CD8^+$ T-cell subsets than with the $CD4^+$ T cells (Rabinowich *et al*, 1996; Stephens *et al*, 1998). $CD8^+$ T-cell has been shown to produce both Th1 and Th2 cytokines (Sad *et al*, 1995). Similarly, tumour microenvironment has been reported to contain mostly Th2 type of cytokines, i.e. IL-4, IL-10 and TGF-$\beta$ produced by either tumour cells or by tumour infiltrating lymphocytes (Ghosh *et al*, 1995; Rabinowich *et al*, 1996). Such an altered cytokine microenvironment may be explained on the basis of the altered cytokine expressions by $CD4^+$ and $CD8^+$ T-cell subsets. Our result suggest that the development of intraoral squamous cell cancer is associated with the reduced frequency of $CD4^+$ subset as well as $CD4^+$ $IL\text{-}2^+$ T-cell subsets in the peripheral blood, along with an enhanced expression of Th2 cytokine (IL-4) by $CD8^+$ T-cell subsets ultimately skewing the Th1/Th2 balance towards Th2 type resulting in the down regulation of T-cell mediated immune response. Cytokine cross regulation have also been reported in several infectious diseases and in patients with solid tumours. IL-4 inhibited the generation of protective T-cell mediated immunity by suppressing IL-2 production (Powrie and Coffman, 1993) through inhibition of IL-2 gene transcription (Goto *et al*, 1999). Contrarily, Th1 cytokines (IFN-$\gamma$ and IL-2) have been found to enhance proliferation of Th1 cells (Maggi *et al*, 1992) and suppress Th2 cell proliferation (Gajewski and Fitch, 1988). When compared between $CD4^+$ and $CD8^+$ T-cell subsets, $CD8^+$ subsets seems to contribute more towards IL-4 expression than the $CD4^+$ T-cell subsets.

We have also attempted to correlate cytokine expression in peripheral T-cell subsets with the disease activity in these patients. The intracellular staining of cytokines showed a reduced frequency of IL-2 expressing T-cell subsets (both $CD4^+$ and $CD8^+$) in oral cancer patients with advanced stage tumours while frequency of IL-4 expressing $CD4^+$ and $CD8^+$ T-cell subsets remained consistently higher in both the groups. It seems therefore, that the late stage of the intraoral cancer is associated with a down regulation of IL-2 secreting T-cell subsets and concomitant up regulation of IL-4 secreting $CD8^+$ T-cell subsets leading to skewing of the immune response towards Th2 type. Progression of the tobacco-related intraoral squamous cell carcinoma to the advanced stage seems to be associated with the down regulation of IL-2 production by $CD4^+$ and $CD8^+$ T-cell subsets. The results confirm our previous observations that the polarization of Th2 response is associated with increased disease activity in patients with intraoral squamous cell carcinoma (Agarwal *et al*, 2003). Immunosuppression in head and neck squamous cell carcinoma has been postulated to occur in a hierarchal manner where the primary tumour region is the most affected site followed by draining lymph nodes (Wang *et al*, 1991). It has also been proposed that metastatic tumour cells in the lymph node may alter the trafficking of lymphocytes between lymph node and peripheral blood. Increased IL-4 has been reported in primary as well as metastatic site of the tumour (Pellegrini *et al*, 1996; Woods *et al*, 1998) indicating that the tumour cells provide Th2 type of microenvironment. Such environment could also be responsible for differentiation of Th0 type of naive T-cell into Th2 cytokine producing T cells.

The down regulation of IL-2 expressing T-cell subsets in patients with intraoral squamous cell carcinoma could be supported by the earlier observations where a signalling defect has been reported in circulating or tumour infiltrating lymphocytes of cancer patients (Lai *et al*, 1996; Rabinowich *et al*, 1996) including that in the oral cancer (Reichert *et al*, 2002). One of the important observations has been that the NF-$\kappa$B/rel family transcription factors that are associated with expression of IFN-$\gamma$ and IL-2 is abnormally expressed in tumour bearing host (Li *et al*, 1994). Other reports have indicated absence or abnormal expression of T-cell receptor associated $\zeta$ chain and $p56^{lck}$ that are important for activation of $CD4^+$ and $CD8^+$ T cells (Rabinowich *et al*, 1996; Whiteside, 1999). The expression of $\zeta$ chain was also reported to be significantly decreased in T-cell subsets and $CD16.56^+$ NK Cells (Kuss *et al*, 1999) in patients with head and neck carcinoma. Down regulation of $\zeta$ chain expression in peripheral blood lymphocytes obtained from patients with head and neck cancer or melanoma was further found to be associated with spontaneous apoptosis in the higher proportion of T cells (Whiteside, 1999). However, it is yet to be seen if the altered cytokine profile in patients with intraoral squamous cell carcinoma is because of the abnormal expression of T-cell receptor associated proteins and/or abnormal expression of down stream signalling molecules involved in the regulation of cytokine gene expression. Taken together our results suggest a differential regulation of IL-2 and IL-4 cytokines in $CD4^+$ and $CD8^+$ T-cell subsets in patients with tobacco-related intraoral squamous cell carcinoma, which is skewed from protective Th1 to immunosuppressive Th2 type.

## Acknowledgements

The Department of Biotehnology, Ministry of Science and Technology, Government of India partly supported this study under M. Biotechnology teaching programme. The authors thank the Director, Institute of Pathology (ICMR), New Delhi, for permitting to use Flow Cytometer and Laser Confocal Microscope.

## References

Agarwal A, Rani M, Saha GK *et al* (2003). Disregulated expression of the Th2 cytokine gene in patients with intraoral squamous cell carcinoma. *Cancer Invest* **32:** 15–28.

Chang DM, Su WL, Chu SJ (2002). The expression and significance of intra-cellular T-helper cytokines in systemic lupus erythematosus. *Immunol Invest* **31:** 1–12.

Coffman RL, Varkila K, Scott P, Chatelain R (1991). Role of cytokine in the differentiation of $CD4^+$ T-cell subsets in vivo. *Immuno Rev* **123:** 189–197.

Das SN, Khanna NN, Khanna S (1983). Delayed hypersensitivity in patients with squamous cell carcinoma of the oral cavity. *Ann Acad Med Singapore* **12:** 412–416.

Das SN, Khanna NN, Khanna S (1986). In vivo and in vitro observation of cellular Immune parameters in squamous cell cancinoma of the oral cavity and its correlation with tumour load and prognosis. *Cancer Invest* **4:** 207–216.

Del Prete GF, DelCarli M, Ricci M, Romagnani S (1991). Helper activity for immunoglobulin synthesis of T-helper type 1 (Th1) and Th2 human T-cell clones; the help of Th1 clones is limited by their cytotoxic capacity. *J Exp Med* **174:** 809–813.

Gajewski TF, Fitch FW (1988). Antiproliferative effects of IFN-$\gamma$ in immune regulation I. IFN-$\gamma$ inhibits the proliferation of Th2 but not Th1 murine helper T-lymphocyte clones. *J Immunol* **140:** 4245–4252.

Ghosh P, Komschlies KL, Cippitelli M *et al* (1995). Gradual loss of T-helper 1 populations in spleen of mice during progressive tumour growth. *J Natl Cancer Inst* **87:** 1478–1483.

Goto S, Sato M, Kaneko R *et al* (1999). Analysis of Th1 and Th2 cytokine production by peripheral blood mononuclear cells as a parameter of immunological dysfunction in advanced cancer patients. *Cancer Immunol Immunother* **48:** 435–442.

Jung T, Lack G, Schauer U *et al* (1995). Allergens, IgE1 mediators inflammatory mechanisms. Decreased frequency of interferon-$\gamma$ and interleukin-2 producing cells in patients with atopic diseases measured at the single cell level. *J Allergy Clin Immunol* **96:** 515–527.

Kalra R, Singh SP, Savage SM *et al* (2000). Effects of cigarette smoke on immune response: chronic exposure to cigarette smoke impairs antigen-mediated signaling in T cells and depletes IP3-sensitive Ca(2+) stores. *J Pharmacol Exp Ther* **293:** 166–171.

Kersey JH, Spector BD, Good RA (1973). Primary immunodeficiency disease and cancer: the immunodeficiency cancer registry. *Int J Cancer* **12:** 333–347.

Khanna NN, Das SN, Khanna S (1982). Serum immunoglobulins in patients with squamous cell carcinoma of the oral cavity. *J Surg Oncol* **20:** 46–48.

Klein G, Boon T (1993). Tumour immunology: present perspectives. *Curr Opin Immunol* **5:** 687–692.

Knerer B, Hulla W, Martinek H *et al* (1996). IL-1 and TNF-$\alpha$ but not IL-2 expression is found in squamous cell carcinomas of the head and neck by RT-PCR. *Acta Otolaryngol* **116:** 132–136.

Kuss I, Sato T, Johnson JT, Whiteside TL (1999). Clinical significance of decreased $\xi$ chain expression in peripheral blood lymphocytes of patients with head and neck cancer. *Clin Cancer Res* **5:** 329–334.

Lai P, Rabinowich H, Crowley-Nowick PA *et al* (1996). Alteration in expression and function of signal transduction proteins in tumour associated NK and T lymphocytes from patients with ovarian CA. *Clin Cancer Res* **2:** 161–173.

Le Cam L, Lagier B, Bousquet J, Pene J (1996). Nicotine does not modulate IL-4 and interferon-gamma release from peripheral blood mononuclear cells and T cell clones activated by phorbol myristate acetate and calcium ionophore. *Int Arch Allergy Immunol* **111:** 372–375.

Li X, Liu J, Park JK *et al* (1994). T-cells from renal cell carcinoma patients exhibit an abnormal pattern of $\kappa$B-specific DNA binding activity: a preliminary report. *Cancer Res* **54:** 5424–5429.

Lotze MT (1992). T-cell growth factors and the treatment of patients with cancer. *Clin Immunol Immunopathol* **62:** 47–54.

Maggi E, Parronchi P, Manetti R *et al* (1992). Reciprocal regulatory effects of IFN-$\gamma$ and IL-4 on the in vitro development of human Th1 and Th2 clones. *J Immunol* **148:** 2142–2147.

Maino VC, Picker LJ (1998). Identification of functional subsets by flowcytometry: Intracellular detection of cytokine expression. *Cytometry* **34:** 207–215.

Meyaard L, Hovenkamp E, Keet IPM *et al* (1996). Single cell analysis of IL-4 and IFN-$\gamma$ production by T-cells from HIV-infected individuals. *J Immunol* **157:** 2712–2718.

Olkowski ZL, Wilkins SA (1975). T-lymphocyte levels in the peripheral blood of patients with cancer of the head and neck. *Am. J. Surg* **130:** 440–444.

Palmer RM, Wilson RF, Hasan AS, Scott DA (2005). Mechanism of action of environmental factors-tobacco smoking. *J Clin Peridontol* **32:** 210–213.

Pellegrini P, Berghella AM, Del Beato T (1996). Disregulation in Th1 and Th2 subsets of $CD4^+$ T-cells in peripheral blood of colorectal cancer patients and involvement in cancer establishment and progression. *Cancer Immunol Immunother* **42:** 1–8.

Petro TM, Schwartzbach SD, Zhang S (1999). Smokeless tobacco and nicotine bring about excessive cytokine responses of murine memory T-cells. *Int J immunopharmacol* **21:** 103–114.

Powrie F, Coffman R (1993). Interleukin-4 and interleukin-10 synergize to inhibit cell mediated immunity *in vivo*. *Eur J Immunol* **23:** 2223–2229.

Rabinowich H, Suminami Y, Reichert TE *et al* (1996). Expression of cytokine genes or proteins and signalling molecules in lymphocytes associated with human ovarian carcinoma. *Int J Cancer* **68:** 276–284.

Reichert TE, Strauss L, Wagner EM, Gooding W, Whiteside TL (2002). Signaling abnormalities, apoptosis and reduced proliferation of circulating and tumour-infiltrating lymphocytes in patients with oral carcinoma. *Clin Cancer Res* **8:** 3137–3145.

Sad S, Marcotte S, Mosmann TR (1995). Cytokine induced differentiation of precursor mouse $CD8^+$ T-cells into cytotoxic $CD8^+$ T-cells secreting Th1 or Th2 cytokines. *Immunity* **2:** 271–297.

Saxena RK, Choudhry V, Nath I *et al* (2004). Normal ranges of some select lymphocyte subpopulations in peripheral blood of normal healthy individuals. *Curr. Sci* **66:** 969–975.

Schantz SP, Guillamondegui OM (1988). Developing perspective in head and neck tumour immunology. *Prob Gen Surg* **5:** 99–111.

Scully C (1982). The immunology of the cancer of the head and neck with particular reference to oral cancer. *Oral Surg* **52:** 157–169.

Seder RA, Paul WE (1994). Acquisition of lymphokine producing phenotype by $CD4^+$ T-cells. *Ann Rev Immunol* **12:** 635–673.

Stephens M, Lim K, Stephens P, Thomas DW, Lim SH (1998). Molecular characterization of tumour infiltrating lymphocytes in oral squamous cell carcinoma. *Cancer Immunol. Immunother* **46:** 34–40.

Wanebo HJ, Jun MY, Strong EW, Oettgen HD (1975). T-cell deficiency in patients with squamous cell carcer of the head and neck. *Am J Surg* **130:** 445–451.

Wang MB, Lichenstein A, Mickel RA (1991). Hierarchical immunosuppression of regional lymphnodes in patients with head and neck squamous cell carcinoma. *Otolaryngol Head Neck Surg* **105:** 517–527.

Wang Q, Rodovan C, Tubbs R *et al* (1995). Selective cytokine gene expression in renal cell carcinoma tumour cells and tumour infiltrating lymphocytes. *Int J Cancer* **61:** 780–785.

Whiteside TL (1999). Signalling defects in T-lymphocytes of patients with malignancies. *Cancer Immunol Immunother* **48:** 346–352.

Woods KV, El-Naggar A, Clayman GL, Grimm EA (1998). Variable expression of cytokines in human head and neck squamous cell carcinoma cell lines and consistent expression in surgical specimens. *Cancer Res* **58:** 3132–3141.