Cancer Immunity (12 June 2008) Vol. 8, p. 10
Submitted: 29 January 2008. Resubmitted: 17 April 2008. Accepted: 23 May 2008.

CANCER IMMUNITY
Article

Dysregulation in immune functions is reflected in tumor cell cytotoxicity by peripheral blood mononuclear cells from head and neck squamous cell carcinoma patients

Anamika Bose[1], Tathagata Chakraborty[1], Krishnendu Chakraborty[1], Smarajit Pal[2] and Rathindranath Baral[1]

[1]*Department of Immunoregulation and Immunodiagnostics, Chittaranjan National Cancer Institute, Kolkata, India*
[2]*Unit of Clinical Biochemistry, Chittaranjan National Cancer Institute, Kolkata, India*

Communicated by: PK Srivastava

We assessed the immunological status of stage III and IV head and neck squamous cell carcinoma (HNSCC) patients and age-matched healthy individuals. In HNSCC patients, the total leukocyte count was lower and the proliferating ability of PBMCs against phytohemagglutinin (PHA) was significantly downregulated. These cells showed lower expression of the early activation marker CD69. Within this PBMC population, the proportion of CD4+, CD8+ T cells, CD3- CD56+, CD16+ NK cells and CD3+ CD56+ NK-T cells was seriously downregulated. However, the proportion of CD4+ CD25+ Foxp3+ regulatory T cells having suppressor function was upregulated. Other immune cells, like CD14+ monocytes/ macrophages and CD20+ B cells, were also fewer in number, although this difference was not statistically significant. Assessment of the cytokine secretory status of PBMCs revealed suppressed levels of Th1 cytokines (IFN-γ, IL-12 and TNF-α) and elevated secretion of Th2 cytokines (IL-4 and IL-10) for HNSCC PBMCs whereas just the opposite was seen for PBMCs from healthy individuals. Dysregulation in the profile of immunocompetent cells and cytokine secretion was reflected in the suppressed cytotoxic function of HNSCC PBMCs, as tested on KB (oral cancer), MCF7 (breast cancer), COLO205 (colon cancer), Jurkat (T cell leukemia), K562 (erythroleukemia) and U937 (monocytic lymphoma) cell lines. The observed decreased cytotoxicity of HNSCC PBMCs may be due to the downregulated expression of cytotoxic molecules (perforin, granzymeB and FasL) in HNSCC PBMCs. Assessment of the extent of immune dysfunction might help design immunotherapeutic protocols by incorporating any agent having immunomodulatory function.

Keywords: human, head and neck cancer, leukocytes, peripheral blood mononuclear cells, immunologic cytotoxicity

Introduction

The immune functions of patients suffering from advanced head and neck squamous cell carcinoma (HNSCC) are frequently suppressed (1, 2), thus these patients may not be considered potential subjects for any therapy targeting the immune system (3). Currently, interferon-alpha2b is considered a promising mode of treatment for various types of cancers (2). This therapy is applied particularly in those cancers where patients are immunocompetent, e.g., melanoma, renal cell carcinoma, etc. (4). Unfortunately, due to the immunosuppression seen in HNSCC, this disease is rarely considered suitable for treatment with such therapy. After our initial success in immunopotentiation with interferon-alpha2b on peripheral blood mononuclear cells from HNSCC patients in *in vitro* studies, we have planned to use this therapy on HNSCC patients attending a cancer clinic at Kolkata, India. The primary objective is to stimulate the suppressed immune system to co-participate in tumor cell cytotoxic functions of chemotherapy and radiotherapy. However, methodical analysis of the degree and extent of immunosuppression of HNSCC patients is still lacking.

In this perspective, the present study was designed to analyze the immune status of HNSCC patients in detail to obtain a clear idea of their innate, humoral and cellular immune functions. This introductory study will set the groundwork to initiate a clinical study to modulate the suppressed immune functions of HNSCC patients by interferon-alpha2b.

Results

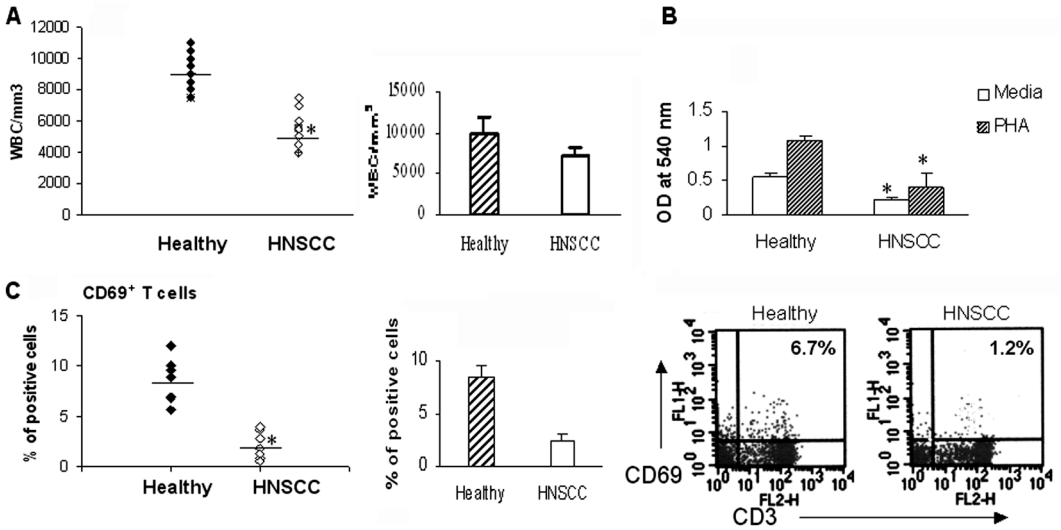
The proportion of leukocytes and their activation is reduced in HNSCC patients

Venous blood was collected from healthy individuals and HNSCC patients. The total leukocyte (WBC) count was determined and PBMCs separated. The total WBC count in HNSCC patients was significantly less than that in healthy individuals (Figure 1A). PBMCs isolated from the blood collected were incubated with or without PHA for 96 hours and proliferative activity was assessed by MTT assay. It is apparent from Figure 1B that proliferation of HNSCC PBMCs was significantly less than that of PBMCs from healthy individuals. After PHA induction, the increase in proliferation of HNSCC PBMCs was relatively less than that of PBMCs from healthy individuals. PHA stimulated HNSCC PBMCs demonstrated less activation, as evidenced by lower expression of the early activation marker CD69 (Figure 1C).

Various immunocompetent cells are dysregulated in HNSCC

PBMCs obtained from HNSCC patients and healthy individuals were stained with surface markers for T cell subsets (CD4+ helper, CD8+ cytotoxic and CD4+ CD25+ Foxp3+ regulatory cells), B cells (CD20+), NK cells (CD3- CD56+, CD16+), NK-T cells (CD3+ CD56+), and monocytes (CD14+).

Figure 1



Leukocyte count, proliferation and activation of PBMCs. (A) Total WBC counts from HNSCC patients (n = 10) and healthy individuals (n = 10) presented in the form of individual (left) and cumulative (right) data. (B) PBMCs isolated from untreated HNSCC patients and healthy individuals (n = 10 in each case) were cultured *in vitro* for 96 h. Proliferation was then determined by MTT assay. (C) Activation status of PBMCs assessed after 48 h by flow cytometry. Individual data (left), cumulative data (middle), as well as a representative figure of CD69 staining (right), are presented. For cumulative data, the mean ± SD are given in the bar diagram. *P<0.0001.

The percentage of CD4+ and CD8+ T cells was much lower in the patient population than in the healthy subjects (Figure 2A) but the ratio of CD4+/CD8+ cells was not significantly different between the two groups. In the case of HNSCC patients, the percentage of CD56+ and CD16+ NK cells (CD3-) and CD3+ CD56+ NK-T cells within the lymphocyte gated population was significantly lower than that seen for healthy individuals (Figure 2B). The CD56+ population could be subdivided into cytotoxic dim and secretory bright population. The CD56+ dim population was significantly decreased in HNSCC PBMCs in comparison to PBMCs from healthy individuals (Figure 2B). On the other hand, the percentage of CD4+ CD25+ Foxp3+ regulatory T cells was significantly higher among HNSCC PBMCs than in PBMCs from healthy individuals (Figure 2A). The percentage of CD20+ B cells and CD14+ monocytes was also slightly less in the HNSCC patient population (Figure 2C).

HNSCC is associated with downregulated Th1 cytokine secretion by PBMCs

PBMCs of HNSCC patients and healthy individuals were cultured *in vitro* for 72 hours and culture supernatants were collected to assess the level of the secreted Th1 cytokines IL-12, IFN-γ and TNF-α (Figure 3). The levels of IL-12, IFN-γ and TNF-α were significantly lower in HNSCC patients (IL-12: 13.09 ± 6.75 pg/ml; IFN-γ: 169.68 ± 59.88 pg/ml; TNF-α: 202.5 ± 68.14 pg/ml) than in healthy subjects (IL-12: 22.58 ± 2.95 pg/ml; IFN-γ: 409.56 ± 143.07 pg/ml; TNF-α: 285.62 ± 64 pg/ml). The same culture supernatants were assessed for the secreted Th2 cytokines IL-4 and IL-10 (Figure 4). The level of IL-4 was much higher in most of the fifteen patients studied (mean IL-4: 14.05 ± 5.25 pg/ml in patients vs. 9.33 ± 2.87 pg/ml in healthy individuals). The production of IL-10 was abnormally high (>130 pg/ml) in four patients, with the median value being much higher in the patient population than in healthy subjects (104.92 ± 29.27 pg/ml in patients vs. 66.88 ± 23.11 pg/ml in healthy individuals).

HNSCC PBMCs are less cytotoxic towards various tumor cells

PBMCs isolated from the blood of HNSCC patients and healthy individuals were incubated with various cancer cell lines (KB, MCF7, COLO205, Jurkat, K562 and U937) to assess the degree of cytotoxicity (Figure 5). The mean percent specific lysis of HNSCC PBMCs towards KB, MCF7, COLO205, Jurkat, K562 and U937 cells was 8.7%, 9.8%, 0.9%, 12.9%, 5.5% and 11.2%, respectively, significantly less than that seen with PBMCs from healthy subjects (27.0%, 31.9%, 27%, 22%, 17.9% and 34.5%, respectively).

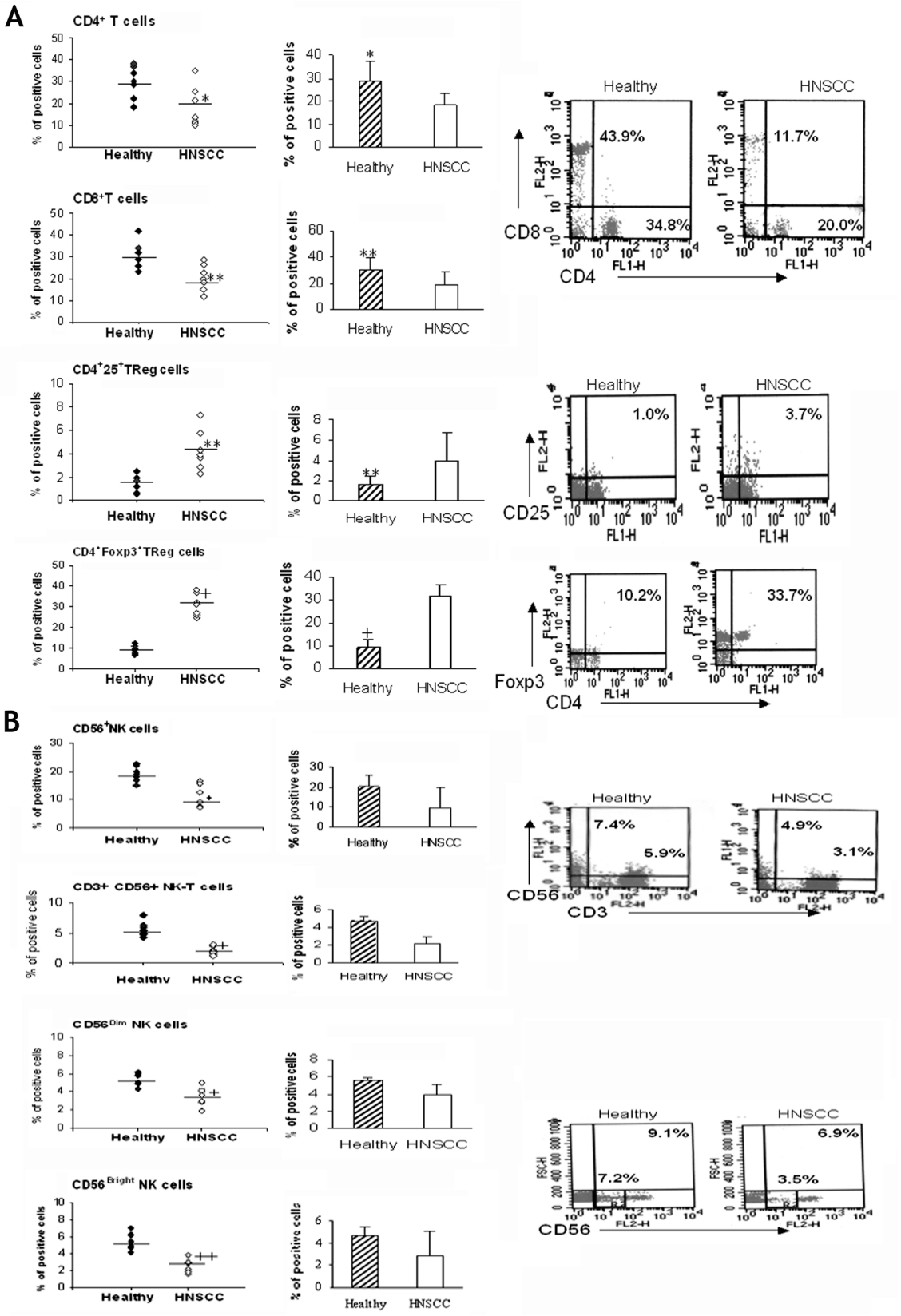
Poor cytotoxicity may be associated with downregulated expression of perforin, granzymeB and FasL in HNSCC PBMCs

Following-up on our observation of decreased cancer cell cytotoxic efficacy of HNSCC PBMCs in comparison to PBMCs from healthy individuals, we assessed the status of various cytotoxic molecules (perforin, granzymeB and FasL) to try to elucidate the basis of this decreased cytotoxicity. Intracellular expression of perforin, granzymeB and surface expression of FasL were studied on both CD56+ NK and CD8+ T cells (Figure 6, Table 1). In the case of PBMCs from HNSCC patients, the mean fluorescence intensity (MFI) of perforin- and granzymeB-expressing CD8+ T cells and CD56+ NK cells was significantly less than that of cells from healthy individuals. Expression of FasL on CD8+ T cells and CD56+ NK cells was also significantly less for HNSCC PBMCs than for PBMCs from healthy individuals.

Discussion

It is not a new observation for hemato-oncologists that cancer patients have comparatively lower counts of white blood cells (leukocytes). This condition is aggravated when patients are exposed to cancer treatments, including chemo- and radiotherapy. In HNSCC patients this situation is more pronounced as this type of malignancy is more

Figure 2


A
CD4+ T cells
% of positive cells
Healthy
HNSCC
CD8+T cells
CD4+25+TReg cells
CD4+Foxp3+TReg cells
Healthy
HNSCC
43.9%
34.8%
11.7%
20.0%
CD8
CD4
1.0%
3.7%
CD25
10.2%
33.7%
Foxp3
B
CD56+NK cells
CD3+ CD56+ NK-T cells
CD56Dim NK cells
CD56Bright NK cells
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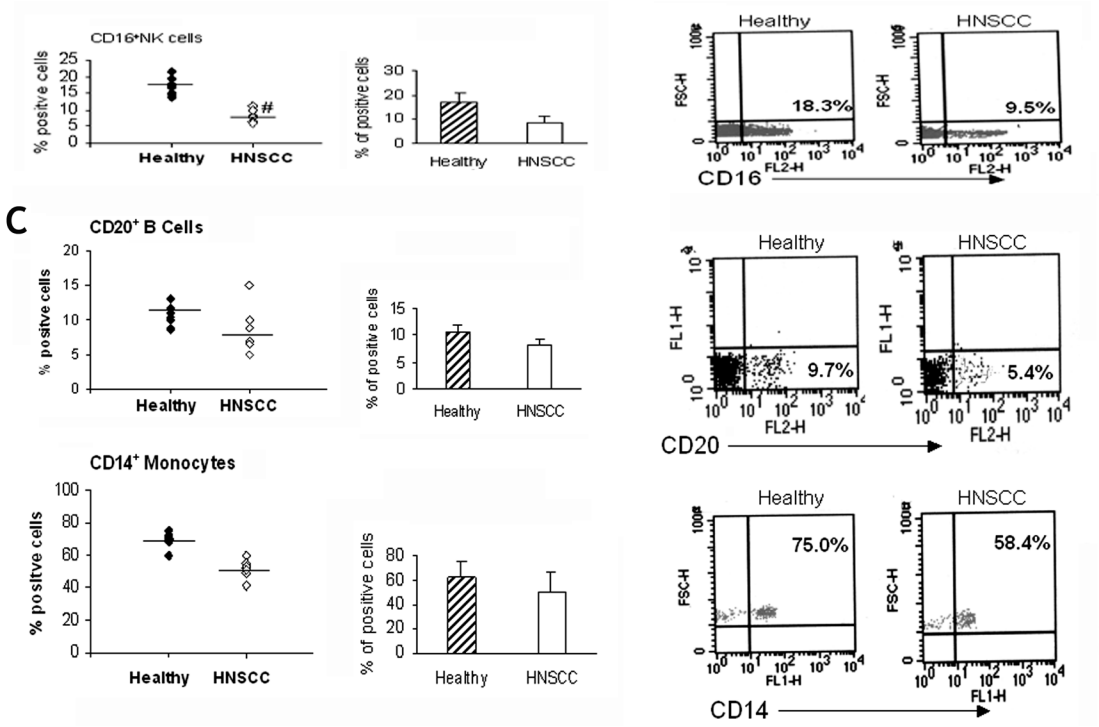
9.1%
7.2%
6.9%
3.5%



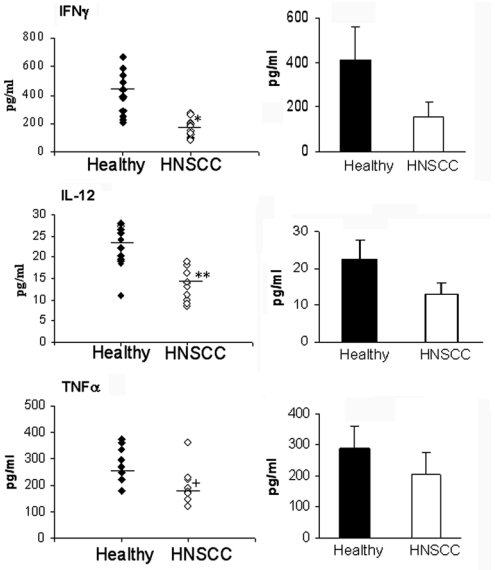
Analysis of surface phenotypic markers of various immune effector cells. PBMCs were isolated from HNSCC patients and healthy individuals (n = 10 in each case). Cells were labeled with different fluorescence-conjugated antibodies and the percentage of positive cells monitored by flow cytometric analysis. For each marker, data representing the mean ± SD from ten individuals (left), cumulative data (middle) and a representative figure (right) are shown. P values are as follows: *, 0.0118; **, 0.0230; +, 0.001; diamond, 0.0221; ++, 0.0014; #, 0.0014.

immunosuppressive than other types of cancers. In this context, optimum clinical outcome may be obtained by successful immunomodulation of HNSCC patients with an agent having immunostimulatory property. To define the point of immunomodulation and to select the proper immunomodulator, it is important to have an idea of the immune status of HNSCC patients. Some observations have already been published (5, 6), however without any report defining the complete immune status of HNSCC patients, including all cell types involved.

With the objective to implement an immunotherapeutic protocol for HNSCC by inducing tumor cell cytotoxicity by immune cells present in leukocytes or other immune compartments, we have first analyzed the complete immune status of patients suffering from HNSCC. In an effort to check the total white blood cell count from HNSCC patients, we observed a significant reduction in the leukocyte count in HNSCC patients. PBMCs present in this leukocyte population demonstrated less proliferative ability against PHA. These cells, while undergoing proliferation, were less active as demonstrated by the poor expression of the CD69 marker. Inactive PBMCs in reduced number definitely cause a reduction in the tumor killing activity. Moreover, the migratory ability of these PBMCs from HNSCC patients was significantly less than that of PBMCs from healthy subjects (data not shown). Dysregulated chemokine signaling in HNSCC may be the reason of impaired cellular migration (unpublished observation). Rectification of the migratory behavior of HNSCC PBMCs would be a vital step for immunomodulation.

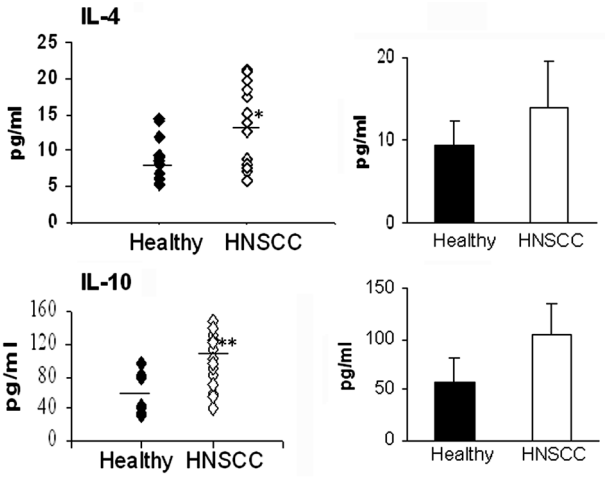
Next we have assessed the status of immunocompetent cells that may participate in the migration to the tumor site. Our results clearly defined that all classes of cytotoxic cells are significantly downregulated, with enhancement of the suppressor regulatory T cells. This situation reduces the tumor cell killing by immune cells and inactivates those cells still available for tumor killing. Mukhopadhaya *et al.* (7) studied the immune reactivity of lymphocytes from HNSCC patients and reported that tumor infiltrating lymphocytes showed a significant less proportion of CD3+ and CD4+ T cells. The percentage of NK cells (CD3- CD56+) was uniformly less in both tumor infiltrating lymphocytes and lymph node lymphocytes in HNSCC patients. In addition to NK cells (CD3- CD56+), we also found that the number of NK-T cells (CD3+ CD56+) was significantly reduced in HNSCC patients. NK-T cells make a functional link between innate and adaptive antitumor immunity (8) by sharing some receptors and functions between NK cells and αβ TCR-bearing T lymphocytes (9). NK-T cells exhibit characteristics of both Th1 and Th2 CD4+ T lymphocytes, together with NK cell activities, and thus may have a dual function in immune regulation and immune surveillance against tumors (10). Downregulation in this NK-T cell population definitely deteriorates various immune signaling cascades in HNSCC patients. This proportional and functional decrease in immunocompetent cells may be influenced by upregulation of CD4+ CD25+ Foxp3+ regulatory T cells. This special type of cells is not only involved in functional downregulation of T, NK or NK-T cells, but also seriously interferes with the antigen presentation function of various cells, including dendritic cells (11). Therefore upregulation of

Figure 3



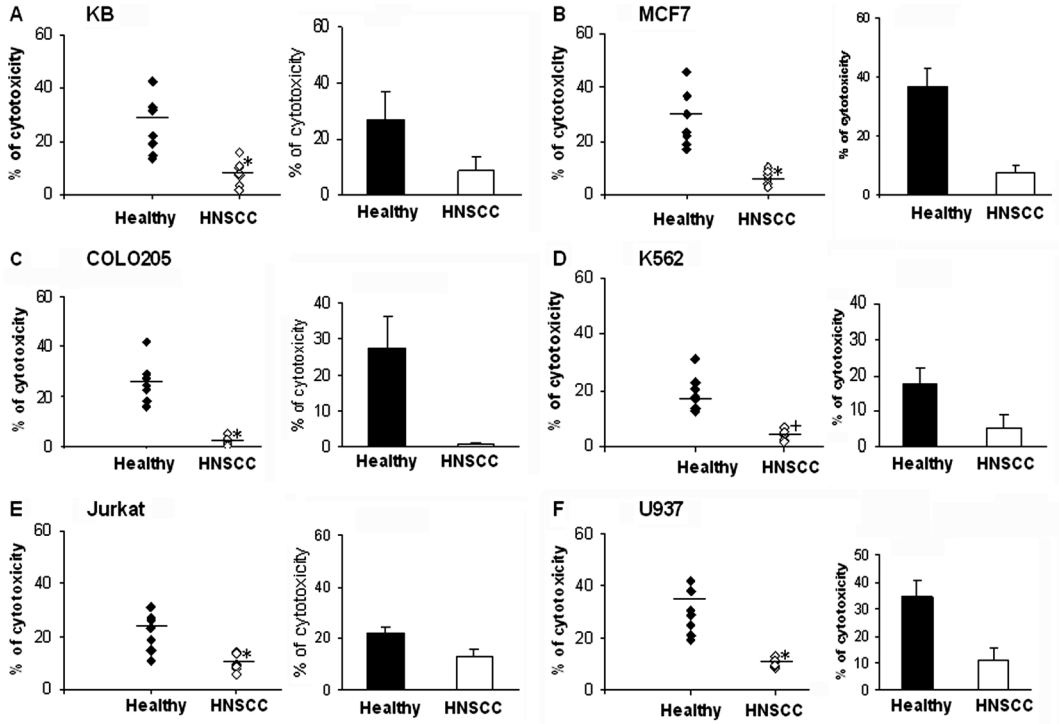
Secretory pattern of Th1 cytokines. PBMCs isolated from HNSCC patients and healthy individuals ($n = 15$ in each cases) were stimulated *in vitro* and culture supernatants were collected after 72 h to measure the levels of IFN-γ, IL-12 and TNF-α by ELISA. The mean ± SD from fifteen individuals (left) and cumulative data (right) are presented. Levels of Th1 cytokines in HNSCC patients were significantly lower than in healthy individuals. *P* values are as follows: *, 0.0001; **, 0.0007; +, 0.0247.

Figure 4



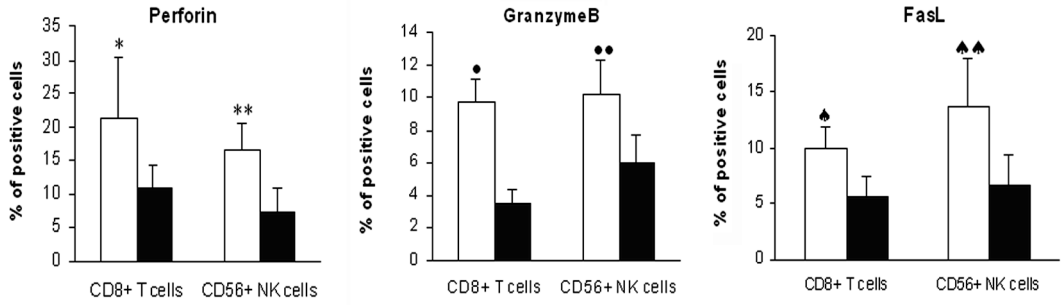
Secretory pattern of Th2 cytokines. PBMCs from HNSCC patients and healthy individuals ($n = 15$ in each case) were stimulated *in vitro* and culture supernatants were collected after 72 h to measure the levels of IL-4 and IL-10 by ELISA. The mean ± SD from fifteen individuals (left) and cumulative data (right) are presented. Levels of Th2 cytokines in HNSCC patients were significantly higher than healthy individuals. *P* values are as follows: *, 0.0226; **, 0.0047.

Figure 5



Cytotoxicity of PBMCs towards different cancer cells. PBMCs were isolated from HNSCC patients and healthy individuals ($n = 10$ in each case) and incubated with KB (A), MCF7 (B), COLO205 (C), K562 (D), Jurkat (E) and U937 (F) cells for 4 h. Cytotoxicity was then determined by the LDH release assay. The mean ± SD from ten individuals (left) and cumulative data (right) are presented. *P* values are as follows: *, <0.001; +, 0.0018.

Figure 6



Status of the cytotoxic molecules perforin, granzymeB and FasL. PBMCs isolated from HNSCC patients and normal individuals (n = 8 in each case) were cultured *in vitro* for 24 h. Cells were washed and stained for intracellular perforin or granzymeB (by anti-human perforin-FITC or anti-human granzymeB-FITC, respectively) along with PE-conjugated anti-CD8 or anti-CD56 antibodies. Similar cells were also stained with anti-human FasL along with PE-conjugated anti-CD8 or anti-CD56 antibodies. Staining status was assessed by flow cytometric analysis. The bar diagrams show the percentage of double positive cells from 8 HNSCC patients (white bar) and normal (black bar) individuals. For the comparison of PBMCs from HNSCC patients and normal individuals, P values are as follows: *, 0.0086; **, 0.0001; single diamond, <0.0001; two diamonds, 0.0006; single spade, 0.0004; two spades, 0.0020.

Table 1
Status of cytotoxic molecules.

Cytotoxic Molecule[1]	Healthy Individuals		HNSCC Patients	
	CD8+ T cells	CD56+ NK cells	CD8+ T cells (*P* value)	CD56+ NK cells (*P* value)
Perforin	71.15 ± 8.85	65.16 ± 13.00	56.56 ± 9.54 (0.0068)	40.15 ± 9.47 (0.0006)
GranzymeB	13.66 ± 3.60	11.69 ± 2.90	9.78 ± 2.54 (0.0259)	7.32 ± 2.10 (0.0039)
FasL	20.79 ± 7.20	13.56 ± 3.40	11.48 ± 6.30 (0.0156)	7.16 ± 3.60 (0.0026)

[1]Values represent the mean fluorescence intensity in each case.

immunocompetent cells, as well as downregulation of the regulatory T cells, is an important phenomenon to be considered for any immunotherapeutic strategy.

In our study, we found decreased secretion of Th1 cytokines (IL-12, IFN-γ and TNF-α) in all HNSCC patients studied compared to healthy controls. All of these cytokines are necessary for the potentiation of the cytotoxic activity of T cells and NK cells (12), and decreased secretion of these cytokines by HNSCC PBMCs is the potential reason of impaired cytotoxic function. IL-12 is released from activated monocytes and has been shown to promote the development of Th1 type T cells and to induce secretion of IFN-γ by activating T cells and NK cells. IFN-γ in turn activates macrophages and monocytes to release IL-12 (13). Furthermore, both IFN-γ and TNF-α possess direct cytotoxic and cytostatic activity towards tumor cells (14, 15). Lathers *et al.* (16) reported that cytokine levels in HNSCC patients suggested a shift to a Th2 bias, as levels of the Th2 cytokines IL-4, IL-6 and IL-10 were increased and the levels of the Th1 cytokine IFN-γ were decreased. These observations are largely the same as our results when we examine the production of IL-10 and IL-4 from PBMCs. In the patient population, both of these cytokines were at much higher levels than in healthy individuals. IL-10 is mainly produced by T helper 2 cells that inhibit cytokine synthesis by Th1 cells (17), an effect attributed to inhibition of the accessory function of macrophages, including downregulation of MHC class II expression, leading to impaired antigen presentation to reactive T cells (18). A much higher level of IL-4 than the mean levels of healthy individuals was observed in six out of seven patients and was correlated with IL-10 levels. Moreover, significant depletion of NK cells in HNSCC patients may participate in the development of M2 condition of macrophages, thereby enhancing the secretion of the Th2 cytokines IL-4 and IL-10 (19). The combination of IL-4 and IL-10 is able to temporarily suppress the generation of IFN-γ producing cells without affecting the polarity of T cells (20). IL-10-mediated decreased secretion of IFN-γ may impair the cytotoxic ability of HNSCC PBMCs. Monocyte-derived IL-10 also stimulates the functions of regulatory T cells which indirectly potentiate tumor growth (11).

This cytokine microenvironment, along with immune cellular dysregulation, may affect tumor cell cytotoxic functions. To obtain evidence in favor of this hypothesis, we have compared the cytotoxic ability of HNSCC PBMCs towards six different cancer cell lines with that of PBMCs from healthy subjects. HNSCC PBMCs were less cytotoxic to all cancer cells tested in comparison to PBMCs from healthy individuals. Dysregulated immune functions in HNSCC patients directly interfere with various cytotoxic functions, including non-specific macrophage/NK-mediated cytotoxicity, antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity and cytotoxic T lymphocyte reaction. Participating immune cells in these cytotoxic reactions are regulated by different cytotoxic molecules. In order to determine the basis of the suppressed tumor cell cytotoxicity by HNSCC PBMCs, we have assessed the status of perforin, granzymeB and FasL in effector cells. Our experimental data clearly show that these cytotoxic molecules are seriously downregulated in CD8+ T cells and CD56+ NK cells from HNSCC patients, thus being the basis of the impaired cytotoxicity of HNSCC PBMCs. In T cell-mediated responses, a critical balance exists between direct cytotoxicity mediated by

perforin and IFN-γ secretion that dictates the immune homeostasis (13, 14). In tumor models, perforin and IFN-γ has each been demonstrated to contribute to tumor incidence and metastasis (17, 18, 19). The IFN-γ secretory status in HNSCC patients is seriously downregulated and may cause cytotoxic impairment. In addition to these fundamental effector functions, cytokines also mediate their cytotoxic functions towards tumor cells. Therefore, an ideal immunotherapeutic strategy should target these key players of immune suppression to obtain optimum clinical benefit with the least toxicity.

Abbreviations

HNSCC, head and neck squamous cell carcinoma

Acknowledgements

We wish to thank Dr. Jaydip Biswas, Director of the CNCI, for providing the necessary facilities. AB and TC gratefully acknowledge the financial assistance given by University Grants Commission (UGC), India, through CSIR/UGC-NET fellowship grants. We also acknowledge the financial assistance in part from the Council of Scientific and Industrial Research, New Delhi (Project # 27(0162)/06/EMR-II).

References

1. Chen Z, Malhotra PS, Thomas GR, Ondrey FG, Duffey DC, Smith CW, Enamorado I, Yeh NT, Kroog GS, Rudy S, McCullagh L, Mousa S, Quezado M, Herscher LL, Van Waes C. Expression of proinflammatory and proangiogenic cytokines in patients with head and neck cancer. *Clin Cancer Res* 1999; **5:** 1369-1379. (PMID: 10389921)

2. Bose A, Ghosh D, Pal S, Mukherjee KK, Biswas J, Baral RN. Interferon alpha2b augments suppressed immune functions in tobacco-related head and neck squamous cell carcinoma patients by modulating cytokine signaling. *Oral Oncol* 2006; **42:** 161-171. (PMID: 16249117)

3. Qin H, Valentino J, Manna S, Tripathi PK, Bhattacharya-Chatterjee M, Foon KA, O'Malley BW Jr, Chatterjee SK. Gene therapy for head and neck cancer using vaccinia virus expressing IL-2 in a murine model, with evidence of immune suppression. *Mol Ther* 2001; **4:** 551-558. (PMID: 11735339)

4. Belardelli F, Ferrantini M, Proietti E, Kirkwood JM. Interferon-alpha in tumor immunity and immunotherapy. *Cytokine Growth Factor Rev* 2002; **13:** 119-134. (PMID: 11900988)

5. Shibuya TY, Kim S, Nguyen K, Parikh P, Wadhwa A, Brockardt C, Do J. Covalent linking of proteins and cytokines to suture: enhancing the immune response of head and neck cancer patients. *Laryngoscope* 2003; **113:** 1870-1884. (PMID: 14603040)

6. Woods KV, El-Naggar A, Clayman GL, Grimm EA. Variable expression of cytokines in human head and neck squamous cell carcinoma cell lines and consistent expression in surgical specimens. *Cancer Res* 1998; **58:** 3132-3141. (PMID: 9679981)

7. Mukhopadhyay R, Tatake RJ, Krishnan N, Rao RS, Fakih AR, Naik SL, Gangal SG. Immunoreactivity of lymphocytes from draining lymph nodes, peripheral blood and tumor infiltrates from oral cancer patients. *J Clin Lab Immunol* 1989; **30:** 21-25. (PMID: 2641790)

8. Yokoyama WM, Kim S, French AR. The dynamic life of natural killer cells. *Annu Rev Immunol* 2004; **22:** 405-429. (PMID: 15032583)

9. Lanier LL. NK cell receptors. *Annu Rev Immunol* 1998; **16:** 359-393. (PMID: 9597134)

10. Brutkiewicz RR, Sriram V. Natural killer T (NKT) cells and their role in anti-tumor immunity. *Crit Rev Oncol Hematol* 2002; **41:** 287-298. (PMID: 11880205)

11. Zou W. Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol* 2006; **6:** 295-307. (PMID: 16557261)

12. Young MR, Young ME, Wright MA. Myelopoiesis-associated suppressor-cell activity in mice with Lewis lung carcinoma tumors: interferon-gamma plus tumor necrosis factor-alpha synergistically reduce suppressor cell activity. *Int J Cancer* 1990; **46:** 245-250. (PMID: 2143498)

13. Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-gamma. *Annu Rev Immunol* 1997; **15:** 749-795. (PMID: 9143706)

14. Ruggiero V, Latham K, Baglioni C. Cytostatic and cytotoxic activity of tumor necrotic factor on human cancer cells. *J Immunol* 1987; **138:** 2711-2717. (PMID: 3031163)

15. Herskind C, Fleckenstein K, Lohr J, Li CY, Wenz F, Lohr F. [Antitumoral action of interferons and interleukins in combination with radiotherapy. Part I: immunologic basis.] [Article in German]. *Strahlenther Onkol* 2004; **180:** 187-193. (PMID: 15057428)

16. Lathers DM, Achille NJ, Young MR. Incomplete Th2 skewing of cytokines in plasma of patients with squamous cell carcinoma of the head and neck. *Hum Immunol* 2003; **64:** 1160-1166. (PMID: 14630398)

17. de Waal Malefyt R, Yssel H, Roncarolo MG, Spits H, de Vries JE. Interleukin-10. *Curr Opin Immunol* 1992; **4:** 314-320. (PMID: 1418711)

18. Lüscher U, Filgueira L, Juretic A, Zuber M, Lüscher NJ, Heberer M, Spagnoli GC. The pattern of cytokine gene expression in freshly excised human metastatic melanoma suggests a state of reversible anergy of tumor-infiltrating lymphocytes. *Int J Cancer* 1994; **57:** 612-619. (PMID: 8181865)

19. Geldhof AB, Van Ginderachter JA, Liu Y, Noël W, Raes G, De Baetselier P. Antagonistic effect of NK cells on alternatively activated monocytes: a contribution of NK cells to CTL generation. *Blood* 2002; **100:** 4049-4058. (PMID: 12393627)

20. Peng JK, Lin JS, Kung JT, Finkelman FD, Wu-Hsieh BA. The combined effect of IL-4 and IL-10 suppresses the generation of, but does not change the polarity of, type-1 T cells in Histoplasma infection. *Int Immunol* 2005; **17:** 193-205. (PMID: 15642955)

21. Bain BJ. Basic hematological techniques. In: *Practical Hematology.* 8th ed. Dacie JV, Lewis SM. (Eds.) Churchill Livingstone; Edinburgh; 1995; 49-82.

22. Baral RN, Chattopadhyay U. Neem (Azadirachta indica) leaf mediated immune activation causes prophylactic growth inhibition of

murine Ehrlich carcinoma and B16 melanoma. *Int Immunopharmacol* 2004; **4:** 355-366. (PMID: 15037213)

23. Bose A, Baral RN. Natural killer cell mediated cytotoxicity of tumor cells initiated by neem leaf preparation is associated with CD40-CD40L-mediated endogenous production of interleukin-12. *Hum Immunol* 2007; **68:** 823-831. (PMID: 17961770)

Materials and methods

HNSCC patients

Patients (30 male and 11 female) with histopathologically confirmed stage III and IV head and neck squamous cell carcinoma (HNSCC) and age-matched healthy individuals (11 male and 7 female) were included in this study before initiation of any treatment. Blood samples from cancer patients and healthy individuals were collected after their informed consent.

Tumor cell lines

KB (oral cancer) and MCF7 (breast cancer) cells were maintained in minimum essential medium (Life Technologies, NY, USA), supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine and gentamycin (0.052 mg/ml) at 37°C with 5% CO_2. COLO205 (colorectal adenocarcinoma), Jurkat (leukemic T cell), K562 (erythroleukemic cell) and U937 (lymphoma) cells were maintained in RPMI 1640 (Life Technologies, NY, USA), supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine and gentamycin (0.052 mg/ml) at 37°C with 5% CO_2.

Leukocyte count

Blood was collected from HNSCC patients and healthy individuals and the total leukocyte (WBC) count determined. Total WBC count was performed using WBC diluting fluid (3% glacial acetic acid) according to a standard procedure using a hemocytometer (21).

PBMC culture

PBMCs from HNSCC patients and healthy donors were isolated from heparinized venous blood by density gradient centrifugation over Ficoll-Hypaque. Isolated PBMCs were cultured in RPMI 1640 medium (Life Technologies, NY), supplemented with 10% FBS, penicillin (50 units/ml), streptomycin (50 µg/ml) and gentamycin (0.052 mg/ml) at 37°C in a humidified atmosphere with 5% CO_2, with phytohemagglutinin (PHA) (5 µg/ml). Seventy-two hours after culture, supernatants were collected, stored at -80°C and used for the measurement of cytokine secretion.

PBMC proliferation

PBMCs isolated from healthy individuals and HNSCC patients were taken in RPMI 1640 medium containing 10% FBS penicillin (50 units/ml), streptomycin (50 µg/ml) and gentamycin (0.052 mg/ml). Cells (2×10^5) were plated in a 96-well round-bottom microtiter plate with or without phytohemagglutinin (PHA) (5 µg/ml) and incubated for 96 h at 37°C in a humidified atmosphere with 5% CO_2. Proliferation was checked by MTT assay as described earlier (22). In brief, the plate containing the cell culture was centrifuged and the medium was partially aspirated. An aliquot of MTT solution (20 µl of a 5 mg/ml solution) was then added to each well and the plates were incubated for 4 h at 37°C. The medium was removed by aspiration and the purple-colored formazon precipitate was dissolved in DMSO (100 µl) and the absorbance measured at 540 nm using a microplate reader (Tecan Spectra, Grodig, Austria).

Flow cytometric analysis of immune cellular markers and FasL

Flow cytometric analysis of the surface phenotypic markers of PBMCs was performed by direct staining of whole blood. Blood (100 µl) was labeled with 20 µl of different anti-human fluorescence-conjugated antibodies (single staining with CD4-FITC, CD8-PE, CD56-PE, CD16-PE, CD20-PE, CD14-FITC, CD69-FITC and double staining with CD3-PE/CD56-FITC from BD Pharmingen, San Diego, CA) and CD178-FITC (e-Biosciences, San Diego, CA) for 30 min as per the manufacturer's recommendations. After labeling, the RBCs were lysed with FACS lysing solution (BD Pharmingen, San Diego, CA), washed and then fixed in 1% paraformaldehyde in PBS and cytometry was performed by using Cell Quest software on a FACScan flow cytometer (Becton Dickinson, Mountainview, CA). Suitable negative isotype controls were used to rule out the background fluorescence. The data was generated by cytofluorometric analyses of 10,000 events. The percentage of the population positive for each marker was determined using quadrant statistics.

Flow cytometric analysis of regulatory T cells

PBMCs of HNSCC patients and healthy individuals were cultured for 24 h, non-adherent cells were washed and surface-stained with either both anti-CD4-FITC and anti-CD25-PE, or with anti-CD4-FITC and the intracellular component stained for Foxp3. After fixation and permeabilization using CytoFix/CytoPerm solutions (BD-Pharmingen, San Diego, CA), the cells were stained for intracellular Foxp3 with PE-conjugated antibody (anti-human Foxp3-PE). Cells were analyzed on a FACSCalibur using CellQuest software. Suitable negative isotype controls were used to rule out the background fluorescence. The data was generated by cytofluorometric analyses of 10,000 events. The percentage of the population which was double positive was determined using quadrant statistics.

Extracellular secretion of cytokines

Th1 cytokines (IFN-γ, TNF-α and IL-12) and Th2 cytokines (IL-4 and IL-10) were measured in PBMC culture supernatants by ELISA using commercially available kits (OptEIA™, BD Pharmingen). In brief, 96-well microtiter plates were coated with capture antibodies (anti-IFN-γ/anti-TNF-α/anti-IL-12/anti-IL-4/anti-IL-10), incubated overnight at 4°C and blocked for 1 h. After washing, 100 µl of cell-free supernatant was added to each well and incubated for 2 h. Bound cytokines were detected using biotinylated mouse anti-human IFN-γ/TNF-α/IL-12/IL-4/IL-10, and subsequently avidin-horseradish peroxidase. Color was developed with TMB substrate solution (OptEIA™, BD-Pharmingen, San Diego, CA). Reaction was stopped with 2 N H_2SO_4 solution and absorbance was measured at 450 nm using a microplate reader (Tecan Spectra, Grodig, Austria).

Cytotoxicity assay

The cytotoxicity of cultured PBMCs (48 h) against different cancer cells was determined by LDH release assay (23) using a commercially available cytotoxicity detection kit (Roche Diagnostics, Mannheim, Germany). In brief, adherent KB, MCF7, COLO205 and Jurkat cells (1×10^4) were plated

overnight as target. Nonadherent K562 and U937 cells (1×10^4) were also taken in plates. PBMCs (1×10^5) from individual HNSCC patients ($n = 6$) and healthy individuals ($n = 6$) were added as effectors to each well and co-cultured for 4 h. Cell-free supernatants were used to measure the level of released LDH. The percentage cytotoxicity was determined as follows: % Cytotoxicity = [(Lysis from effector-target mixture - Lysis from effector only) - Spontaneous lysis] / [Maximum lysis - Spontaneous lysis] x 100.

Intracellular assessment of cytotoxic molecules, perforin and granzymeB

PBMCs of HNSCC patients and healthy individuals were cultured for 24 h, non-adherent cells were washed and stained with anti-CD8-PE or anti-CD56-PE. After fixation and permeabilization using CytoFix/CytoPerm solutions (BD-Pharmingen, San Diego, CA), the cells were stained for intracellular perforin (anti-human perforin-FITC) or granzymeB (anti-human granzymeB-FITC) antibodies. Cells were analyzed on a FACSCalibur using CellQuest software. Suitable negative isotype controls were used to rule out the background fluorescence. The data was generated by cytofluorometric analyses of 10,000 events. The percentage of the population which was doubly positive was determined using quadrant statistics.

Statistical analysis

Results representing data for individuals, as well as averages for the two study classes (HNSCC patients and healthy individuals), are presented as indicated in the figures. Triplicate assays were performed in case of ELISA and LDH release assay. Statistical significance was established by performing an unpaired *t*-test using INSTAT 3 Software (GraphPad Software Inc. San Diego, USA).

Contact

Address correspondence to:

Rathindranath Baral, Ph.D.
Department of Immunoregulation and Immunodiagnostics
Chittaranjan National Cancer Institute (CNCI)
37, S. P. Mookherjee Road
Kolkata-700026
India
Tel.: + 91 33 2476-5101, Ext. 334
Fax: + 91 33 2475-7606
E-mail: rbaral@hotmail.com