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Short Communication

Production of Interleukin-10 by Human Bronchogenic Carcinoma

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Interleukin-10 (IL-10) is a recently characterized cytokine with suppressive activity against various aspects of the cellular immune response. Our laboratory has previously demonstrated that another anti-inflammatory cytokine, IL-1 receptor antagonist (IRAP) is produced and secreted by human bronchogenic carcinomas. We speculated that tumor production of IRAP may mitigate host responses and confer increased tumor viability. In this study, we investigated the capacity of human bronchogenic tumors to produce IL-10 as another possible mechanism to attenuate host defenses. We found increased levels of antigenic IL-10 in tissue homogenates of human bronchogenic carcinomas compared with normal lung tissue (13.69 ± 2.87 versus 5.84 ± 0.84 ng/mg total protein). Immunohistochemical staining of tumors illustrate primary localization of antigenic IL-10 to individual tumor cells. Analysis of supernatants of several unstimulated human bronchogenic cell lines in vitro demonstrated the ability of tumor cells to constitutively produce IL-10. Functional studies of mononuclear cells, cultured in the presence of conditioned medium from a bronchogenic cell line, demonstrated their increased tumor necrosis factor and IL-6 production with the addition of neutralizing antibodies to IL-10. These findings demonstrate that human bronchogenic carcinomas elaborate functional IL-10, which may significantly impair immune effector cell function and enable the tumor to evade host defenses. (Am J Pathol 1994, 145:18–25)

Human bronchogenic carcinoma displays an extremely aggressive clinical course and represents the leading cause of malignancy-related mortality in the United States.[1] This behavior may reflect an increased capacity to evade detection and containment by host immune responses. Potential mechanisms that tumors may use to avoid host defenses include the expression of antigens that lack sufficient immunogenicity, the down-regulation of tumor cell surface major histocompatibility complex (MHC) molecules, rapid growth kinetics that may produce resistant cell lines, and the masking or shedding of tumor antigens.[2] Finally, tumors may also secrete factors that directly suppress inflammatory responses and specific immune cell functions.[2]

Interleukin-10 (IL-10) is a recently characterized cytokine that demonstrates varied immunosuppressive bioactivity. Since its initial isolation by Mosmann et al[3,4] in 1988, investigations have elucidated many of the immunological properties of this cytokine. Originally identified as a product of $CD4^+$ T cells, IL-10 is also produced by monocytes, macrophages, B cells, certain populations of $CD8^+$ T cells, and Epstein-Barr Virus (EBV)-transformed lymphoblastoid cells lines.[5–8] Recent work has

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demonstrated that keratinocytes may also elaborate IL-10.[9] A particularly interesting finding is that a previously uncharacterized open reading frame, BCRF-1, in the EBV genome bears striking homology with IL-10.[6,10] Functional studies reveal that IL-10 has profound effects on monocytes, resulting in alterations in cell morphology and cytotoxicity, down-regulation of the expression of MHC class II antigens, and inhibition of proinflammatory cytokine production.[11–15] Furthermore, IL-10 also exerts direct effects on the growth and function of T cells, B cells, and mast cells.[4,16–20] These specific actions result in the capacity for IL-10 to attenuate a wide range of effector immune responses, including T cell cytokine production and antigen-specific proliferation, B cell immunoglobulin synthesis, and the elaboration of tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) by natural killer cells.[19,21–26] IL-10 may play an important role in homeostasis under normal circumstances and speculation has arisen that IL-10 biology may also have important consequences for certain infectious diseases, organ transplantation, immune tolerance, and cancer immunotherapies.

Previous work from this laboratory has demonstrated the capacity of human bronchogenic tumors to elaborate significant quantities of another immunosuppressive cytokine, IL-1 receptor antagonist protein (IRAP).[27] We have proposed that tumor secretion of IRAP may impair host responses and result in an increased ability of tumors to evade host defenses. Our earlier findings led us to further examine the immunosuppressive capacities of tumors and to postulate that bronchogenic carcinomas may also produce and secrete IL-10 as an additional means of attenuating normal host immune responses.

In this report we describe increased antigenic levels of IL-10 in homogenates of human bronchogenic carcinomas compared with normal lung tissue. Immunohistochemical staining of tumor tissue graphically localizes antigenic IL-10 to individual tumor cells, suggesting that the bronchogenic carcinomas rather than any infiltrating mononuclear cells are the primary source of increased IL-10 in tissue homogenates. Analysis of supernatants of human bronchogenic carcinomas maintained *in vitro* under unstimulated conditions demonstrate measurable quantities of IL-10 and confirm the capacity of tumor cells to produce and secrete IL-10. Finally, functional studies of mononuclear cells cultured in the presence of conditioned medium from a tumor cell line demonstrate increased mononuclear cell IL-6 and TNF-α production with the addition of neutralizing antibodies to IL-10.

Materials and Methods

Tissue Samples

Lung tissue specimens were obtained in a prospective fashion from 58 consecutive patients undergoing thoracotomy for resection of suspected primary bronchogenic carcinoma. Two specimens were obtained from each patient; one from the tumor and one from an area of normal lung distal to the tumor. After recovery from the operating room, tissue samples were promptly homogenized and processed for protein isolation. Additional specimens were fixed in 4% paraformaldehyde for 24 hours before transfer to 70% ethanol and subsequent paraffin embedding. Final pathological diagnoses were determined by review of specimen slides by university hospital pathologists. Specimens of squamous cell carcinomas and adenocarcinomas were included in our study. Those of mixed cellularity or other pathological histology were excluded, leaving 47 specimens to be included in our evaluation.

Reagents

Polyclonal anti-human IL-10-, IL-6-, and TNF-α-specific antiserum were produced by the immunization of rabbits with human recombinant IL-10 (Pepro Tech Inc., Rocky Hill, NJ), IL-6 (R&D Systems, Minneapolis, MN), and TNF-α (Genentech, San Francisco, CA), respectively, in multiple intradermal sites with complete Freund's adjuvant. The specificity of each of these resulting antibodies was assessed by Western blot analysis against human recombinant IL-10, IL-6, and TNF-α. Antibodies were specific in our sandwich ELISA without cross-reactivity to a panel of 12 human recombinant ILs, including IL-1α, IL-1β, IRAP, IL-2, IL-4, IFN-γ, and members of the C-C or C-X-C chemokine family.

Cytokine ELISA

Antigenic IL-10, TNF-α, and IL-6 were quantitated using a modification of a double ligand method as previously described.[28] Briefly, flat-bottom 96-well microtiter plates (Nunc Immuno-Plate I 96-F) were coated with 50 μl/well of the appropriate polyclonal antibodies (1 ng/μl in 0.6 M NaCl, 0.26 M H_3BO_4, and 0.08 N NaOH, pH 9.6) for 24 hours at 4 C and then washed with phosphate-buffered saline (PBS), pH

7.5, 0.05% Tween 20 (wash buffer). Microtiter plate nonspecific binding sites were blocked with 2% bovine serum albumin in PBS and incubated for 60 minutes at 37 C. Plates were rinsed three times with wash buffer and diluted (neat and 1:10) and samples (50 µl/well) were added, followed by incubation for 1 hour at 37 C. Plates were washed three times and 50 µl/well of biotinylated polyclonal rabbit or the appropriate anti-human IL-10, IL-6, or TNF-α antibodies (3.5 ng/µl in PBS, pH 7.5, 0.05% Tween 20, and 2% fetal calf serum added and plates incubated for 45 minutes at 37 C.

Plates were washed three times, streptavidin-peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA) added, and the plates incubated for 30 minutes at 37 C. Plates were washed three times and chromogen substrate (Bio-Rad Laboratories, Richmond, CA) added. The plates were incubated at room temperature to the desired extinction and the reaction terminated with 50 µl/well of 3 M H_2SO_4 solution. Plates were read at 490 nm in an automated microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). Standards were 1/2 log dilutions of recombinant IL-10, IL-6, and TNF-α from 100 ng to 1 pg/ml (50 µl/well). This ELISA method consistently detected specific cytokine concentrations in a linear fashion greater than 50 pg/ml. All tissue homogenate specimens were run in parallel for total protein content (Pierce, Rockford, IL).

Immunohistochemical Localization of Antigenic IL-10

Paraffin-embedded tissue was processed for immunohistochemical localization of IL-10 protein using a modification of our previously described technique.[27,29] Briefly, tissue sections were dewaxed with xylene and rehydrated through graded concentrations of ethanol. Tissue nonspecific binding sites were then blocked using normal goat serum (BioGenex, San Ramon, CA). Tissue sections were then washed and incubated with optimal concentrations of rat monoclonal (subclone JES3-9D7; PharMingen, San Diego, CA) antibodies to human IL-10 or equivalent concentrations of the same subclass rat IgG (rat IgG1; PharMingen). The tissue sections were washed and then incubated for 60 minutes with secondary goat anti-rat biotinylated antibodies (BioGenex). The tissue sections were then washed twice in TRIS-buffered saline and incubated with alkaline phosphatase conjugated to streptavidin (BioGenex). Fast Red (BioGenex) reagent was used for chromogenic localization of IL-10 antigen. After optimal color development, tissue sections were immersed in sterile water, counterstained with Mayer's hematoxylin, and coverslipped using an aqueous mounting solution.

Tumor Cell Culture Supernatants

The human bronchogenic carcinoma cell lines A549, A427, and Calu-6 (ATCC, Rockville, MD) were cultured in 35-mm tissue culture plates under standard conditions and overlaid with 1 ml of recommended medium. RPMI 1640 medium with 100 U/ml penicillin, 100 mg/ml streptomycin, 25 mM HEPES, 1 mM L-glutamine, and 10% fetal calf serum was used for A549 cells, whereas the A427 and Calu-6 lines were maintained in Eagle's minimum essential medium with nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% fetal calf serum.

Cell cultures were grown to 90% confluence, washed twice with appropriate medium, then overlaid with fresh medium (1 ml) and allowed to incubate for an additional 24 hours. Trypan blue staining demonstrated >90% viability. Conditioned medium was harvested and centrifuged 15 minutes at 600 *g* to remove any cellular debris then frozen at −20 C until further analyzed by specific ELISA. An additional, larger quantity of A549-conditioned medium for mononuclear cell studies was produced by culturing cells in 225-cm^2 flasks until confluent then washing twice and overlaying cells with fresh medium overnight before harvesting, centrifuging, and freezing the resulting conditioned medium.

Mononuclear Cell Isolation and Culture

Human peripheral blood mononuclear cells were isolated from healthy volunteers. Briefly, heparinized venous blood was obtained then processed by Ficoll-Hypaque centrifugation. Mononuclear cells were then resuspended in complete medium and washed three times. After counting cells with a hemacytometer using light microscopy, cells were washed again and resuspended at a concentration of 5×10^6 cell/ml in conditioned medium from A549 tumor cells in culture. Mononuclear cells were then cultured for 24 hours in 35-mm well plates alone and with 1:10 concentrations of either rabbit anti-human IL-10 or control sera. Supernatants were harvested, centrifuged, and frozen until analyzed by specific ELISA for IL-6 and TNF-α content.

Statistical Analysis

Data were analyzed by a Macintosh IIfx computer using the Statview II statistical package (Abacus Concepts, Inc., Berkeley, CA). Data were expressed as

mean ± SEM and compared using a Student's two-tailed *t*-test. Data were considered statistically significant if P values ≤ 0.05.

Results

We postulated that a potential mechanism that human bronchogenic carcinomas may use to attenuate host immune responses is the production and secretion of the immunoregulating cytokine IL-10. To test our hypothesis, we first analyzed and compared tissue homogenates of normal lung and lung tumors for the presence of IL-10. Our initial findings demonstrated 2.3-fold elevations in the levels of antigenic IL-10, normalized to total protein (TP), from bronchogenic carcinoma tissue compared with normal lung tissue homogenates ($P < 0.005$). As shown in Figure 1, tumor tissue-derived IL-10 was 13.69 ± 2.87 ng/mg TP compared with 5.84 ± 0.84 ng/mg TP for normal lung tissue ($n = 47$ patients). Subgroup analysis by specific histology revealed no difference in elevations of antigenic IL-10 for squamous carcinomas ($n = 21$) and adenocarcinomas ($n = 26$) with IL-10 levels of 15.41 ± 3.70 ng/mg TP and 12.29 ± 4.28 ng/mg TP, respectively. IL-10 content varied within normal and tumor tissue, as demonstrated by scattergram in Figure 1, B, with some degree of overlap.

To determine the cellular source of antigenic IL-10 in tumor tissue we used immunohistochemistry. Samples of normal lung and lung tumor tissue were fixed and immunostained with specific monoclonal antibodies for IL-10. Our results demonstrated that immunoreactive IL-10 in tumor tissue was primarily localized to individual tumor cells in a heterogeneous staining pattern for all tumors studied (Figure 2, C and D). In contrast, samples of normal lung tissue demonstrated no staining for IL-10 (Figure 2, B). Staining with control rat IgG demonstrated the absence of significant nonspecific staining of tumor tissue (Figure 2, A). In addition, stains using HAM56 (Enzo Diagnostics, Inc., Farmingdale, NY), a murine monoclonal antibody against human mononuclear cells,

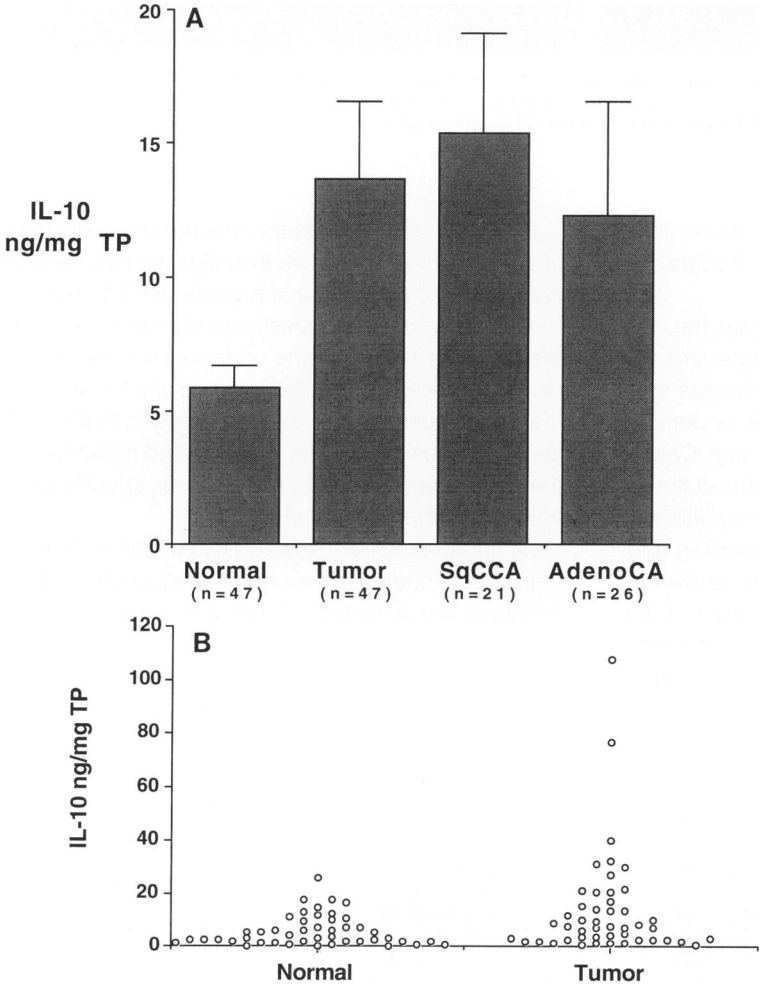

Figure 1. A: *Antigenic determinations of IL-10 in tissue homogenates for normal lung and human bronchogenic carcinoma tumor tissue* (n = 47). *Normal lung tissue IL-10 content = 5.84 ± 0.84 ng/mg TP. Tumor tissue IL-10 content = 13.69 ± 2.87 ng/mg TP. Tumor subgroup analysis by final histological diagnosis also included demonstrating squamous cell carcinoma* (n = 21) *and adenocarcinoma* (n = 26) *with IL-10 levels of 15.41 ± 3.70 and 12.29 ± 4.28 ng/mg TP, respectively.* B: *Scattergram demonstrating distribution of IL-10 content of normal and tumor specimens.*

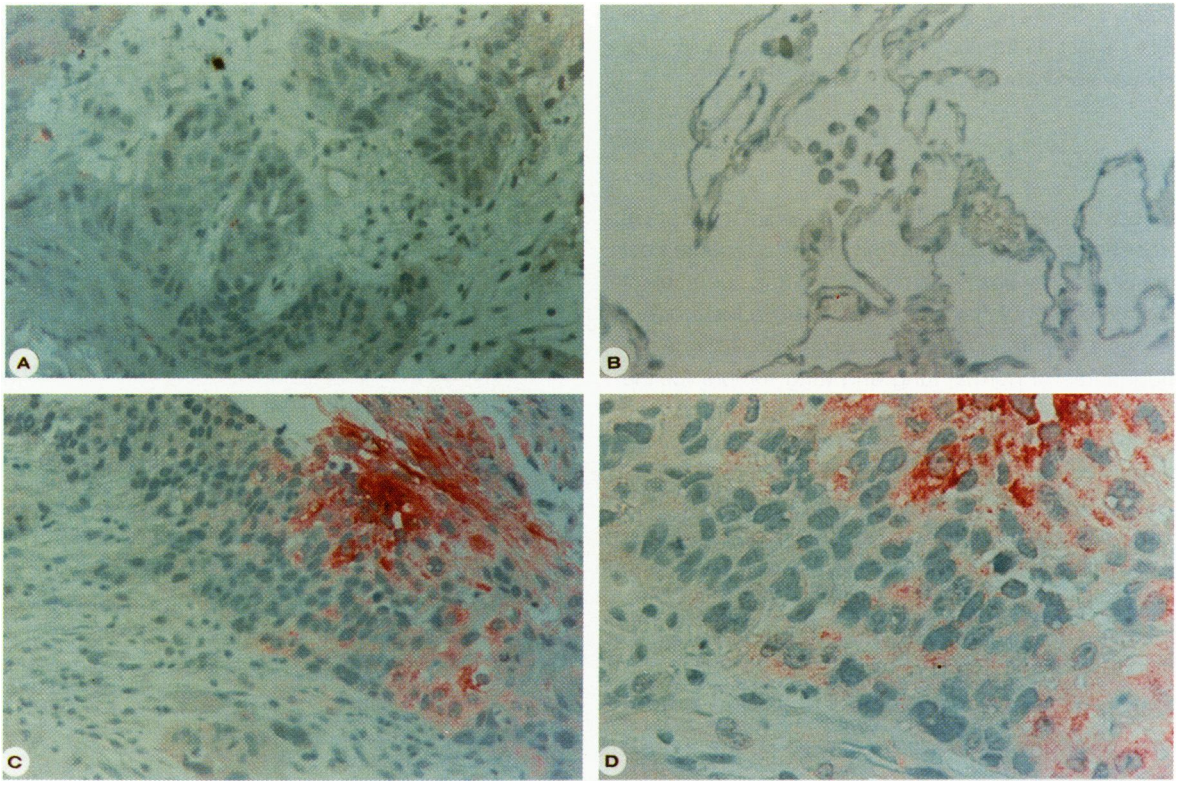

Figure 2. *Representative photomicrographs of immunohistochemical staining of IL-10 in human bronchogenic carcinoma. Demonstrates positive staining with specific anti-IL-10 antibody at ×200 and ×400 (***C** *and* **D***, respectively) and negative control (×200) incubated with preimmune serum (***A***). Staining of normal lung tissue for IL-10 demonstrates the absence of detectable antigenic IL-10 at this level of sensitivity (***B***).*

confirmed that tumor cells rather than immune cells were the primary source of antigenic IL-10 (data not shown).[30]

Furthermore, to definitively demonstrate the capacity of bronchogenic tumors to elaborate and secrete constitutive IL-10 we used *in vitro* studies with several available bronchogenic cell lines under unstimulated conditions. The A549, A427, and Calu-6 ATCC bronchogenic tumor cell lines were cultured unstimulated for 24 hours to generate conditioned medium. The supernatants were then harvested and assessed for IL-10 by specific ELISA. As shown in Table 1, analysis of cell-free conditioned medium from these cells maintained under unstimulated conditions demonstrated 6.27 ± 1.08, 1.87 ± 0.97, and 7.56 ± 1.07 ng/ml antigenic IL-10, respectively, for A549, A427, and Calu-6 cell lines.

Table 1. *Constitutive IL-10 Production from Bronchogenic Tumor Cell Lines*

Cell Line	IL-10 (*ng/ml*)
A549 (CCL 185)	6.27 ± 1.08
A427 (ATCC HTB 53)	1.87 ± 0.97
Calu-6 (ATCC HTB 56)	7.56 ± 1.07

Finally, to demonstrate the functional significance of tumor-derived IL-10, we investigated the effect of conditioned medium from a bronchogenic tumor cell line on the cytokine expression of mononuclear cells. We used the A549 cell line under unstimulated conditions to generate conditioned medium. Isolated human mononuclear cells were cultured in this conditioned medium also under unstimulated conditions in the presence of control antibodies or specific neutralizing antibodies to IL-10.

As shown in Table 2, the addition of anti-IL-10 neutralizing antibodies to the conditioned medium of the A549 cell line at the time of exposure to mononuclear

Table 2. *Cytokine Production from Mononuclear Cells Cultured in the Presence of Conditioned Medium from A549 Tumor Cells*

	TNF (ng/ml)	IL-6 (ng/ml)
Mononuclear cells alone	0.41 ± 0.04	2.11 ± 0.37
Mononuclear cells + control Ab	0.19 ± 0.21	1.87 ± 0.13
Mononuclear cells + anti-IL-10	1.74 ± 0.26	3.49 ± 0.63

cells resulted in increased monocyte TNF-α production compared with the addition of control antibodies. The resulting TNF-α levels in mononuclear cell supernatants were 1.74 ± 0.26 and 0.19 ± 0.21 ng/ml, respectively, for cells cultured with anti-IL-10 and control antibodies ($P < 0.05$). Similar, although not statistically significant, results were obtained when supernatants were evaluated for IL-6 production. Mononuclear cells cultured with control antibodies produced 1.87 ± 0.13 ng/ml IL-6, whereas cells cultured with anti-IL-10 generated 3.49 ± 0.63 ng/ml IL-6. The addition of control antibodies to mononuclear cell cultures resulted in no significant differences in IL-6 or TNF-α production.

Discussion

The clinical response to the treatment of human bronchogenic carcinoma is dismal. Despite extensive research and numerous clinical trials using various multimodality therapies, the 5-year survival rate remains less than 15%.[31] Although results to date have been mostly discouraging, most immunologists and cancer biologists feel that immunotherapy modalities represent the greatest promise for successful therapy for bronchogenic carcinoma and many other malignancies. A greater understanding of the interactions between tumors and the immune system is essential for the development of successful approaches with immunotherapy. Once the precise mechanisms used by tumors to evade host responses are understood, specific strategies can be used to circumvent these mechanisms and maximize immune effector cell activity.

Our study has noted significant increases in antigenic IL-10 detected in homogenates of human non-small cell bronchogenic carcinomas compared with normal lung tissue. This disparity was consistent for tumors of squamous cell and adenocarcinoma histologies. Further studies using immunohistochemical staining techniques graphically demonstrated individual tumor cells to be the primary source of antigenic IL-10. Investigations identifying mononuclear cells infiltrating tumor tissue revealed that local IL-10 was primarily tumor derived rather than a product of immune cells. The detection of significant quantities of IL-10 in the supernatants of several bronchogenic carcinoma cell lines definitively demonstrates the capacity of bronchogenic carcinomas to elaborate IL-10. Importantly, viability studies of these cell lines suggests that IL-10 may be actively secreted by these cells. Finally, functional studies examining the effects of conditioned medium from a bronchogenic carcinoma cell line on the capacity of mononuclear cells to elaborate cytokines revealed a specific inhibitory action by tumor-derived IL-10, which importantly was attenuated by the addition of anti-IL-10.

These results demonstrate that biologically active IL-10 is constitutively produced and is probably secreted by human bronchogenic carcinomas. Elevations of IL-10 within tumors may dramatically impair local host immune responses at a number of locations along immune pathways. Sentinel macrophages infiltrating tumor tissue would encounter increasing levels of IL-10. Sufficient levels of IL-10 may then exert inhibitory actions on these mononuclear cells, resulting in a marked diminution of their cytokine producing and antigen presentation capacities. Impaired macrophage function also indirectly inhibits T cell responses due to ineffective cell-to-cell communications. Finally, tumor-derived IL-10 may also directly affect T cell and natural killer cell activity. Thus, at nearly every level of immune defense to malignancy, IL-10 may represent a potent threat to impair host responses.

Our findings support the concept that human bronchogenic carcinomas may produce IL-10 as a means of escaping host defenses. Other workers have observed IL-10 in certain lymphoproliferative and dermal malignancies.[32–34] Additional studies are needed to definitively establish the functional significance of tumor-derived IL-10. Subsequent investigations regarding the expression of tumor-derived IL-10 may reveal potential regulatory networks leading to the production and regulation of IL-10. These networks may exploit local tissue responses and cytokine production by both resident stromal cells and infiltrating immune effector cells. As the complexities of tumor and immune cell interaction are further elucidated potential new sites for therapeutic intervention and immunomodulation may be discovered. Ultimately, it is hoped that these insights will lead to the development of effective therapies to counter tumor defenses and impact the significant mortality due to bronchogenic carcinomas and other malignant diseases.

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