Characteristics of PBMC obtained from leukapheresis products and tumor biopsies of patients with non-small cell lung cancer

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Abstract. The current study characterized peripheral blood mononuclear cells (PBMC) obtained from leukapheresis products of patients with non-small cell lung cancer (NSCLC) for cytokine release, the ability to incorporate tritiated thymidine following stimulation using PHA as well as the levels of both CD4 and CD8 regulatory T cells (Tregs) as defined by FoxP3 expression. Results were compared to normal donor PBMC obtained from buffy coat products. Heterogeneous levels of Th1 (γ interferon and IL-2), Th2 (IL-10 and IL-13), pro-inflammatory (TNF-α and IL-6) and the hematopoietic inducing cytokine GMCSF were detected from both populations of PBMC as measured using ELISA. Overall, we observed that combined levels of Th1 and Th2 cytokines were higher in lung cancer patients compared to that seen in normal donor PBMC. The increased cytokine production was coupled with an observed decrease in the ability of lung cancer patient PBMC to incorporate tritiated thymidine. Furthermore, cytokine containing supernatants obtained from patients inhibited the incorporation of tritiated thymidine from PBMC obtained from normal donors. Thus, the combined cytokines which included high levels of IL-10, appeared to exhibit suppressive functional activity. While not statistically significant, the overall trend toward a Th2 cytokine environment was supported by an increased level of Tregs observed in the leukapheresis products of lung cancer patients. These levels were variable and were accompanied by higher than normal levels of CD8$^+$ cells co-expressing FoxP3. Finally, tumor biopsies were examined from lung cancer patients along with autologous normal adjacent tissue (NAT). In these studies, both γ interferon and IL-10 were detected. The levels of IL-10 in the LPS stimulated cultures were statistically greater from the cancer biopsies compared to the NAT. The current study confirms many earlier results in a comprehensive manner and extends the analysis to leukapheresis products. An environment is described in cancer patients which is characterized by increased cytokine production and decreased proliferative potential likely under the influence of a significant population of regulatory T cells (Tregs). Taken together, these results are discussed as they relate to the potential implications in lung cancer patients immune response to their disease.

Introduction

Lung cancer is the leading cause of death due to cancer in both men and women (1,2). Of these cases of lung cancer, approximately 80% are non-small lung cancer (NSCLC) (1). When diagnosed, the disease is often at an advanced stage and difficult to treat. The accepted therapy for NSCLC depends upon the stage in which the patients present (3). The treatments include: surgery for early-diagnosed and localized disease; radiation therapy for residual disease in the surgical field as well as un-resectable lesions; and/or chemotherapy for systemic and micro-metastatic disease. Unfortunately, 5-year survival following these conventional approaches still remains low, estimated in the range of 5-20% (4,5).

As is the case with most forms of cancer, NSCLC tumors initiate and progress despite potential for the development of both innate and acquired anti-tumor immune responses. Tumor antigen specific cytotoxic T lymphocytes (CTL) (6,7) and antibodies (8,9) have been described and their anti-tumor activities well documented. However, these anti-tumor effector mechanisms are regulated and many times limited in their effectiveness by two types of suppressor cells. A lymphoid component, regulatory T cells (Tregs) (10-12) and a myeloid component, the myeloid derived suppressor cell (MDSC) (13). These cell types are operational in both afferent and efferent phases of immune responses. Suppressor activity is mediated through either direct cell contact or the release of cytokines such as IL-10 and TGF-ß. Combinations of these and other cytokines, released by immune effector cells, connective tissue cells and tumor cells, condition the micro-environment and promote or inhibit the development of an effective anti-tumor immune response.

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Cytokines, the soluble mediators of immunity, were grouped by Mosmann *et al* (14), into two types, T helper 1 and T helper 2, whose activity correlated with responses to different microbiologic infection (15). Th1 responses are characterized by γ interferon release from T cells when challenged with intracellular pathogens including viruses and some bacteria. Th2 responses are characterized by IL-4 release effective against extracellular bacteria and parasites. Recently a family of T helper 17 (Th17) cytokines (16) were described and are important in responses to extracellular pathogens and in the induction of inflammation (17). Interestingly, Th1 and Th2 cytokines such as γ interferon and IL-4 respectively can inhibit each other's action (18,19). A typical example is the inhibition of Th2 or humoral responses by Th1 cytokines and the subsequent inhibition of Th1 or cell mediated immune (CMI) responses by Th2 cytokines. Thus an inherent balance is built into the system preventing over-responsiveness to both intra- and extracellular organisms. In the response to tumors, where Th1 or CMI responses are crucial, studies using ELISA or flow cytometric analysis of intracellular cytokines have demonstrated that PBMC from cancer patients release the Th2 cytokines IL-4 and IL-10 preferentially when compared to PBMC obtained from normal donors (20-22). Thus, the suggestion has been that the micro-environment conditioned by these cytokines is inhibitory to effective Th1 anti-tumor responses.

The present study was done to compare the cytokine environment conditioned by PBMC obtained from leukapheresis products of patients with NSCLC. Twenty-nine NSCLC patients included in a dendritic cell (DC) vaccine trial performed by our group were chosen for analysis (23,24). PBMC of these patients were compared to PBMC obtained from 10-normal donor buffy coats. The PBMC were analyzed for cytokine release to both T cell and CD14+ cell stimuli. Overall, the levels of cytokines secreted from lung cancer patient PBMC were greater than that observed from normal donors. A trend, although not significant, was observed in that higher levels of both Th1 and Th2 cytokines increased as the stage of the cancer increased. This was coupled with decreased thymidine incorporation of T cells in patients as their stage increased from I to III. In addition, flow cytometric analysis revealed higher levels of regulatory T cells (Tregs) in the leukapheresis products of NSCLC patients. Finally, tumor biopsies from NSCLC patients were compared to autologous normal adjacent tissue for their ability to secrete cytokines in response to both T cell and CD14 cell stimuli. The cytokine environment described in the current report is one characterized by high levels of cytokines with potential for inhibition due to the levels of IL-10 observed. This coupled with the high levels of Tregs suggests that immune response to lung cancer could be diminished. In addition, implications are discussed when considering the use of leukapheresis products as a source of anti-tumor effector cells to generate reagents for immunotherapy studies.

Materials and methods

Patients. Leukapheresis products were obtained from NSCLC patients. All stages (I-IV) and histological types (adenocarcinoma, squamous cell carcinoma, bronchio-alveolar cancer,

Table I. Dendritic cell vaccine patient characteristics.

Patient[a]	Histology[b]	Stage[c]	Treatment[d]
DC1	Sq	IIIA	Sg, Ch, XRT
DC2	Ad	IIIA	Ch, XRT
DC3	Sq	IIIA	Sg
DC4	Ad	IIB	Sg
DC5	Sq	IIIA	Ch, XRT
DC6	Ad	IB	Sg
DC7	Ad	IIIA	Sg
DC8	BAC	Multifocal	Sg
DC9	Ad	IA	Sg
DC10	Ad/N	IB	Sg, Ch, SRT
DC11	Ad	IB	Sg
DC12	Ad	IIIB	Ch, XRT
DC13	Sq	IIIA	SG, XRT
DC14	Ad	IIIB	Ch, XRT
DC15	Ad	IIIA	Ch, Sg, XRT
DC16	Ad	IIIA	Sg, Ch, XRT
DC17	LC	IB	Sg
DC18	Ad	IIIB	Ch, XRT
DC19	Sq	IIIB	Ch, XRT
DC20	Ad	IA	Sg
DC22	Sq(BAC)	IIIA	Ch, XRT
DC23	Ad/Sq	IB	Sg, Ch, XRT
DC24	LC	IIIA	Ch, XRT
DC25	Sq	IIIA	Ch, XRT
DC26	Ad	IA	Sg
DC27	BAC	IA	Sg
DC28	LC	IB	Sg, Ch, XRT
DC29	Sq	IIB	Sg, Ch
DC30	Ad	IIIB	Ch, XRT
DC31	Ad	IA	Sg
DC32	BAC	IB	Sg, Ch
DC33	Ad	IIIA	Sg, Ch, XRT
DC34	Sq	IIIB	Ch, XRT
DC35	Ad	IIIA	Sg
DC36	BAC	Multifocal	Ch

[a]Patients included in the current study were part of a vaccine clinical trial conducted by our group as previously described by Hirschowitz *et al* (23). [b]Histology of the tumors included: Ad, adenocarcinoma; Sq, squamous cell carcinoma; LC, large cell carcinioma, BAC, bronchio-alveolar cancer; N, neuroendocrine; when 2 are listed it is a mixed disease. [c]Stage of cancer at entry into the protocol. [d]Prior therapy when patient entered the protocol. Sg, surgery; Ch, chemotherapy; XRT, radiation therapy.

large cell carcinoma and neuroendocrine) associated with the disease were represented in the study. Most patients had conventional therapies including surgery, chemotherapy, and/or radiation therapy for their disease (Table I). The PBMC were obtained at least 6 weeks after the delivery of conventional therapies and at least 3 weeks prior to receiving immuno-

therapy. The immunotherapy consisted of autologous DCs pulsed with apoptotic bodies derived from the allogeneic NSCLC cell line 1650 as previously described (23,24).

Preparation of PBMC. Leukapheresis products were obtained from the University of Kentucky Hospital under local IRB approval and normal donor buffy coats were purchased from the Kentucky Blood Center. Both products were subjected to Ficoll Hypaque purification as previously described (7). In brief, cell products were diluted 1:1 in sterile PBS and layered onto Ficoll Hypaque in 50-ml sterile centrifuge tubes (13 ml Ficoll Hypaque; 30 ml cell containing solution). Tubes were centrifuged at 400 x g for 30 min. The buffy coats were removed and washed four times in medium containing FBS. Peripheral blood mononuclear cells were counted and assessed for cell viability using Trypan Blue analysis. PBMC were cryopreserved as previously described (25) for future immunological analysis.

Preparation of lung tumors and normal adjacent tissue. Fresh NSCLC tumor biopsies and normal adjacent lung tissue were obtained from the Cooperative human tissue network (CHTN). In a few cases, samples were obtained from the University of Kentucky Hospital under IRB approval. The tumor and NAT were given an identifier (UKY plus a number). The samples were minced to 3-mm^3 chunks and enzyme digested at 37˚C (5% CO_2 in air environment) to single cell suspensions as previously described (26). The diced tissue samples were mixed with enzyme medium (40 ml of enzyme medium per gram of tissue) (27) and placed in sterile bottles containing a magnetic stir bar for 12-18 h. The enzyme medium was comprised of RPMI-1640 (Gibco-BRL) supplemented with DNAse (30,000 units/ml), collagenase (1 g/l), hyaluronidase (100 mg/l), penicillin (50 μg/ml), streptomycin (50 μg/ml), amphotericin (50 μg/ml), and gentamycin sulfate (10 μg/ml). Following digestion, the samples were strained through a sterile 70-μm mesh filter to remove clumps of fibrous material, centrifuged to collect cells and washed with PBS containing 5% fetal bovine serum (FBS) to neutralize residual enzymes in the medium. The cells were resuspended in X-VIVO 15 serum free medium, counted and assessed for cell viability using Trypan Blue exclusion technique. Single cell suspensions of solid tumor NAT which were <60% viable were cleaned up using Ficoll Hypaque gradients as described above.

Cytokine release studies. Single cell suspensions of NSCLC tumor biopsy, NAT or PBMC obtained from NSCLC patients or normal donors were placed in cell culture at 1.0x10^6 cells per ml of culture medium, 1 ml/well of 24-well sterile cell culture plates. The medium was X-VIVO 15 serum free medium containing penn/strep and gentamycin. The cultures were either established alone or in the presence of plastic bound anti-CD3 (OKT3; 1 μg/ml of binding buffer) and 10 μg/ml soluble anti-CD28 mAb. PBMC from NSCLC patients and normal donors were also stimulated with the anti-TLR4 agonist LPS (10 μg/ml). The culture supernatants were removed after 24 h, centrifuged at 400 x g to remove residual cells, and cryopreserved until analyzed using commercial ELISA kits. The ELISA kits utilized were all obtained from BD PharMingen. The cytokines and their assay sensitivities were: γ interferon, 4.7-300 pg/ml; IL-2, 7.8-500; TNF-α, 7.8-500; GMCSF, 4.7-300 pg/ml; IL-6, 4.7-300 pg/ml; IL-10, 8-500 pg/ml; IL-13, 1.56-44.4 pg/ml; and IL-17, 0.75-100 pg/ml.

Cell surface phenotyping: assessment of Tregs. Regulatory T cells (Tregs) were assessed by direct surface and intracellular staining with fluorescent antibodies detected using flow cytometry. Expression of Foxp3, a member of the forkhead/winged-helix family of transcription factors, is considered a phenotypic marker for Tregs (28-31). PBMC, and cells extracted from tumor and normal adjacent lung tissue, were washed, counted, and resuspended at 1x10^6 per tube in 100 μl staining buffer (PBS containing 1% FBS and 0.1% sodium azide). Cells were surface stained with CD8 FITC, CD25 PE and CD4 PerCP (BD PharMingen) at 4˚C in the dark for 30 min, then washed three times with 2 ml cold staining buffer. Intracellular staining was performed with APC anti-human Foxp3 staining set (eBioscience). Briefly, cells were resuspended in 1 ml freshly diluted Fix/Perm solution, incubated at 4˚C in the dark for 30 min, washed three times with permeabilization buffer and stained at 4˚C in the dark for an additional 30 min. Tubes were washed twice, resuspended in 100 μl staining buffer and analyzed on a BD FACScalibur with CellQuest software. Lymphocytes were gated by FSC/SSC and data are presented as percent positive of this gate. In addition, standard 2-color analysis was done to assess leukocyte subsets in the PBMC samples using direct FITC and PE labeling. The antibodies included: anti-CD3 (T cells), anti-CD4 (helper T cells), anti-CD8 (cytolytic T cells) and anti-CD14 (monocytes).

Assessment of tritiated thymidine incorporation by PHA stimulated PBMC. The ability of PBMC from patients and normal donors to respond to the proliferative effects of PHA were measured by studying the incorporation of tritiated thymidine. In brief, PBMC from either normal donors or patients with NSCLC were plated at 1.0x10^5/well of 96-well microtiter plates in a volume of 200 μl of X-VIVO 15 serum free medium. Conditions included PBMC alone or PBMC in the presence of 1 or 5 μg/ml of PHA. After 3 days, 1 μCi of tritiated thymidine was added and the plates harvested 16-18 h later using a Packard cell harvester, Meriden Cn.

In order to test the functional activity of cytokine containing supernatants, PHA responsiveness was tested in the presence or absence of supernatants obtained from both normal donors and patients with NSCLC. The cytokine supernatants obtained following 18-24-h incubation as described above were harvested, centrifuged at 400 x g to remove residual cells and pooled and frozen until use. Normal donor sups were pooled from 10 normal donor experiments and the NSCLC sups were also pooled from 10 random donors with lung cancer. For PHA stimulation, PBMC obtained from 2 normal donors were tested at 1.0x10^5 cells per well in 200 μl of medium in 96-well flat bottom microtiter plates. PHA was added at 1 μg per well. After 4 days, 1 μCi of tritiated thymidine was added per well. After 18-24 h, the plates were harvested and bound tritiated thymidine counted using a Packard Top Count microplate scintillation counter
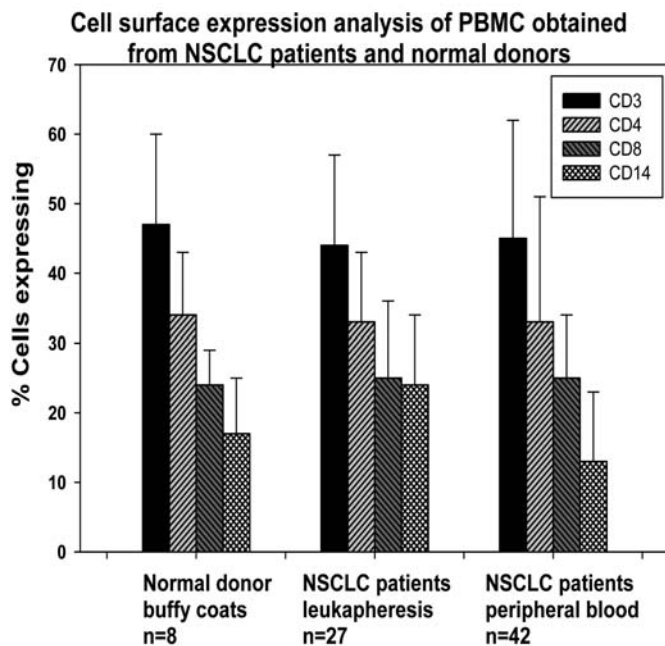
Figure 1. PBMC from normal donor buffy coats and NSCLC patients' peripheral blood and leukapheresis products were analyzed using direct cell surface phenotyping. The cells were stained with FITC or PE labeled antibodies to the T cell antigens CD3, CD4, CD8 and CD14 on monocytes. All samples were processed using Ficoll Hypaque separation medium. Note that the levels of each cell type were not significantly different from one another.

(Packard Instrument Company, Downers Grove, IL). The data were presented as either total cpm or stimulation index which was obtained by dividing the total experimental cpm by cpm for lymphocytes alone.

Results

Components of NSCLC patient leukapheresis products and normal donor buffy coats. Twenty-nine leukapheresis products and 42 peripheral blood bleeds obtained at post vaccine intervals were studied from patients enrolled in a dendritic cell vaccine trial being conducted by our group (23,24). Ten buffy coats, prepared by the Kentucky Blood Center, were utilized as normal donor controls. Peripheral blood mononuclear cells (PBMC) obtained from the three sources following Ficoll Hypaque purification were dual stained for direct cell surface phenotyping. Observe similar populations of PBMC from patient leukapheresis, peripheral blood draws and normal donor buffy coat products (Fig. 1). CD3+ cells comprised >40% of the whole cell population. CD4+ cells outnumbered CD8+ cells and CD14+ monocytes were slightly elevated in the leukapheresis products of NSCLC patients (24% for patients; 18% for normal donors). There were no significant differences in the levels of any of the PBMC subtypes thus validating the comparisons of cytokine release presented below.

Patterns of cytokines secreted by PBMC from NSCLC patients and normal donors. PBMC from leukapheresis products and normal donor buffy coats were established in overnight culture either: i) alone; ii) in the presence of the

T cell receptor (TCR) stimulants plastic bound anti-CD3 monoclonal antibody (OKT3 mAb) and soluble CD28; or iii) the CD14+ cell stimulant TLR-4 agonist Lipopolysaccharide (LPS). Cytokines were measured after 24 h using standard ELISA. We examined levels of Th1 (IL-2, γ interferon), Th2 (IL-10, IL-13), pro-inflammatory (TNF-α, IL-6), Th17 (IL-17) and hematopoietic cytokine (GMCSF) from T cells and CD14+ monocytes.

Following TCR stimulation of PBMC, similar levels of γ-INF, TNF-α, GMCSF and IL-17 were observed in both normal donors and patients with NSCLC. Significantly higher levels of the cytokines IL-2 ($p<0.01$), IL-10 ($P<0.001$), IL-13 ($p<0.0001$) and IL-6 ($p<0.01$) were observed from NSCLC patients compared to normal donor PBMC (Fig. 2). When the PBMC were stimulated with the TLR-4 agonist LPS, similar levels of IL-6 and GMCSF were observed. Interestingly, significantly higher levels of γ-INF ($p<0.01$), IL-10 ($p<0.001$) and TNF-α ($p<0.02$) were found in the super-natants of NSCLC patient PBMC (Fig. 3). In the analysis of individual cytokines, significance was determined using Student's t-test.

Comparison of total Th1 and Th2 cytokines secreted by stage I-III NSCLC patients and normal donors. In Figs. 2 and 3, we compared levels of both Th1 and Th2 cytokines, individually, between 29 patients with NSCLC and 10 normal donors. While we observed differences between the 2 groups, we wanted to compare total levels of cytokines as a comparison. Protein cytokine levels (pg/ml per million cells released in 24 h) of Th1, Th2, GMCSF, and Pro-inflammatory cytokines were totaled, grouped and plotted for each donor group (normal donor and NSCLC patients) showing the hetero-geneity in cytokine release. The levels of Th1 cytokines (IL-2 and γ interferon, solid bars) (Fig. 4) increased from normal donors through stage I and III NSCLC patients (range of means: 407 to 803 to 958 pg/million PBMC). Interestingly, the increase in Th1 cytokines between normal donor and stage I patients was significant ($P<0.038$) as was the difference between normal donor and stage III patients ($P<0.044$). There was no significant difference in Th1 cyto-kines between stages I and III. More striking were the total levels of Th2 cytokines (IL-10 and IL-13) as they increased between normal donors (502 pg) to stage I patients (1250 pg) to stage III patients (1591 pg). Again, statistically significant differences were noted between normal donor and stage I ($P<0.041$) and stage III ($P<0.0048$) NSCLC patients. Less striking was the increase in levels of the pro-inflammatory cytokines IL-6 and TNF-α (Fig. 4) with levels being higher but not statistically significant in stage III patients (7685 pg) compared to stage I (7283 pg) and normal donors (6075 pg). The levels of GMCSF, however, remained similar between normal donors (1570 pg) and patients (stage I, 1611 pg; stage III 1816pg). The numbers of patients with stage II disease were not numerous enough (n=2) to allow comparisons. In the analysis of total cytokines, significance was based on Wilcoxon rank sum statistics.

Tritiated thymidine incorporation by PBMC. PBMC from both normal donors and patients with NSCLC were then tested for their ability to incorporate tritiated thymidine

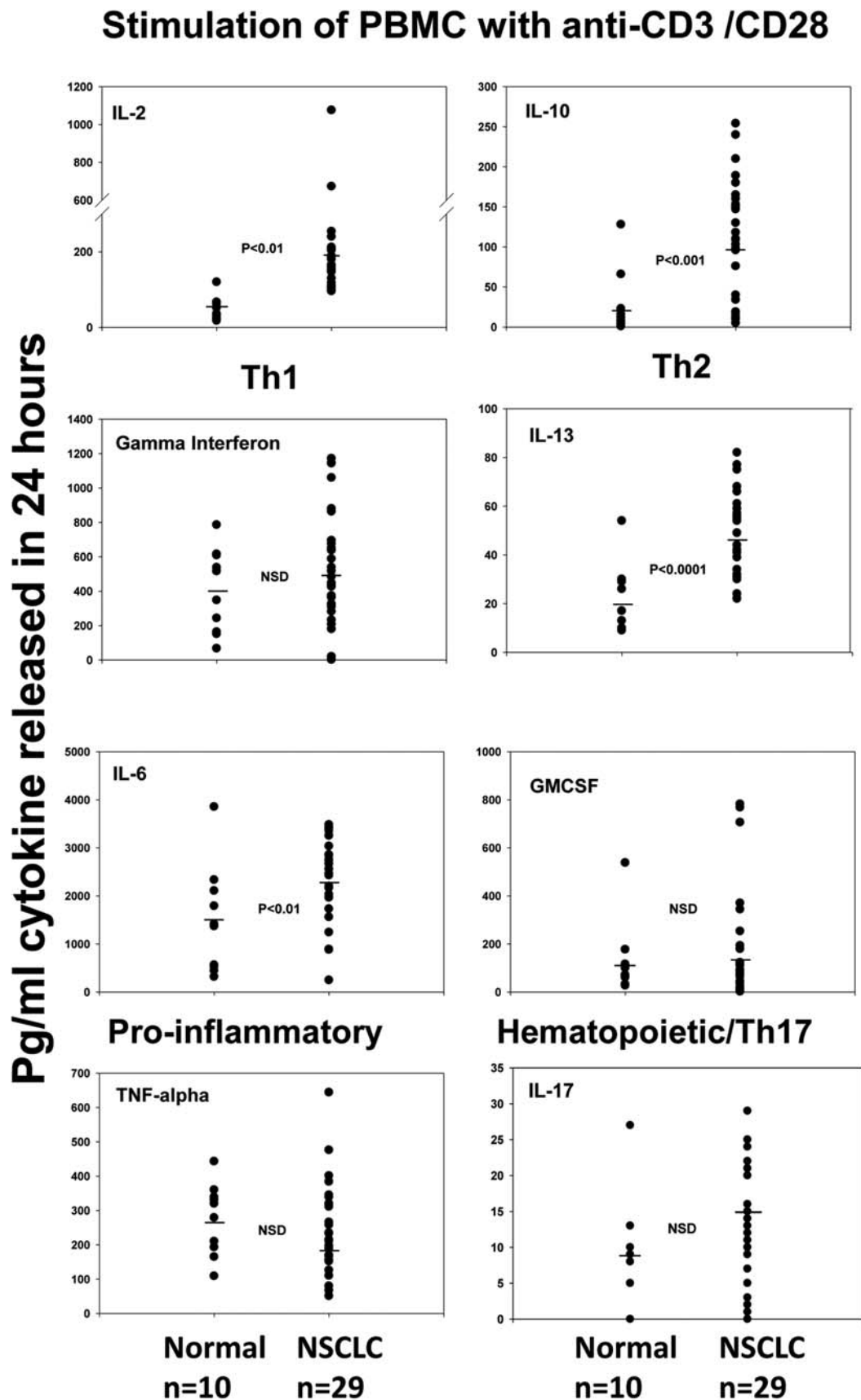## Stimulation of PBMC with anti-CD3 /CD28

Figure 2. Cytokine release from T cells measured using ELISA as described in Materials and methods. Cytokines measured were: Th1, γ interferon and IL-2; Th2, IL-10 and IL-13; Pro-inflammatory, IL-6 and TNF-α; Hematopoietic, GMCSF; and Th17, IL-17. Samples of PBMC obtained from NSCLC patient leukapheresis products (n=29) were compared to PBMC obtained from normal donor buffy coats (n=10). As described, 1x10^{6} PBMC were cultured overnight either alone or in the presence of 1 μg plastic bound anti-CD3 (OKT3) and soluble anti-CD28. Supernatants were removed and assayed for cytokine and results presented as pg/ml cytokine released per million PBMC in 24 h. Note that there was no significant difference (NSD) between the groups in the levels of: γ interferon, TNF-α, GMCSF, and IL-17. However, differences as determined using Student's t-test were significant for IL-2 ($p<0.01$), IL-6 ($p<0.01$), IL-10 ($p<0.001$) and IL-13 ($p<0.0001$).
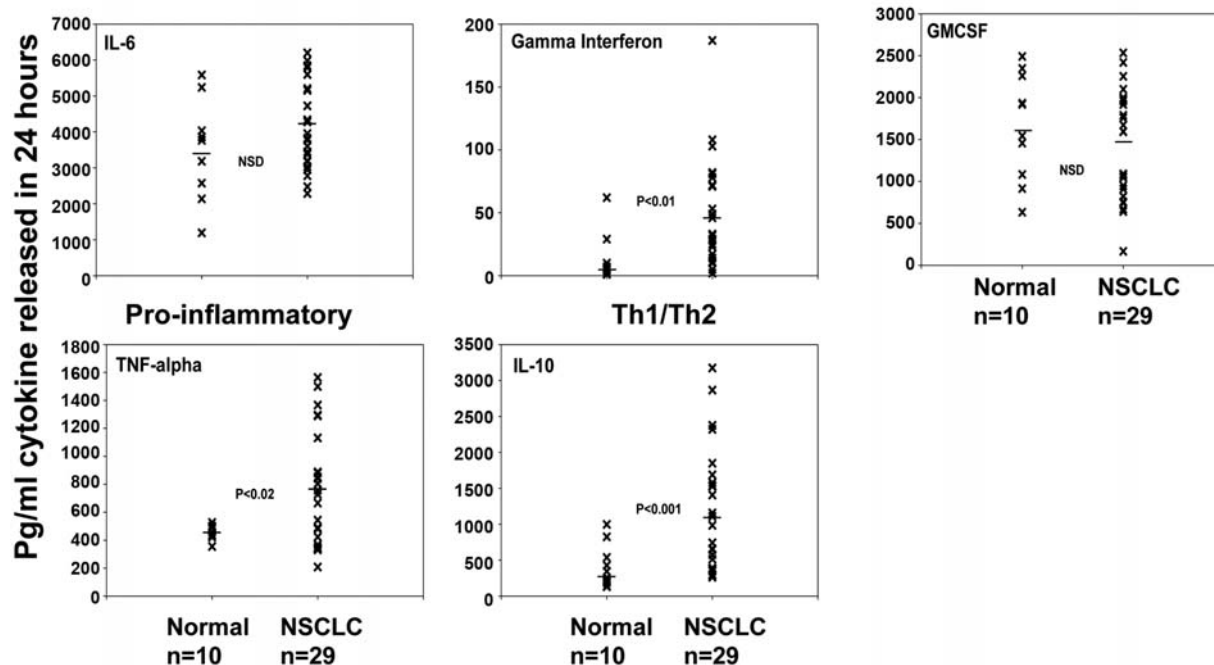
Stimulation of PBMC with LPS

Figure 3. Cytokine release measured from monocytes using ELISA as described in Materials and methods. Cytokines measured were: Th1, γ interferon; Th2, IL-10; Pro-inflammatory, IL-6 and TNF-α; and Hematopoietic, GMCSF. Samples of PBMC obtained from NSCLC patient leukapheresis products (n=29) were compared to PBMC obtained from normal donor buffy coats (n=10). As described, $1x10^6$ PBMC were cultured overnight either alone or in the presence of 10 μg/well LPS. Supernatants were removed after 24 h and assayed for cytokine and results presented as pg/ml cytokine released per million PBMC in 24 h. Note that there was no significant difference (NSD) between the groups in the levels of: IL-6 and GMCSF. However, differences were significant for TNF-α (p<0.02); γ interferon (p<0.01); and IL-10 (p<0.001).
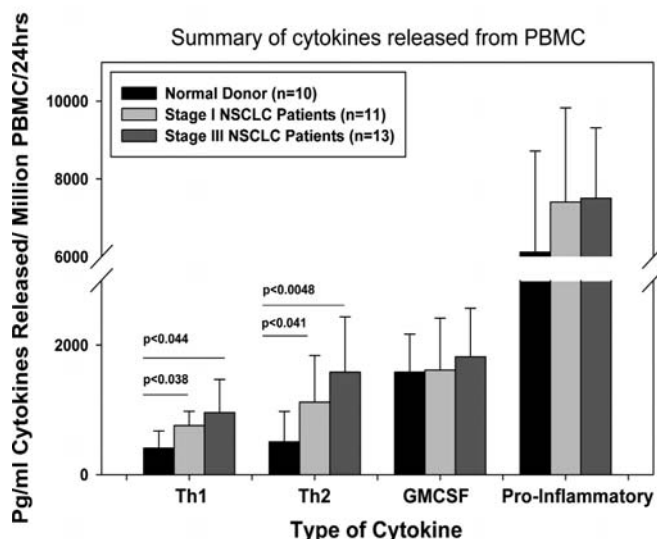

Figure 4. The amounts of secreted cytokines were compared between normal donors and patients with NSCLC. Total cytokine levels were obtained by adding the amounts of each type of cytokines released by both T cells and LPS stimulated monocytes in order to compare potential differences in cytokine microenvironments. The total cytokine levels were plotted comparing normal donors to stage I and III NSCLC patients enrolled in a clinical trial conducted by our group. Note that in general, levels of Th1 and Th2 cytokines were significantly higher in patients when compared to normal donors. While not significant, a trend also existed showing that levels of Th1 and Th2 cytokines increased as the stage of the patients increased. Levels of GMCSF did not appear to increase while levels of pro-inflammatory cytokines increased to a slight degree. Levels of significance were determined using Wilcoxon rank sum statistics.

following exposure to either 1 or 5 μg/ml PHA. The PBMC were obtained from normal donor buffy coats or leukapheresis products of patients with NSCLC as described in Table I. Note in Fig. 5A that PBMC from normal donors incorporated more tritiated thymidine than both stage I and stage III NSCLC patients using either 1 or 5 μg/ml PHA. These data are presented as stimulation indices with a significant difference (p<0.05) observed in the SI between normal donor and stage III patients.

Effect of supernatants on normal donor PBMC response to PHA. Supernatants obtained from normal donor PBMC (n=10) and NSCLC patients (n=29) stimulated with anti-CD3 and soluble anti-CD28 (Fig. 5A) were each pooled, filtered to remove cells and added at 25% volume/volume concentration to test the effect on PHA stimulated PBMC from 2 normal donors. In Fig. 5B, note that supernatants from NSCLC patients stimulated with anti-CD3 and CD28 were inhibitory (p<0.0001) to the PHA response of normal donor PBMC as measured by tritiated thymidine incorporation. Thus, cytokines contained in the supernatants appeared to be functionally active, in most cases, inhibitory to T cell functional response as measured by tritiated thymidine incorporation assay.

Patterns of cytokines secreted by NSCLC tumor biopsies compared to normal adjacent tissue (NAT). Nineteen paired samples of NSCLC tumor biopsies (generally 0.1-1.0 g of tissue obtained) and normal adjacent tissue (NAT) were obtained from the Cooperative Human Tissue Network
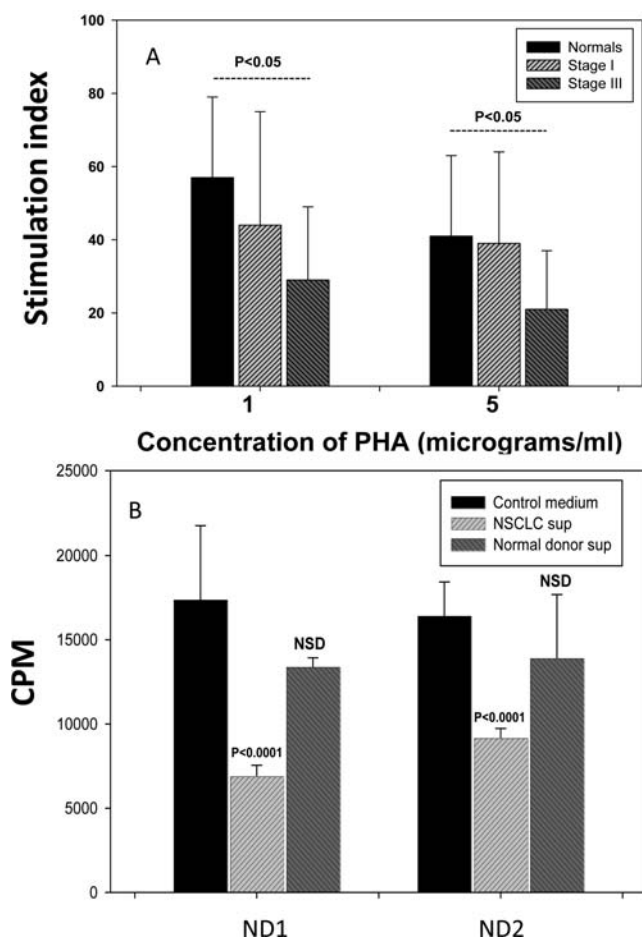
Figure 5. PBMC obtained from normal donors (n=10) and PBMC obtained from NSCLC patients (n=29) were compared for their ability to respond to stimulation with either 1 or 5 μg/ml of the T cell mitogen PHA (A). The cultures were maintained for 3 days at which point 1 μCi/ml tritiated thymidine was added per well and the culture continued overnight. Following 18-24 h, the wells were harvested and analyzed as described in Materials and methods. Note that higher levels of tritiated thymidine were obtained when cultures were stimulated with 1 μg PHA compared to 5 μg. Note at both concentrations, that PBMC obtained from NSCLC patients incorporated more thymidine than PBMC obtained from patients with NSCLC. Interestingly, PBMC from stage I patients had higher levels of thymidine incorporation compared to patients with stage III disease. While the levels were higher in normal donor PBMC, the only significant differences were noted when stage III NSCLC patients were compared to the thymidine incorporation by normal donor PBMC (P<0.05). Results are presented as stimulation index which was obtained by dividing the amount of cpm incorporated by experimental cultures (1 or 5 μg PHA) by control cultures (no PHA condition). In (B), thymidine incorporation of normal donor PBMC and NSCLC patient PBMC were studied in the presence of supernatants containing cytokines obtained from patient and normal donor PBMC stimulated with OKT3 and anti-CD28. The volume of supernatant used was 25% of control medium. The assay was the same as described for the control experiments presented in (A). Note, that PBMC obtained from 2 separate normal donors incorporated thymidine similarly. However, when supernatant obtained from NSCLC patient PBMC were added to the normal donor cultures, the level of thymidine incorporation was significantly reduced (P<0.0001) for both donors. Supernatant from normal donor PBMC cultures, while reduced, were not significantly different than the control cultures.

(CHTN). The samples were enzyme digested overnight and treated similarly to PBMC as described above. Single cell suspensions of NAT and tumor were cultured either alone, with anti-CD3 monoclonal antibody (OKT3) or LPS for 24 h.

The results of cytokine release assays for the Th1 cytokine γ interferon and the Th2 cytokine IL-10 are presented in Table II. In general, cytokine release was variable with both γ interferon and IL-10 detected in all samples studied. While nanogram levels of γ interferon were observed from CD3 stimulated cells contained in tumor tissue, paired NAT samples also showed increased levels (see samples # 123, 138, 139, 163, 164 and 186). While the mean for γ interferon release was increased, the difference was not significant based on the range of levels detected. Similarly, IL-10 was secreted to some degree in both NAT and tumor tissue. A significant difference in IL-10 release was noted between LPS stimulated tumor and LPS stimulated NAT (P<0.001; compare bold columns). Tumors and NAT cultures stimulated with anti-CD3 did not show appreciably higher levels of IL-10 when compared to cells incubated in the absence of stimulation.

Levels of CD4 and CD8 regulatory T cells (Tregs) in different anatomic compartments of NSCLC patients: peripheral blood and tissue biopsies. Tregs were analyzed in patient leukapheresis products, normal donor buffy coats and tumors obtained from the CHTN. Paired normal adjacent lung samples were also analyzed. The cells were stained for surface expression of CD4 or CD8 and CD25. The double positive cells were then analyzed for expression of intracellular FoxP3. In Fig. 6A, observe that normal donors expressed low levels of both CD4 and CD8 cells co-expressing CD25 and intracellular FoxP3 (<5%) when compared to NSCLC patient samples. Note that all leukapheresis products obtained from NSCLC patients contained high levels of Tregs ranging from 4 to 25%. The levels of Tregs were significantly higher (p<0.05) in stage I patients compared to normal donors and also significantly higher (p<0.03) in stage III patients compared to normal donors. No differences, however, were noted between stage I and stage III patients. While CD4 levels were strikingly high, levels of CD8s expressing the regulatory phenotype were also elevated but not to the level of CD4 Tregs. When analyzing tumor vs. NAT, in 2 out of 3 cases levels of CD4CD25FoxP3$^+$ cells were higher in tumor vs. NAT but in UKY113, higher levels of Tregs were observed in the NAT than the tumor biopsy.

Discussion

In the current study, immunologic analysis was performed on PBMC obtained from leukapheresis products of NSCLC patients. PBMC contained in normal donor buffy coats were used as comparative controls. We examined cytokine secretion from PBMC using ELISA, thymidine incorporation as an indicator of cell proliferation, and cell surface phenotyping to quantitate Tregs. In additional studies, we examined IL-10 and γ interferon secretion from lung tumor biopsies and autologous normal adjacent lung. In the overall analyses of these data, NSCLC patients produced more cytokines, contained higher levels of Tregs in circulation and had a reduced lymphoid proliferative response than that observed in normal donors. The results in the current study are important and provide insight into the interactions that occur *in vivo* between growing cancers and both lymphoid and myeloid components

Table II. Secretion of cytokines[a] from single cell suspensions of normal adjacent tissue (NAT) and NSCLC tumor biopsies.[b]

| | γ interferon | | | | | | IL-10 | | | | | |
| | NAT | | | Tumor | | | NAT | | | Tumor | | |
Biopsy #	Alone[c]	OKT3	LPS	Alone	OKT3	LPS	Alone	OKT3	LPS	Alone	OKT3	LPS
UKY-62	10	12	11	5	136	5	26	29	**34**	137	218	**255**
UKY-63	28	301	210	26	2480	62	201	178	**224**	171	401	**565**
UKY-64	80	1849	144	27	433	38	125	140	**118**	183	126	**664**
UKY-65	4	441	9	22	91	14	37	36	**189**	69	37	**192**
UKY-68	20	697	12	20	276	47	23	22	**12**	23	19	**328**
UKY-70	15	319	12	9	551	16	287	257	**165**	158	143	**215**
UKY-73	21	244	18	16	192	22	210	102	**154**	450	157	**482**
UKY-74	10	54	12	13	51	38	14	26	**14**	217	92	**464**
UKY-93	16	156	23	15	182	16	374	289	**585**	522	367	**1575**
UKY-99	6	110	14	5	175	6	4	16	**81**	24	38	**266**
UKY-115	17	76	20	23	163	29	34	36	**35**	57	47	**187**
UKY-123	50	1896	11	64	983	27	532	287	**261**	124	470	**612**
UKY-137	16	74	8	4	70	10	68	77	**140**	15	8	**611**
UKY-138	12	1132	16	24	894	84	94	41	**206**	114	227	**336**
UKY-139	162	7188	72	7	5075	7	35	16	**162**	177	191	**300**
UKY-141	20	764	32	10	81	16	28	29	**15**	15	9	**545**
UKY-163	106	1661	279	59	9393	228	157	235	**370**	133	247	**331**
UKY-164	30	3520	72	14	9839	212	159	155	**215**	93	104	**370**
UKY-186	58	2520	210	29	7107	56	127	122	**157**	62	77	**181**
Mean[d]	36	1211	62	21	2009	49	133	110	**165**	144	157	**446**
SD	41	1750	84	16	1874	64	140	97	**139**	136	137	**316**
												P<0.001

[a]Cytokines IL-10 and γ interferon were measured using ELISA as described in Materials and methods. [b]Samples were obtained from CHTN. Both tumor samples and NAT were processed as described in Materials and methods. [c]Conditions included: OKT3, anti-CD3 mAb; LPS or alone. Single cell suspensions were cultured 18-24 h in 1 ml of culture medium. The cells were established at 1.0×10^6 /ml and supernatants collected and cryopreserved as described. [d]Mean ± the standard deviation. All groups were analyzed for statistical significance using Student's t-test. No differences were observed except for IL-10 secretion from NAT vs. tumor biopsy (p<0.001).

of the immune system. PBMC from lung cancer patients are clearly different than those obtained from normal donors, and likely reflect the influence of tumor *in vivo*. Since many investigators use leukapheresis products as a source of precursor cells for immunotherapy; consideration of these differences might lead to improved protocols for effector cell generation.

Previous *in vitro* studies examined cytokines secreted from stimulated and un-stimulated populations of PBMC obtained via venipuncture (21,22). Venipuncture provides both plasma and cellular components obtained directly from venous circulation which is the easiest access for preparing clinical reagents and assessing immunotherapies. The current study extends these observations by examining PBMC obtained from lung cancer patient leukapheresis products. Leukapheresis is performed at the beginning of many immunotherapy protocols, provides larger numbers of PBMC and can be adjusted for preferentially collecting subsets of leukocytes (myeloid vs. lymphoid). The large scale processing of blood using closed collection procedures in leukapheresis machines provides adequate precursors for the generation of antigen pulsed DCs (32) and anti-tumor lymphocytes (33). Historically, leukapheresis was integral to lymphokine activated killer cell protocols (LAK) which provided the basis for many immunotherapies being tested today (34). Leukapheresis products, drawn from a central or peripheral line, undergo differential centrifugation returning plasma to the patient while enriching mononuclear cells. Despite differences in the manner used to obtain PBMC, we are confident in the similarity between the products based on the cell surface phenotyping results (Fig. 1). Overall, we believe the results presented provide important insight into the tumor-microenvironment conditioned by PBMC contained in leukapheresis products. PBMC derived cytokines likely influence both positively and negatively the differentiation of therapeutic effector cell precursors both *in vivo* or *in vitro*.
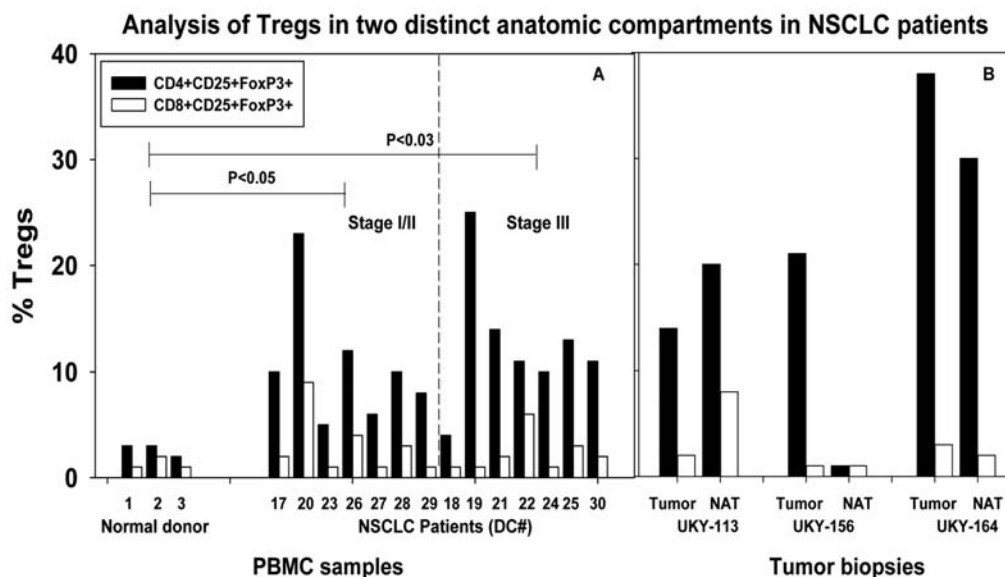
Figure 6. Levels of Tregs (CD4^{+} or CD8^{+}/CD25^{+}/FoxP3^{+}) were measured from normal donor buffy coats (n=3), NSCLC patient leukapheresis products (n=14) and biopsies obtained from lung cancer patients (n=3). Note that in general, the levels of Tregs from NSCLC patients were higher than those obtained from normal donors. In addition, in most cases, as expected, the levels of CD4 cells co-expressing CD25 and FoxP3 were higher than CD8 cells co expressing these markers. Also note in 2/3 cases, the % of CD4^{+} Tregs was higher in lung cancer biopsies (UKY-156 and UKY-164) compared to autologous normal adjacent tissue. Observe in 113, however, a higher % of Tregs was obtained from NAT compared to autologous tumor tissue.

The original classification of cytokines by Mosmann using murine T cell clones reflected cell of origin and the elicited cellular function (i.e., proliferation, differentiation, activation). This study attributed IL-2, γ interferon and lympho-toxin secretion to T helper 1 cells (Th1) and IL-4, -5, -6, -10 and -13 secretion to Th2 cells (14). Similar patterns were reported for human T cell subsets (35). Certain cytokines including GMCSF, TNF-α and IL-3 were found to be released by both Th1 and Th2 cells. Reviewing the literature, how-ever, reveals inconsistencies in the interpretation of these criteria with many investigators modifying the schema based on their own model systems. For instance, some studies list TNF-α as a Th1 cytokine and exclude IL-6 as a Th2 cyto-kine (15,19,36). It is essential that standards be conformed to in order for studies to be comparable. In the present report, we developed a consensus based on the cancer literature. Thus we considered: IL-2 and γ interferon as Th1; IL-10 and IL-13 as Th2; and TNF-α and IL-6 as pro-inflammatory cytokines. Using a similar schema, a study in prostate cancer showed both TNF-α and IL-6 were elevated along with PSA in hyperplasic prostate tissue (37). Finally, we included GMSCF as a hematopoietic cytokine and IL-17, a component of inflammation and autoimmunity, as a representative of the Th17 family.

Using ELISA, bulk levels of cytokines can be quantitated as they are released from PBMC *in vivo* or *in vitro*. *In vivo* release is measured in collected plasma, or plasma derived serum samples. In this fluid anatomic site, soluble cytokine levels are considered to reflect the *in vivo* steady state and ongoing immune reactions (38-40). These reactions are mediated by immune effector cells interacting with connective tissue cells, other supporting cells and in some cases tumor cells. When PBMC themselves are monitored by ELISA after 18-24-h culture, some protein may be carried over from the

in vivo state. More importantly, Fan *et al* showed cytokines presented in the current study (i.e., γ interferon, IL-2, IL-10, IL-6 and TNF-α), are produced between 1 and 8 h following receptor stimulation *in vitro* (PHA and anti-CD3) (41). Thus, the quality and quantity of cytokines measured is likely a reflection of pre-committed lymphoid and myeloid subsets mobilized to the periphery which have a phenotype deter-mined by the health of the donor. TCR or TLR stimulation results in cytokine release from cells expanded *in vivo* providing insight into various disease states (42-46). These studies have shown predictable levels of both Th1 and Th2 cytokines in acute and chronically ill patients. The levels of cytokines observed differed from those obtained in age and sex matched normal donor controls.

In the current study, levels of some but not all Th1 and Th2 cytokines were significantly higher in NSCLC patients compared to normal donors. Only IL-10 was significantly different from TCR and TLR-4 stimulated PBMC. When the total levels of cytokines were presented, it was clear that both Th1 and Th2 cytokines were elevated in NSCLC patients over normal donors. A trend was also observed for an increase in both Th1 and Th2 cytokines between stage I and III patients, although the difference was not significant based on the sample size. In some previous studies of cancer, the levels of Th1 cytokines were reported to be similar between normal donors and cancer patients (melanoma, basal cell carcinoma, colorectal and prostate cancer) while significant differences were noted in the level of Th2 cytokines (47-50). The present study extends this observation measuring PBMC derived cytokines from leukapheresis products. It is important to note that the cytokines considered to be Th1 and Th2 between the present study and others is sometimes different, thus it is difficult to make direct comparisons. In NSCLC, prior studies showed increased Th2 cytokine levels such as

IL-4, IL-6 and -10 comparing levels of mRNA (21) as well as RT-PCR analysis (20). This was partially confirmed in the present study since we measured both IL-10 and IL-13, not IL-4 and considered IL-6 a representative of Pro-inflammatory cytokines. Regardless, the combined results describe a micro-environment of cytokines in cancer patients which is elevated compared to normal donors. The potential effect on anti-tumor responses is likely influenced in either a positive or negative way. The effect in tumor lesions is probably dependent upon particular cytokines present at the interface of tumor and lymphoid and/or myeloid cells.

Pro-inflammatory cytokines TNF-α and IL-6, also studied in the present report, were shown to be associated with the pathogenesis of cancer and the related cachexia (51). In NSCLC, Fortunati *et al* reported an increase in the serum levels of both TNF-α and IL-6 in lung cancer patients. While TNF-α secretion was associated with PBMC, the same was not true for IL-6. The source of IL-6 was suggested to be the tumor mass. The current study supports the TNF-α finding but also showed that IL-6 cytokine was actively transcribed in receptor stimulated T cells; with nanogram quantities per million cells detected by ELISA studies.

In an antigen independent system, we compared T cells from normal donors to those obtained from NSCLC patients for their ability to incorporate tritiated thymidine into DNA in response to PHA. While thymidine incorporation in the strictest sense is not a direct measure of T cell proliferation and expansion, increased thymidine incorporation was accompanied by an increase in the number of blastic lymphoid colonies observed in the microtiter wells (data not shown). The highest level of thymidine incorporation was noted in the PBMC obtained from normal donors. Levels of incorporation decreased in stage I NSCLC patients and was significantly reduced in stage III patients. This finding suggested that NSCLC patients have a decreased proliferative response that changes to a less favorable state as the stage of the patient increases. Earlier studies in head and neck cancer showed a similar decreased proliferative response (52). In lung cancer, the response to PHA was also reduced as the stage of disease increased from I to III (53). Monocyte depletion partially restored the thymidine incorporation, suggesting monocyte derived cytokines were contributing to the decreased T cell response. In the current study, supernatants from stimulated T cell cultures were inhibitory to the thymidine incorporation observed. We speculate that higher levels of Th2 cytokines could contribute to reduced thymidine incorporation observed. While not tested directly, this is corroborated by prior studies (54,55). Unfortunately, in a prospective follow-up study, thymidine incorporation could not provide an accurate assessment of the overall status of cellular immunity in cancer (56), even though T cell response to T cell mitogens and TCR stimulation is often used as a measure of immune competence in young vs. aged individuals (57). Perhaps, decreased thymidine incorporation, taken together with higher levels of Th2 cytokines observed in cancer patients, could together predict immune responsiveness. Such a study should be done prospectively in order to validate the approach.

The progression of cancer from a non-vascularized aggregate of transformed cells into a mature vascularized tumor lesion occurs in the presence of both innate and acquired immune components. In spite of this, the tumor evolves as a result of evading recognition through both tumor-derived and host-derived soluble mediators (cytokines) and cells. The immunosuppressive cells are comprised predominantly of two types, myeloid-derived suppressor cells (MDSCs) (13) and Tregs (11,12). While MDSCs were not evaluated in the current report, evidence exists that they are potent inhibitors of immunity to lung cancer. Tregs, which comprise 5-10% of all CD4+ cells in normal donors (58) can range between 20-25% in cancer patient peripheral circulation (12). Tregs have also been identified in tumor tissue, pleural effusions, and in draining lymph nodes (11,58-60). Interestingly, CD4+CD25+FOXP3+ cells were found to be selectively drawn to tumor sites (tumor Tregs) by the expression of CCL22 by tumor macrophages and tumor cells (61). This accumulation was correlated with poor clinical outcome. In the present study, Tregs were found to be more numerous in the leuka-pheresis products of NSCLC patients compared to normal donors. It is fair to speculate the levels of Th2 cytokines and the reduced proliferative potential of lymphocytes is caused in part by the influences exerted by the higher levels of Tregs. Their presence in lung tumors have been shown to decrease lymphocyte proliferative potential (12). In addition, while statistically significant differences in Tregs were noted between normal donors and NSCLC patients, their increased levels were not observed between stages I and III as was shown for the reduction in proliferative potential. This suggests that a threshold level of Tregs are generated in early stages of cancer and appear to be functionally maintained throughout the disease state.

The peripheral blood contains leukocytes newly emigrated from the bone marrow as well as cells committed to antigen in peripheral tissues. These circulating PBMC provide protection against systemic disease. Tumor sites contain variable levels of leukocytes which have been implicated in disease prognosis; that is, more leukocytes, better predicted outcomes (62,63). In NSCLC, their presence was correlated with improved survival (64). Intratumoral leukocytes may be found directly adjacent to tumor cells as well as in peritumoral regions in the parenchyma and around tumor vasculature. Tumor associated leukocytes include variable numbers of tumor associated macrophages (Tam), DCs, granulocytes as well as lymphocytes termed tumor infiltrating lymphocytes (TIL). In a number of different tumor histologies, TIL have been shown to have anti-tumor reactivity (65). Interestingly, using RT-PCR it was shown that high levels of the immunosuppressive cytokines IL-10 and TGF-ß could be localized to tumor infiltrating cells (66). These high levels of Th2 cytokines were also found in patient sera which was inhibitory to T cell proliferation. In NSCLC, using *in situ* hybridization, it was shown that both pleural fluid and tumor samples contained large numbers of IL-4, IL-10, and TGF-ß secreting cells with remarkably lower number of cells secreting the Th1 cytokines IL-2, IL-12, IL-18 and γ interferon (67-69).

In the current study, we measured both γ interferon and IL-10 from 19 NSCLC tumor samples and paired autologous normal adjacent tissue. We examined cytokine release over 24 h in the absence and presence of either CD3 or TLR-4 stimulation and detected heterogeneous quantities of cytokine in each sample examined. This heterogeneity in

γ interferon and IL-10 noted in both NAT and tumor probably reflected the variability in leukocyte numbers and subsets in each site at the time of biopsy (data not shown). In samples stimulated with anti-CD3, there appeared to be more γ interferon in tumor samples when compared to normal adjacent tissue (NAT) but it did not reach a level of significance. In contrast, tumor biopsies stimulated with LPS released consistently higher levels of IL-10 than autologous NAT samples (p<0.001). But, variable levels of IL-10 were noted in the NAT. A contributing factor could be PBMC emigrating from the blood since we have shown that blood derived PBMC were a potent source of IL-10. It was also not clear that NAT samples received by the lab were tumor free. Either case, the overall microenvironment of tumor derived from lung was observed to be a Th2 in nature. With that said, the different levels of γ interferon observed affirm that Th1/Th2 balance is not rigid but variable from lesion to lesion. Considering the metastatic nature of many solid cancers; Th2 cytokines are likely associated with more progressive disease within the synapse between immune cells and tumor. In these cases, tumor progression may be favored rather than the development of the more desirable anti-tumor immunity.

An experimental anti-tumor therapy, immunotherapy, pioneered in the early 1980s using IL-2 and lymphokine activated killer cells (LAK), has evolved to include tumor antigen-specific effector mechanisms. Immunotherapy has been delivered alone or together with conventional therapies. Vaccines are currently the focus of immunotherapy utilizing tumor associated antigens delivered with autologous dendritic cells as antigen presenting cells. Vaccines are commonly used to increase the precursor frequency of specific anti-tumor effector cells and/or plasma cells secreting antibodies. There have been some cases of tumor regression (23,24,67,70,71). Considering that the source of DCs is usually leukapheresis products, it is plausible that limited successes are due in part to the nature of cancer patient derived PBMC as described in the current report. Perhaps reducing the immunosuppressive nature of these PBMC could amplify potential anti-tumor responses. Murine studies have shown that the depletion of Tregs *in vivo* resulted in maximal tumor rejection (72) and, depletion of human Tregs enhanced CD8 T cell responses following DNA immunization (73). Recent reports from the NCI combined cellular therapy with attempts to reduce the levels of regulatory T cells in melanoma (74). Improved response rates were reported. Remaining cognizant of the suppressive nature of PBMC obtained in leukapheresis products (levels of IL-10 and Tregs, reduced proliferative potential) and designing *in vitro* and *in vivo* means to neutralize this suppression, could be beneficial to researchers and allow the development of more potent immunotherapeutic approaches.

Acknowledgements

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