Procathepsin D and Cytokines Influence the Proliferation of Lung Cancer Cells

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Abstract. *Enzymatically inactive procathepsin D (pCD) has been established as an important factor in the development of lung cancer. In addition to serving as a growth factor, pCD is also involved in communication between cancer cells and surrounding cells. In the present investigation, a possible combination of autocrine and paracrine actions of pCD was investigated. pCD initiated secretion of interleukin (IL)-4, IL-8, IL-10 and IL-13 from lung cancer cells. These cytokines participated in the proliferation of the cancer cells, as demonstrated both by adding the cytokines and by inhibition using specific anti-cytokine antibodies. Using several clones of lung cancer cells differing in production of activation peptide, the pCD/cytokine cooperation and action was shown to be dependent on pCD secretion. Further proof that pCD is one of the key molecules involved in cancer development is presented.*

Procathepsin D (pCD) is a major secreted protein in several types of cancer cells, including lung and breast cancer cell lines (1, 2). The role of PCD has been demonstrated in at least 12 different types of cancer (3), however, lung cancer has not been vigorously studied. However, higher cathepsin D (CD) activities have been observed in lung tumor cells (4) and could be used for prognosis (5). In addition, the use of lung cancer cells with genetically modified secretion of pCD revealed that the level of pCD expression correlated with carcinogenic properties (2).

In addition to the mitogenic effects of pCD on cancer cells, numerous studies demonstrated the involvement of pCD in cancer invasion and metastasis. Several authors have also suggested that CD is involved in the regulation of blood vessel formation, especially in solid tumors. Stromal CD expression correlated with microvessel density in ovarian tumors: significant association between CD expression of host stromal cells and vascular density was also described in breast cancer tumours (6). Based on these data, several models of the mechanism of pCD action have been proposed (7) in which the overexpressed pCD escapes normal targeting pathways and is secreted out of the cancer cells. Subsequently, pCD interacts with surrounding proteins and is recognized *via* its activation peptide (AP) by an as yet unidentified cell surface receptor. This interaction releases a signal that results in differential expression of cancer-promoting genes, including various cytokines that in turn stimulate tumor growth. pCD secreted by cancer cells is also captured by stromal cells and promotes fibroblast proliferation, motility and invasion that results in cancer progression (8).

Tumorigenesis is a complex process involving not only growth of the primary tumor cells (9), but also communication with surrounding tissues and cells, including stromal cells and fibroblasts. Extensive *in vitro* and *in vivo* research has demonstrated that this communication can promote the growth of cancer cells (10, 11). Secretory proteins of different families play an important part in these processes, including a complex system of intercellular communication and regulation.

pCD has been found to possess strong autocrine (review (7)) and paracrine proliferative properties (8). Berchem's group found that pCD stimulated not only parent cancer cell proliferation, but also tumor angiogenesis by a paracrine mechanism (12). Supporting evidence came from work by the Liaudet-Coopman group who demonstrated that pCD was able to stimulate the proliferation, survival, motility and invasion of fibroblasts (8). Our group has found a correlation between ER$^+$ breast cancer cells and cytokine production (13) and later showed that pCD initiated the secretion of cytokines such as interleukin (IL)-4, IL-8, IL-10, IL-13 and MIP-1β from such tumor cells. The used of specific antibodies against individual cytokines proved the effects of secreted cytokines on the proliferation of the cancer cells. In addition, expression of cytokine receptors on tested cell lines corresponded to the effects of individual cytokines. An analogous pattern was also observed for fibroblasts, which, under physiological conditions, are the cells in closest contact with the tumor tissue and play a role in tumor growth and invasion (14).

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This study aimed to better elucidate the possible relations between pCD and cytokine secretion by evaluating cytokine release after the addition of AP.

Materials and Methods

Chemicals. RPMI 1640 medium, Iscoves's modified Dulbecco's medium, HEPES, antibiotics, glutamine and transferrin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fetal calf serum (FCS) was procured from Hyclone Laboratories (Logan, UT, USA). Recombinant human IL-4, IL-8, IL-10 and IL-13 were purchased from Biosource International (Camarillo, CA, USA).

Antibodies. Monoclonal antibodies against individual cytokines were purchased from B&D Systems (Minneapolis, MN, USA). Control MOPC-21 IgG antibody was purchased from Sigma Chemical Co.

Peptide. The 44 amino acid-long peptide analogous to the AP of pCD was synthesized at the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic in Prague. Purity of the AP was confirmed using high pressure liquid chromatography (HPLC), amino acid analysis and by mass spectrometry. The HPLC method showed purity of more than 95%. The amino acid analysis confirmed the amino acid composition. The mass spectrometry results were in accordance with the proposed molecular structure. Additionally, the purity of the peptide was also verified by *N*-terminal sequencing using an automated system where the first nine *N*-terminal amino acids were in agreement with the designed structure.

Cell culture. Human lung cancer cell line NCI-H23 and fibroblasts Detroit 573 were obtained from the ATCC (Manassas, VA, USA). The cell lines were maintained in RPMI 1640 medium containing HEPES buffer supplemented with 10% heat-inactivated FCS, 100U/ml penicillin and 100 µg/ml streptomycin, in plastic disposable tissue culture flasks at 37°C in a 5% CO_2/95% air incubator.

Cytokine array. Tested cell lines were incubated for various time intervals with AP in serum-free conditions. The supernatant was collected, filtered through 0.22 µm filter and stored in a –80°C freezer. For the cytokine analysis, the protein microarray services provided by Allied Biotech (Ijamsville, MD, USA) were used. In brief, the services used a sandwich Ab-based protein detection multiplex assay. A streptavidin-Cy5 conjugate was used for assay detection. The assay was conducted in quadruplicate with positive and negative controls spotted on each microarray. The assay detects cytokines IL-2, interferon (IFN)-γ, TNF-α, IL-8, IL-12 P70, IL-12 P40, IL-4, IL-6, IL-10, IL-5, IP-10, MIP-1β, IL-13, IL-1β.

In vitro cell proliferation assay. For the growth experiments, the cells were harvested and washed six times in Iscoves's modified Dulbecco's medium with HEPES buffer supplemented with glutamine, antibiotics, 10 µg/ml of human transferrin and 0.1% FCS. The cells were seeded in 96-well tissue culture plates at a density of 5×10^4 cells/ml (150 µl/well) in the presence or absence of purified proteins (10 ng/well) in triplicate. After five days in culture, the proliferation was evaluated using a Biotrak cell proliferation ELISA system version 2 (Amersham Pharmacia Biotech,
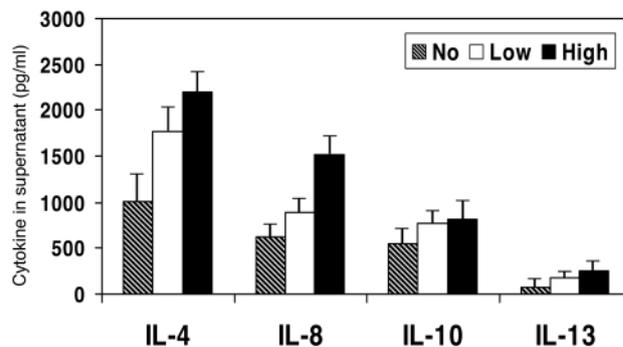

Figure 1. *Levels of four secreted cytokines 4 hours after the addition of AP (10 ng/well) to NCI-H23 lung cancer cell line. The means of three independent experiments±SD are shown. No-, low-, high-pCD secreting NCI-H23 clone.*

Piscataway, NJ, USA) according to the instructions given by the manufacturer. The positive control included NCI-H23 cells in RPMI medium with 10% FCS and the negative control comprised of NCI-H23 cells in RPMI medium without serum.

Statistics. Student's *t*-test was used to statistically analyze the data.

Results

In the first set of experiments, the secretion of cytokines from the NCI-H23 cells 4 hours after the addition of AP was measured. Three different clones of NCI-H23 cells were used – one with no secretion of pCD, one with low secretion and one with high secretion. The preparation and characterization of these clones has been described previously (2). As shown in Figure 1 the addition of synthetic AP caused strong secretion of IL-4, IL-8 and IL-10, whereas production of IL-13 was low. IL-4 and IL-8 secretion was dependent on the level of secreted pCD. None of the other cytokines included in the cytokine array showed any measurable signal. The addition of natural pCD instead of synthetic AP yielded identical results (data not shown).

As shown before, lung cancer cells do not normally grow in serum-free conditions, only clones with high pCD secretion showed slight proliferation (2). The addition of exogeneous AP resulted in similar growth of all three cell clones. The addition of individual cytokines significantly increased the proliferation only in the low and high pCD-producing clones (Figure 2).

To confirm these observations, the effect of the addition of specific anti-cytokine antibodies on cytokine/AP-mediated proliferation was tested. AP was added to the growth medium, and in parallel, antibodies to particular cytokines or mixures of antibodies were added and the cell proliferation in 120 h was measured. The results of these experiments are shown in Figure 3. It was clear that the addition of individual anti-cytokine antibodies slowed the growth of the cells
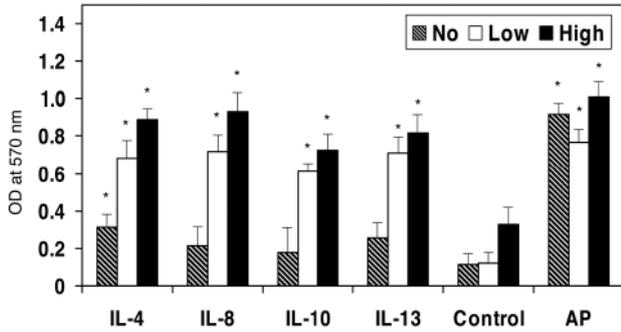
Figure 2. *Growth of NCI-H23 cells in serum-free medium containing either individual cytokines or AP after 5 days in culture. NCI-H23 clones: control cells with no pCD secretion (No), medium pCD secretion (Low) and higher pCD secretion (High). Data are the means of three independent experiments±SD. *Differences between control and treated groups, p<0.05.*
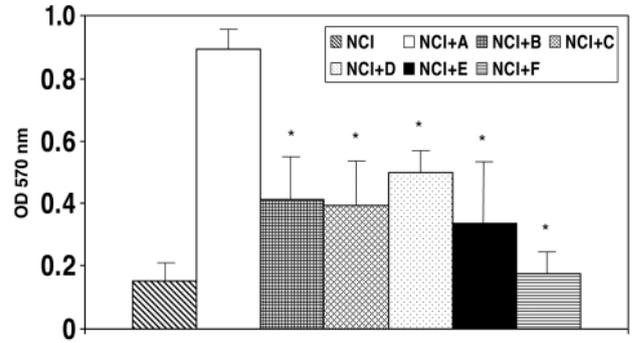

Figure 3. *Effects of antibodies to individual cytokines (1 μg/sample) on proliferation of NCI-H23 (NCI) cells after addition of AP. A, addition of AP; B, AP and antibodies to IL-4; C, AP and antibodies to IL-8; D, AP and antibodies to IL-10; E, AP and antibodies to IL-13; F, AP and antibodies to all four studied cytokines. Cells without pCD secretion were used. Data are the means of three independent experiments±SD. *Differences between goups treated with AP and individual antibodies were significant at the p<0.05 level.*

secreting the cytokine, with the cocktail of all anti-cytokine antibodies showing the strongest effects. The antibodies were tested in the 0.1 to 2 μg/sample range, control MOPC-21 IgG antibody used at the same concentration showed no effects. The data on addition of only antibodies to the medium (with no AP added) are not shown as they had no influence on the proliferation of any of the tested cell lines.

In the last experiment, Detroit 573 fibroblasts were co-cultured with the lung cancer cells NCI-H23 (either low- or high-pCD producing clones). The cells were grown either alone or as a mixture, with the same total number of cells. To evaluate the effects of AP and the cytokines on the cell proliferation, either anti-pCD monoclonal antibodies or a mixture of all four anti-cytokine monoclonal antibodies was added. The growth of the NCI-H23/Detroit 573 cell combination was approximately 3-fold faster in the low pCD-producing clone and almost 4-fold faster in the high pCD-producing clone (Figure 4). Addition of anti-pCD or anti-cytokine antibodies showed strong and comparable inhibition of cell proliferation. Using morphological observation after Giemsa staining, the cell ratio did not change and remained the same throughout the experimental period of five days (data not shown).

Discussion

Our findings showing direct effects of pCD and its AP on growth and invasiveness of lung cancer cells (15) were later further extended by clarifying of these effects by using stable transfected clones. These studies showed that the expression of pCD enhanced the carcinogenic properties of lung cancer cells and that AP was essential for these activities, but the catalytic activity or glysosylation was not involved in the growth-promoting activity.
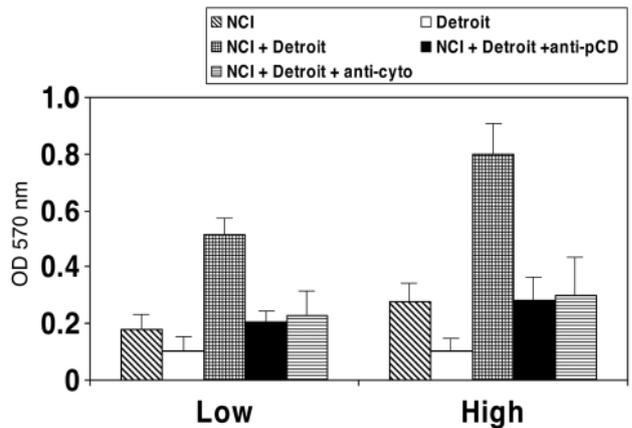

Figure 4. *Proliferation of co-cultured lung cancer cells (NCI-H23) and fibroblasts (Detroit 573). Average value of 3 independent experiments with SD values. Anti-pCD, anti-cyto: monoclonal antibodies to pCD and mixture of all four antibodies to the studied cytokines. Two clones of NCI-H23 were used: medium pCD secretion (Low) and high pCD secretion (High).*

The interaction of AP with lung cancer cells and Detroit 573 fibroblasts (14) led to secretion of a specific group of cytokines including IL-4, IL-8, IL-10 and IL-13. Both here and in our previous work, the effects of AP and pCD were identical (2, 3, 13, 14), therefore only AP was used throughout this study. In addition, the use of a synthetic peptide excludes the possibility of some impurity co-isolated with pCD which could trigger some false cellular response.

Increased accumulation of pCD in the growth medium of various cancer cells leading to their faster proliferation has

repeatedly been demonstrated since the original observation in 1986 (16-18). The secretion of this defined group of cytokines correlated with the level of pCD secreted from the cells. Other cytokines (IL-1β, IL-2, IL-5, IL-6, IL-12 P70, IL-12 P40, IFN-γ, TNF-α, IP-10, and and MIP-1β) were undetected. These cytokines also supported the proliferation of the lung cancer cells, but only in the clones with medium or strong pCD production. The clone with no measurable secretion of pCD was resistent, despite the fact that it reacted to AP. The specificity of this stimulation was further demonstrated by the use of specific anti-cytokine antibodies.

Cytokines have an important role in the intercellular communications between different parts of the immune system. Reports on the role of cytokines in cancer are not completely clear showing both growth inhibitory and growth promoting properties (19). Malignant progression is a complex process where cytokines produced both by malignant and surrounding cells can function both as growth factors and/or immunomodulators. In lung cancer, a variety of lung tumor-derived factors including IL-6 (20) and IL-8 (21) regulate tumor growth. Additional studies have shown that lung cancer cells (similar to fresh lung tumors) produce a distinct type 2 cytokine pattern including IL-4, IL-10 and IL-13 (22).

Cytokines IL-4 and IL-13 are produced by T-cells, mast cells and basophils, with reported cancer promoting and blocking functions. IL-4 enhanced the proliferation of several pancreatic cancer cell lines (23). On the other hand it was shown that IL-4 and IL-13 inhibited the proliferation of estrogen induced breast cancer cell lines (24). In addition, IL-4 regulated 15-PGDH in lung cancer cells (25) and mediated pleiotropic effects in the lung cancer microenvironment (26). Similar data can be gathered for IL-8. In addition to its immunological functions as a neutrophil chemoattractant and activating factor, *in vitro* studies have shown that IL-8 acts as a growth factor taking part in angiogenesis in tumor tissues (27). IL-8 has also been found to promote glioma formation and malignant progression (28) and to function as an autocrine growth factor in ovarian (29), colon (30) and lung cancer (31).

Regulatory functions of IL-10 have been found in the growth and differentiation of many cell types of the immune system as well as keratinocytes and endothelial cells (32). The immunomodulatory and immunosuppressive functions of IL-10 in cancer have been reviewed many times and both positive and negative roles which IL-10 may play in cancer development have been stressed (33, 34). In lung cancer, IL-10 promoted both resistance to apoptosis and metastatic potential (35).

Tumorigenesis is a complex process involving not only growth of the primary tumor and/or tumor stem cells, but also communication with surrounding tissues and cells. In this process, stromal tissue, including the vasculature, adipocytes, immune cells and fibroblasts, participate. All these cells secrete numerous cellular products, including various growth factors and extracellular matrix components.

Tumor fibroblasts probably originate from normal fibroblasts and they are very similar to fibroblasts involved in the wound healing processes. There is clear evidence that fibroblasts communicate with the primary tumor cells and this communication is critical for development of the disease. In this regards, our present co-culture experiment showed much faster proliferation of the cancer cells with the fibroblasts compared to the individual cell lines. This enhancement was further increased by the increased pCD secretion and could be inhibited by the addition of either anti-AP or anti-cytokine antibodies. Thus the pCD secretion observed in many cancer derived cell lines may lead to a secretion of cytokines promoting the growth of both fibroblasts and cancer cells. Therefore, a selective inhibition of pCD interaction with these cells could slow down or even entirely stop this process, highlighting pCD as a potential target for cancer therapy.

Acknowledgements

Conflict of Interests Statement

The Authors have no conflict of interests to declare.

References

1 Rochefort H, Garcia M, Glondu M, Laurent V, Liaudet E and Rey J: Cathepsin D in breast cancer: mechanisms and clinical applications, a 1999 overview. Clin Chim Acta *291*: 157-170, 2000.

2 Vashishta A, Saraswat Ohri S, Proctor M, Fusek M and Vetvicka V: Role of activation peptide of procathepsin D in proliferation and invasion of lung cancer cells. Anticancer Res *26*: 4163-4170, 2006.

3 Vetvicka V, Benes P and Fusek M: Procathepsin D in breast cancer: What do we know? Effects of ribozymes and other inhibitors. Cancer Gene Therapy *9*: 854-863, 2002.

4 Chyczewski L, Plonski A, Chyczewska E, Furman M, Ostrowska H and Niklinski J: Activity and tissue localization of cathepsin D in non-small cell lung cancer. Roc Akad Medyc *42*: 217-229, 1997.

5 Zhixin W and Xiaoyan Z: Expression and prognostic relation of cathepsin D in non-small lung cancer tissues and lymph nodes. Chin J Tuberc Respir Dis *21*: 164-166, 1998.

6 González-Vela MC, Garijo MF, Fernández F, Buelta L and Val-Bernal JF: Cathepsin D in host stromal cells is associated with more highly vascular and aggressive invasive breast carcinoma. Histopathology *34*: 35-42, 1999.

7 Benes P, Vetvicka V and Fusek M: Cathepsin D – many functions of one aspartic protease. Crit Rev Oncol Hematol *68*: 12-28, 2008.

8 Laurent-Matha V, Maruani-Herrman S, Prebois C, Beaujoin M, Glondu M, Noel A, Alvarez-Gonzales ML, Blacher S, Coopman P, Baghdiguian S, Gilles C, Lancarek J, Freiss G, Vignon F and Liaudet-Coopman E: Catalytically inactive human cathepsin D triggers fibroblast invasive growth. J Cell Biol *168*: 489-499, 2005.

9 Jones RJ, Matsui WH and Smith BD: Cancer stem cells: are we missing the target? J Natl Cancer Inst *96*: 583-585, 2004.

10 Ronnov-Jessen L, Petersen OW, Koteliansky VE and Bissell MJ: The origin of the myofibroblasts in breast cancer. J Clin Invest *95*: 859-873, 1995.

11 Horgan K, Jones DL and Mansel RE: Mitogenicity of human fibroblasts *in vivo* or human breast cancer cells. Br J Surg *74*: 227-229, 1987.

12 Berchem G, Glondu M, Gleizes M, Brouillet JP, Vignon F, Garcia M and Liaudet-Coopman E: Cathepsin-D affects multiple tumor progression steps *in vivo*: proliferation, angiogenesis and apoptosis. Oncogene *21*: 5951-5955, 2002.

13 Voborka Z, Vetvicka V, Vetvickova J and Fusek M: Cytokines affect procathepsin D-stimulated proliferation of breast cancer cells. Anticancer Res *22*: 913-919, 2002.

14 Fusek M, Vetvickova J and Vetvicka V: Secretion of cytokines in breast cancer cells: the molecular mechanism of procathepsin D proliferative effects. J Interferon Cytokine Res *27*: 191-199, 2007.

15 Vetvicka V and Fusek M: Procathepsin D and human cancer. Curr Topics in Peptide Protein Res *6*: 93-99, 2004.

16 Vignon F, Capony F, Chambon M, Freiss G, Garcia M and Rochefort H: Autocrine growth stimulation of the MCF 7 breast cancer cells by the estrogen-regulated 52 K protein. Endocrinology *188*: 1537-1545, 1986.

17 Vetvicka V, Vetvickova J and Fusek M: Effect of procathepsin D and its activation peptide on prostate cancer cells. Cancer Lett *129*: 55-59, 1998.

18 Vetvicka V, Vetvickova J and Benes P: Role of enzymatically invasive procathepsin D in lung cancer. Anticancer Res *24*: 2739-2744, 2004.

19 Mocellin S, Wang E and Marincola FM: Cytokines and immune response in the tumor microenvironment. J Immunotherapy *24*: 392-407, 2001.

20 Takizawa H, Ohtoshi T, Ohta K, Yamashita N, Nirohata S, Hirai K, Jiramatsu K and Ito K: Growth inhibition of human lung cancer cell lines by interleukin 6 *in vitro*: a possible role in tumor growth *via* an autocrine mechanism. Cancer Res *53*: 4175-4181, 1993.

21 Smith DR, Polverini PJ, Kunkel SL, Orringer MB, Whyte RI, Burdick MD, Wilke CA and Strieter RM: Inhibition of interleukin-8 attenuates angiogenesis in bronchogenic carcinoma. J Exp Med *179*: 1409-1415, 1994.

22 Huang M, Wang J, Lee P, Sharma S, Mao JT, Meissner H, Uyemura K, Modlin R, Wollman J and Dubinett SM: Human non-small cell lung cancer cells express a Type 2 cytokine pattern. Cancer Res *55*: 3847-3853, 1995.

23 Prokopchuk O, Liu Y, Henne-Bruns D and Kornmann M: Interleukin-4 enhances proliferation of human pancreatic cancer cells: evidence for autocrine and paracrine actions. Brit J Cancer *92*: 921-928, 2005.

24 Blais Y, Gingras S, Haagensen DE, Labrie F and Simard J: Interleukin-4 and interleukin-13 inhibit estrogen-induced breast cancer cell proliferation and stimulate GCDFP-15 expression in human breast cancer cells. Mol Cell Endocrinol *121*: 11-18, 1996.

25 Chi X and Tai HH: Interleukin-4 up-regulates 15-hydroxy-prostaglandin dehydrogenase (15-PGDH) in human lung cancer cells. Exp Cell Res *316*: 2251-2259, 2010.

26 Cui X, Yang SC, Sharma S, Heuze-Vourc'h N and Dubinett SM: IL-4 regulates COX-2 and PGE_2 production in human non-small cell lung cancer. Biochem Biophys Res Commun *343*: 995-1001, 2006.

27 Keping X: Interleukin-8 and human cancer biology. Cytokine Growth Factor Rev *12*: 375-391, 2001.

28 Brat DJ, Bellail AC and Van Meir EG: The role of interleukin-8 and its receptors in gliomagenesis and tumoral angiogenesis. Neuro-oncology *7*: 122-133, 2005.

29 Xu L and Fidler IJ: Interleukin 8: an autocrine growth factor for human ovarian cancer. Oncol Res *12*: 97-106, 2000.

30 Li A, Varney ML and Singh RK: Expression of interleukin 8 and its receptors in human colon carcinoma cells with different metastatic potentials. Clin Cancer Res *7*: 3298-3304, 2001.

31 Boost KA, Sadik CD, Bachman M, Zwissler B, Pfeilschifter J and Muhl H: IFN-gamma impairs release of IL-8 by IL-1beta-stimulates A549 lung carcinoma cells. BMC Cancer *8*: 265, 2008.

32 Moore KW, Waal Malefyt R, Coffman RLO and O'Garra A: Interleukin 10 and interleukin 10 receptor. Ann Rev Immunol 19: 683-765, 2001.

33 Vicari AP and Trinchieri G: Interleukin-10 in viral disease and cancer: Exiting the labyrinth? Immunol Rev *202*: 223-236, 2001.

34 Moncellin S, Marincola SM and Young HA: Interleukin-10 and the immune response against cancer: a counterpoint. J Leukocyte Biol *78*: 1043-1051, 2005.

35 Zeng L, O'Conner C, Zhang J, Kaplan AM and Cohen DA: IL-10 promotes resistance to apoptosis and metastatic potential in lung tumor cell lines. Cytokine *49*: 294-302, 2010.