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Human Lung Carcinoma Cells Directly Interact with Monocytes *in-vitro* to Trigger Robust Release of TNF-α from Monocytes and Autocrine IL-6 Secretion

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ABSTRACT

Inflammation is increasingly being associated with cancer initiation and progression. Inflammatory mediators, mostly cytokines like TNF-α and IL-6, originate typically from tumor-immune cell interactions. Monocytes, precursors of macrophages, form a major chunk of the immune cells infiltrating the tumors and potential source of inflammatory signals. Devising *in-vitro* models to study he role of inflammatory mediators in human tumorigenesis, especially in challenging tumors like that of the lung, is a thrust area. In this study, we mimic *in-vivo* conditions, where lung carcinoma cells would come directly in contact with monocytes, by co-culturing representative human lung carcinoma cell line, A549 with human monocytic cell line, THP-1and evaluate TNF-α and IL-6 expression by immunoassays. TNF-α was detectable in co-culture supernatants as early as 1 hour after co-culturing of two cell types. Following the kinetics of TNF-α expression in the co-culture we observe that TNF-α levels reach to peak levels at 4-6 hours after co-culture before receding to lower levels indicating tight regulation of TNF-α expression. Surprisingly, high amounts of IL-6 were detected in the co-culture, even though THP-1 poorly expresses IL-6 unless pre-activated. We found IL-6 was almost exclusively coming from A549 cells, as only IL-6 of human origin was detected when A549 cells were co-cultured with mouse macrophages, RAW 264.7. The presence of TNF-α and IL-6 even after prolonged co-culture points to

desirable presence of TNF-α and IL-6 for tumor cells. These results indicate that tumor cells are able to interact directly with monocytes generating TNF-α in a regulated manner. This suggests a significant role of varying TNF-α and IL-6 levels in tumor microenvironment during tumorigenesis and in better understanding of inflammatory mechanisms associated with cancer.

Keywords: Inflammation, lung carcinoma, A549, TNF-α, IL-6, monocytes, THP-1, Co-culture, immunoassay

INTRODUCTION

Inflammation is a part of the host defense system originally evolved to protect organisms against invading pathogens. However, it has become apparent that inflammation can also be evoked by intrinsic mediators (endogenous mediators) in the absence of infection.. (Balkwill and Coussens, 2004), A link between inflammation and cancer has been suspected since the 19th century, when Rudolf Virchow first noted that malignant tumors arise at regions of chronic inflammation and contain inflammatory infiltrates (Balkwill and Mantovani, 2001; Coussens and Werb, 2002).Recent studies have clearly shown that chronic inflammation increases the risk of tumor development and progression (Karin, 2005, 2006).Clearly, some tumors evade the immune system and go on to become cancers (Seliger, 2005) Some tumor cells also release products that inhibit the immune response; for example by secreting the cytokine TGF-β, which suppresses the activity of macrophages and lymphocytes (Frumento, 2006). In addition, immunological tolerance may develop against tumor antigens, so the immune system no longer attacks the tumor cells (Seliger, 2005). Paradoxically, macrophages can promote tumor growth (Stix, 2007) when tumor cells send out cytokines that attract macrophages, which then generate cytokines and growth factors that nurture tumor development. In addition, a combination of hypoxia in the tumor and a cytokine produced by macrophages induces tumor cells to decrease production of factor/s that blocks metastasis and thereby assists spread of cancer cells. Tumor-Infiltrating Macrophages (TIMs) are known to constitute a large

part of tumors especially carcinomas (tumors of epithelial origin) and it is established that these TIMs are recruited from the circulating monocyte pool (Chen *et al,.* 2003; Johnson *et al*. 2000). Cytokines like TNF, IL-6, Il-10, TGF-β, IL-17have been implicated with tumorigenesis of same cancers (Montovani and Sica, 2010). However, the source of these inflammatory cytokines in tumors that are not associated with chronic infectionand mechanistic pathways involved therein remain poorly understood (Montovani and Sica, 2010). This is particular to carcinomas or cancers of epithelial origin. There is a need to evaluate inflammatory mediators in appropriate model systems of human carcinomas to give fresh insights into cancer-related inflammation. Here, we report that tumor cells of lung origin (Adernocarcinoma-A549) are able to interact with monocytes (THP-1) in an *in-vitro* co-culture system and initiate release of TNF and IL-6 in a regulated manner.

MATERIALAND METHODS

Cell lines: Human lung carcinoma cell line, A549 (a kind gift from DevinderSehgal, Staff Scientist, National Institute of Immunology) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, USA) supplemented with 10% Fetal Calf Serum (Biological Industries, USA) as recommended. Human monocytic cell line, THP-1 and Mouse Macrophage cell line, RAW264.7 were cultured in recommended RPMI (Roswell Park Memorial Institute) medium and supplemented with 10% Fetal Calf Serum (FCS).

Co-culture: Various Numbers of A549 Cells were plated overenight to allow adhesion and stable culture**.** Overnight culture medium of A549 cells was removed and cells were washed with fresh medium before co-culturing with $2*10^5$ THP-1 cells. The co-cultures were set up in DMEM or RPMI medium supplemented with 10% FCS

Culture supernatants: Culture supernatants were collected at various time points after co-culture of cells. The collected supernatants were centrifuged at 12,000 rpm for 15 minutes to remove any cell debris and then stored at -80 degrees C or

analysed immediately.

Cytokine analysis: Commercial Immunoassay (ELISA) kits for Human TNF-α (BD Biosciences), Human IL-6(BD Biosciences), Mouse TNF (BD Biosciences), Mouse IL-6 (BD Biosciences) were used to evaluate cytokine expression. Immunoassay protocols from the suppliers are strictly adhered for cytokine analysis.

Statistical Analysis: Results were expressed as Mean ± standard error of Mean (SEM) of 5 or 3 experiments in triplicate. Where ever applicable data was subjected to one way analysis of variance (ANOVA). P values of <0.05 were considered statistically significant.

RESULTS

A549 cells initiate TNF secretion from THP-1 cells

TNF is a well-known pro-inflammatory cytokine and secreted upfront in most immune responses. To this end, TNF response was studied in A549cells when co-cultured with THP-1 cells. Tumor cells when co-cultured with THP-1 cells gave robust TNF responses (Figure 1).

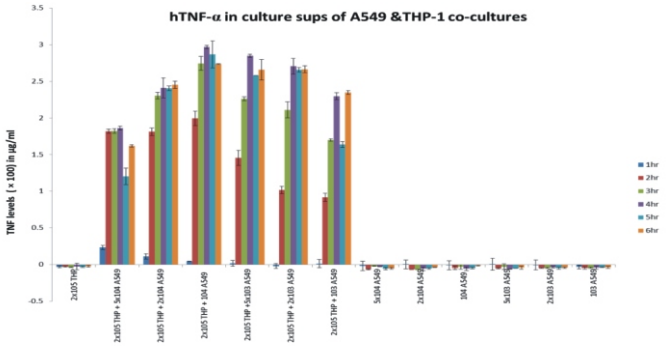


Figure 1. TNF (human TNF or hTNF) expression in A549 (different cell numbers) and THP-1 co-culturesalongwithA549 only, THP-1 only controls at 1 to 6 hour time-points after co-culture.
*representative of atleast 5 experiments with each carried out in duplicates or triplicates. Each value represents statistically significant mean ±SEM where P <0.05 compared to controls

TNF is detected as early as 1 hour post co-culture. It is observed that the optimum tumor cell: monocyte cell ratio in terms of the best TNF response comes nearly to 1:10, which is equivalent to cell ratios found in solid tumors. The response peaks around 4-6 hour time point and recedes thereafter (Figure 2).

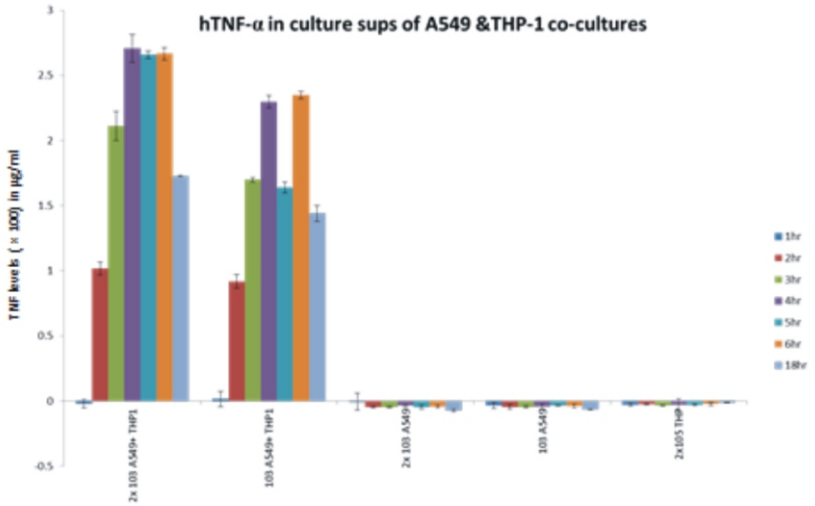


Figure 2. Kinetics of TNF expression in A549/THP-1 co-cultures with controls following several time-points after co-culture.
*representative of atleast 5 experiments with each carried out in duplicates or triplicates.Each value represents statistically significant mean +SEM where P <0.05 compared to controls.

The tumor cell number affects the potency but not the kinetics of the response, probably owing to concentration of the stimulus from tumor cells (Figure 1& 2). The response is faster and as robust, if not more, than the known ligands of TNF activation from THP-1 cells like Flagellin, LPS and Pam3CSK4 (from previous experiments/data not shown). No expression is detected in A549 and THP-1 controls.

TNF expression in co-culture reaches a peak before waning off but persists at later time points

The interesting observation of depleted TNF levels at later time points (30 hours after co-culture) throws the available levels of TNF in the microenvironment into spotlight (Figure 2). This also suggests that TNF inducing factors could actively be regulated from the tumor cells. It also implies that the tumor cells are sensitive to TNF levels throughout.

Robust IL-6 expression is detected and kinetics do not follow TNF secretion

Similar to TNF, Interleukin-6 (IL-6) is detected in high amounts in the co-culture supernatant (Figure 3). Interestingly, IL-6 kinetics indicates that IL-6 is expressed subsequent to TNF expression. At early time points of 1 or 2 hours, negligible or very low levels of IL-6 are detected. Once detected at about 4 hours post co-culture, the levels continue to go up and do not wane off unlike TNF. It is important to note that optimal TNF levels coincide with IL-6 induction (Figure 2 & 3), referring to the likelihood of the two cytokine pathways being inter-related.

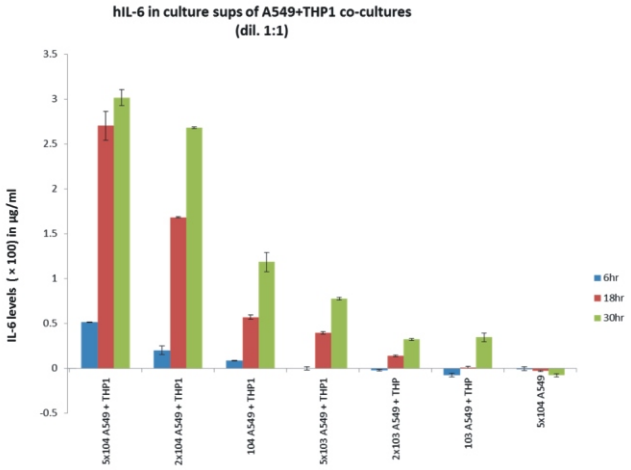
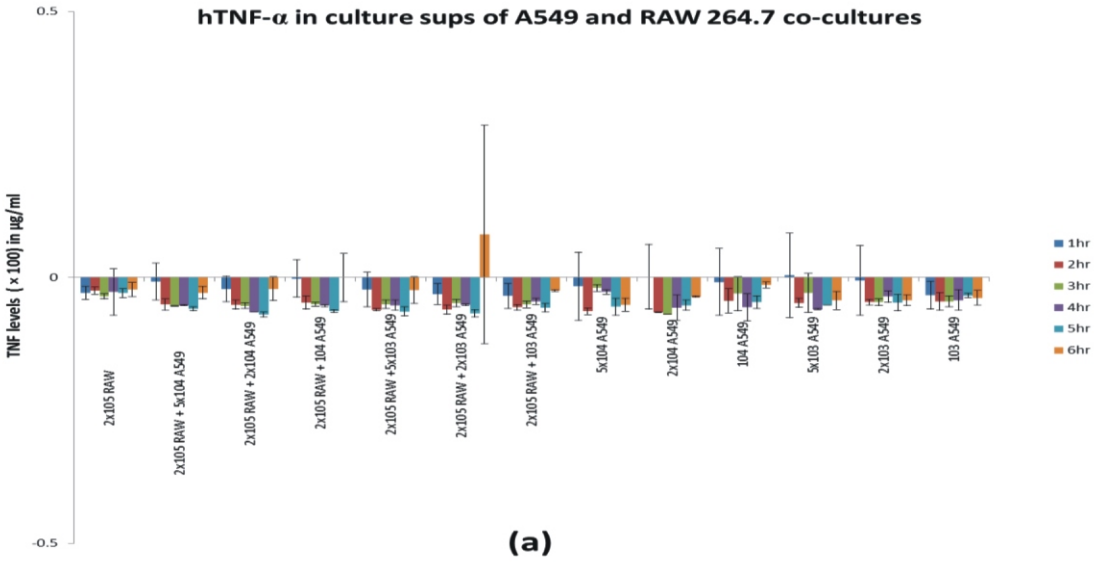


Figure 3. Histogram showing expression of IL-6 at various time pointsin A549/THP-1 co-culture supernatants.
*representative of atleast 5 experiments with each carried out in duplicates or triplicates.Each value represents statistically significant mean +SEM where P <0.05 compared to controls.

TNF-α is secreted by monocytes/macrophages while IL-6 is released by A549 cells

To know more about the source of these cytokines and the nature of factor/s inducing these cytokines, we co-cultured A549 with mouse macrophage cell line, RAW264.7, inter-specific cell co-culture. Analysis of cytokines reveals that the human cells are able to trigger these responses from mouse cells as well, though not with same intensity (Figure 4b). No human TNF is detected on the co-culture (Figure 4a), implying that macrophages are the source of TNF in the co-culture and not A549 cells. Surprisingly, human IL-6 is detected from the co-culture of A549 and RAW264.7 cells (Figure 5). Thus, the two cytokines have separate sources in the co-culture and do not come from the same cell showing an active interaction.



(a)

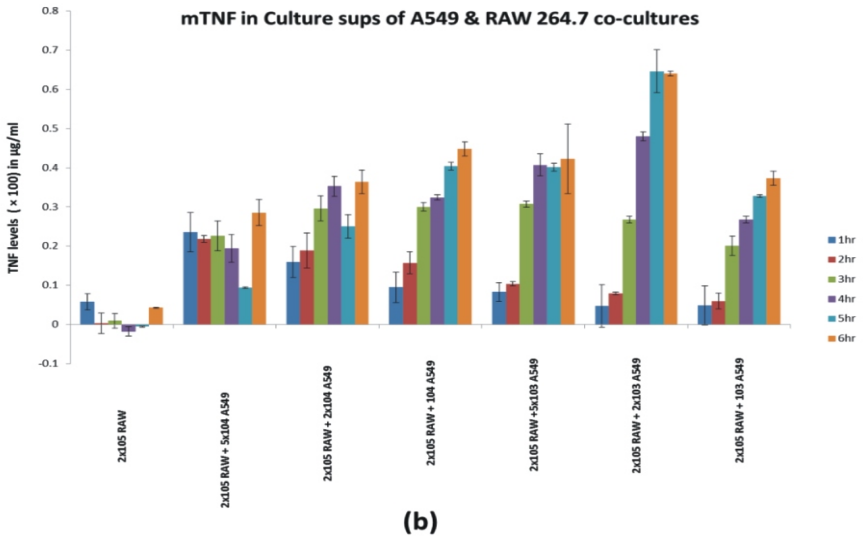


Figure 4. (a) Human TNF and (b) Mouse TNF expression in A549/RAW264.7 (Human/mouse) co-culture *representative of atleast 3 experiments with each carried out in triplicates.Each value represents statistically significant mean +SEM where P <0.05 compared to controls.

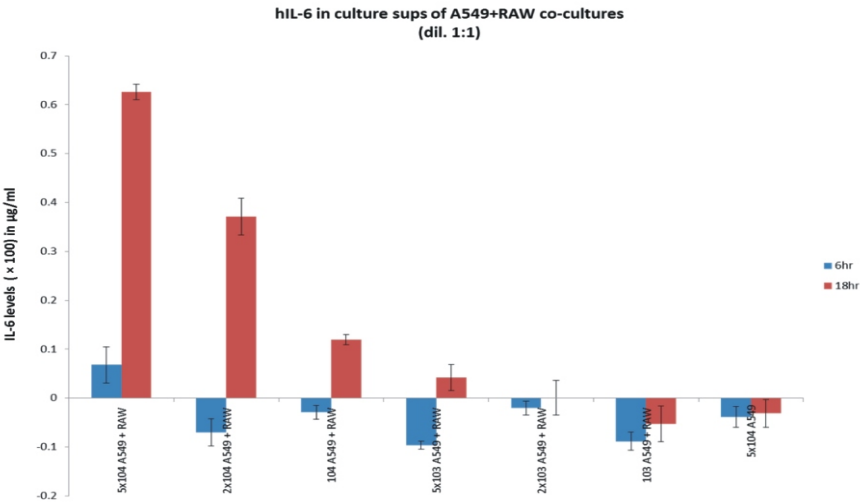


Figure 5. IL-6 levels in co-culture supernatants of A549/RAW264.7
*representative of atleast 3 experiments with each carried out in triplicates.Each value represents statistically significant mean +SEM where P <0.05 compared to controls.

DISCUSSION

TNF (Tumor Necrosis Factor) name would suggest that it counters tumor development, but both,TNF-α and IL-6, are reported to promote tumorigenesis via different pathways (Montovani *et al.,* 2008). Both have been implicated in poor prognosis and chemoresistence (Montovani et al. 2008 and Ohri *et al*. 2009). TNF-α and IL-6 are believed to be critical modulators in Cancer related Inflammation-CRI (Montovani *et. al.,* 2008). They are known to induce pro-proliferative pathways in tumor cells. Here, we show that Human monocytic cells, THP-1 can be modulated by representative lung tumor cells, A549 to generate TNF-α and IL-6 (Figure 1 & 3). This is of considerable importance as monocytes are probably among the first cells encountered by the developing hyperplasia or tumor. Normally, monocytes from the blood would get activated to Macrophages of classical 'M1' type when encountered with tumor antigens which are expressed regularly by tumor cells (Montovani et al., 2006). In orderto evade immune surveillance,tumors are thought to activate macrophages which are more like 'M2' type called Tumor-associated Macrophages or 'TAMs' (Pollard, 2004). This is achieved in majority by the cytokine milieu in the tumor microenvironment (Pollard, 2004).

The expression pattern of TNF in the co-culture system suggests that monocytes do sense the tumor cells instantly and release very high amounts of TNF creating a pro-inflammatory microenvironment (Figure 1 & 2). This inflammatory environment appears to reach a threshold before waning off (Figure 2). The lowering of TNF may be due to TNF exhaustion from the co-culture and/or TNF regulation from monocytes. It appears that the threshold levels are sensed to abate the expression of TNF pointing to possibly active mechanisms involving in TNF expression. Interestingly, IL-6 is expressed when TNF levels reach their peak in the co-culture (Figures 1, 2 & 3). Literature suggests that THP-1 cells do not express IL-6 without activation (Jones et al., 2003) like with PMA (PhorbolMyristic Acid). In order to verify the source of these cytokines, we evaluated cytokine levels in co-cultures of A549 and RAW264.7, mouse

macrophages. The similarity of immune systems in human and mouse was the basis to study this co-culture as a control model. Surprisingly, we detected mouse TNF and human IL-6 in the inter-specific cell co-cultures but no human TNF (Figure 4a, 4b & 5) or mouse IL-6 (data not shown). This indicates that TNF-α is released by macrophages (monocytes) while IL-6 is secreted from tumor cells. This is corroborated in literature where A549 is not known to express TNF-α (Lee et al., 2004) like other cells of epithelial origin. Analysis of the cytokine kinetics invokes a possibility of IL-6 expression in response to TNF presence in the microenvironment from monocytes, this, however, is inconclusive from the results so far. Thus, tumor cells generate TNF *via* a paracrine loop while IL-6 is expressed in an autocrine fashion after appropriate stimulus. Moreover, the ability of tumor cells to trigger similar responses from human as well as mouse monocytes/macrophages underlines the possibility of the similarity of the initiating factor/s. Nevertheless, the expression patternhints at separate inter-regulated cytokine initiating pathways.The persistence of moderate TNF-α and high IL-6 levels indicates existence of chronic inflammation, which is implicated in cancers like gastric, colon etc. (Tu *et al.,* 2008). This microenvironment may be beneficial to tumor cells by evading effector immune responses through altered phenotype of antigen presenting cells like monocytes/macrophages.Further analysis of these and other cytokines likes IL-10, IL-12, TGF-β would provide more insights into the cytokine milieu in tumor microenvironment. Also, identification of pathways that regulate the crosstalk between lung tumor and immune cells will allow detailed understanding of immune evasion by tumors and pursuing possible therapeutic targets for lung cancer.

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REFERENCES

Balkwill, F and Mantovani. A. 2001. Inflammation and cancer: back to Virchow? *Lancet* **357**: 539-545.

Balkwill, F and Coussens, L.M. 2004. Cancer: an inflammatory link. *Nature*, **431**: 405-406.

Chen, J.J.W; Yao P.L; Yuan, A; Hong, T.M; Shun, C.T; Kuo, M.L. 2003.Up-regulation of tumor interleukin-8 expression by infiltrating macrophages: Its correlation with tumor angiogenesis and patient survival in non-small cell lung cancer. *Clin Cancer Res* **9**: 29-37.

Coussens, L.M.and Werb, Z. 2002. Inflammation and cancer. *Nature* **420**: 860-867.

Frumento, G., Piazza T., Di Carlo E and Ferrini S.2006 Targeting tumor-related immunosuppression for cancer immunotherapy *Endocrine, Metabolic & Immune Disorders Drug Targets* **6** (3): 23-37.

Johnson, S., Kerr, K., Chapman, A., Kennedy, M., King, G., Cockburn, J . 2000 Immune cell infiltrates and prognosis in primary carcinoma of the lung. *Lung Cancer* **27**:27-35

Jones, M., Tötemeyer, S., Maskell D., Bryant C. and Barrow P. 2003.Induction of Proinflammatory Responses in the Human Monocytic Cell Line THP-1 by C.jejuni.*Infect. Immun.,* **71** (5) 2626-2633.

Karin, M. 2005. Inflammation and cancer: the long reach of Ras. *Nat. Med.,* **11**: 20-21.

Karin, M. 2006. Nuclear factor-kappaB in cancer development and progression.*Nature.* **441**: 431-436.

Lee, J., Sorbo, L., Uhlig, S., Porro, G., Whitehead, T., Voglis, S., Liu, M., Slutsky, S. and Zhang, H. 2004 Intercellular adhesion molecule-1 mediates cellular cross-talk between parenchymal and immune cells after lipopolysaccharide neutralization. *Journal of Immunology.* **172** (1) 608-616.

Mantovani, A., Schioppa T., Porta C., Allavena, P and Sica, A.2006.Role of tumor-associated macrophages in tumor progression and invasion.*Cancer Metastasis Rev.***25**: 15-22.

Mantovani, A and Sica A. 2010. Macrophages, innate immunity and cancer: balance, tolerance, and diversity. *Curr. Opin. Immuno., l***22** :23-17.

Mantovani, A., Allavena, P., Sica A. and Balkwill, F. 2008. Caner Related Inflammation. *Nature.* **454**, 436-444.

Ohri, C., Shikotra, A., Green, R., Waller, D and Bradding, P. 2009. Macrophages within NSCLC tumour islets are predominantly of a cytotoxic M1 phenotype associated with extended survival. *Eur. Respir., J.* **33**:18-26.

Pollard, J. 2004. Tumour-educated macrophages promote tumour progression and metastasis. *Nat. Rev. Cancer* **4**:718.

Seliger, B. 2005. Strategies of tumor immune evasion. *Bio Drugs.* **19** (6): 47-54.

Stix, G. 2007. A malignant flame.*Scientific American.* **297** (1): 60-67.

Tu, S., Bhagat G., Cui G., Takaishi S., Kurt-Jones E., Rickman B., Betz K., Penz-Oesterreicher M., Bjorkdahl O., Fox J and Wang T. 2008. Overexpression of interleukin-1beta induces gastric inflammation and cancer and mobilizes myeloid-derived suppressor cells in mice. *Cancer Cell,* **14**(5):408-19.