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■■■ RESEARCH ARTICLE

Low cytolytic functions of CD8+ T cells and NK cells and high levels of inflammatory cytokines and chemokines in early diagnosed lung cancer patients

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■■■ ABSTRACT

Background: About 80% to 85% of lung cancers are non-small cell lung cancer (NSCLC), which are believed to secrete mediators that may lead to disorders in the immune system and tumor immune surveillance. This disorder may include dysfunction in CD8+ T cells and NK cells, which play critical role in fighting against cancer. **Aim:** Evaluate the impact of lung cancer on the numbers and function of CD8+ T cells, NK and NK T cells as well as the inflammatory milieu. **Materials and Methods:** Peripheral blood samples were collected from healthy volunteers (n=5) and early diagnosed lung cancer patients (NSCLCs; n=5). The numbers of CD8+ T cells, NK cells and NK T cells were analyzed by flow cytometry after staining with the relevant antibodies. Plasma was prepared from the blood to measure the proinflammatory cytokines and chemokines by Lumiex® protein array. **Results:** Compared to healthy controls, lung cancer patients showed significant decrease in numbers of CD8+ T cells and NK cells. NK cells showed considerable reduction in their expression levels of the cytolytic molecule granzyme-B. Marked higher levels of IL-1 and CCL2 was detected in the plasma of lung cancer patients as compared to healthy controls. **Conclusion:** Early diagnosed lung cancer patients showed dysfunction not only in the numbers of cytolytic immune cells but also their function that correlated with inflammatory microenvironment.

Keywords: Cytotoxic CD8+ T cells; Cytokines; Chemokines; Granzyme; Lung Cancer; Natural Killer; NK; NK T cells; NSCLCs

■■■ ARTICLE INFO

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INTRODUCTION

Lung cancer, in particular NSCLCs, is known to be one of the most serious types of cancer in terms of both incidence and mortality. It accounts for more annual deaths than breast, prostate and colon cancers combined, representing approximately one-third of the total cancer-related mortalities (Wang et al. 2018). The most recent report estimated 2.1 million new lung cancer cases and 1.8 million deaths predicted in 2018 around the world (Ilbawi and Velazquez-Berumen 2018).

Unfortunately, it holds a poor prognosis as its 5-year overall survival (OS) rate ranges between 5-15% (Black and Khurshid 2015). Despite the progress made in the treatment methodologies implied in lung cancer, it is primarily still treated by surgery, chemotherapy or radiation in different combinations. The limited success of these therapeutic interventions has been observed, as only 15%–30% of patients with NSCLCs respond to treatment highlighting the urgent need for novel therapeutic approaches (Aktaş, Öztürk et al. 2018).

Therefore, novel approaches for the treatment of lung cancer have been developed such as immunotherapy and gene therapy (Lara-Guerra and Roth 2016). These new multimodal treatments have the ability to enhance cancer treatment in combination with conventional chemotherapy such as: i) immune checkpoint inhibitors like PD1/PD-L1, ii) antigen-specific vaccines (e.g. mucin-1, L-BLP25, GVAX and GD3 and iii) adoptive T cell transfer (e.g. CAR T-cell) all of which function by augmenting the immune system to kill cancer cells. While very promising, immunotherapy challenges some limitations, including suppressive tumour secretions, physical barrier of trafficking and homing of immune cells into the tumour site and emergence of immunosuppressive cells (Hiraki et al. 2016, Yang et al. 2016).

Recent studies have reviled that development and progression of lung cancer are linked to the weakness of the quality and quantity of adaptive immune responses directed toward cancer cells (Degos et al. 2019). Cytotoxicity plays a vital role in the immune system, which is carried out by various immune cells. Cytotoxic T cells CD8^{+} (CTL) and natural killer cells (NK) conduct this process via two main pathways (Fu et al. 2019). In the first pathway, CTLs use the ligands of the TNF superfamily present on their cell surface to bind and eliminate target cells (Paul and Lal 2017). Meanwhile, the second

Therefore, this study is aimed to address the functionality of CD8^{+} T, NK and NKT cells by measuring granzyme-B in early diagnosed lung cancer patients and correlate it with the cytokine and chemokine profile.

MATERIAL AND METHODS
Subjects populations
Peripheral blood samples were obtained from healthy individuals (n=5: 4 males and 1 female) and from early diagnosed lung cancer patients (n=5:4 males and 1 female) admitted at Oncology Department, Tanta University Hospital, Egypt. For patients, their ages ranged between 35 to 75 years with a median age of (55.52± 4.81) and for controls it ranged between 23 to 30 years with a median age of (26.32 ± 2.430). All patients signed informed consent under a protocol approved by Faculty of Medicine Ethical Committee Review Board,

pathway depends on exocytosis of the cytolytic granular content of the immune cells (e.g. perforin, granzyme and interferon-gamma) into cancer cells leading to their immediate lysis (Street et al. 2004).

In lung cancer, as CTL, NK and NK T cells take part in the immune defense mechanism, the ability of cancer cells to resist these cytolytic mediators is essential for their survival and proliferation. This resistance can be attributed to the release of certain mediators from lung cancer cells such as prostaglandin E2 (PGE2) and transforming growth factor (TGF)-β, which play an important role in assisting tumor cell proliferation, anti-apoptotic properties, angiogenesis and chemotherapeutic resistance (Barrett et al. 2017). In addition, previous studies have correlated the significant reduction of the cytolytic granzyme-B levels in cancer tissues with the decreased cytotoxic activity of immune cells and the establishment of a permissive tumor microenvironment (Hodge et al. 2014). On the other hand, recent studies have reported that in non-treated lung cancer patients, the levels of plasma cytokines and proteins play a key role in shaping the innate and adaptive immunity (Silva et al. 2017). These cytokines are correlated with poor prognosis and decreased OS by altering different constituents of the tumor microenvironment (Mantovani et al. 2018).

Tanta University. Patients were diagnosed according to the World Health Organization (WHO) criteria based on TNM classification (Travis, Brambilla et al. 2015). Classification of subjects was performed after detection of inclusion criteria and patient's performance. The demographic details of these groups have been described in Table1.

Chemical and reagents
Lymphocyte separation medium (Ficoll paqué [TM]) was purchased from Lonza (Basel, Switzerland). Phosphate buffer saline (PBS) was obtained from Verviers, Inc® (Belgium). CD3 (perCP.Cy5•5), CD4 (Allophycocyanin APC-A), CD56 (FITC), granzyme-B (GzB) Phycoerythrin (PE), FACS Perm buffer were purchased from BD Biosciences (BD) (San Jose, CA, USA). Sheath Fluid was purchased from (Luminex Corp, Austin, TX, USA).

PBMCs separation

For plasma separation, blood samples was collected from 5mL peripheral blood on K_2EDTA tubes and samples were centrifuged and plasma was collected. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll Paqué™ technique using break off centrifugation. Cells were washed twice using cold PBS and counted using hemocytometer to check the viability. The numbers of PBMCs in each sample was determined before to culture them.

Surface and intracellular staining of PBMCs

After separation of PBMCs as described above, cells were incubated with anti-CD3 (perCP.Cy5.5), anti-CD4 (APC-A) and anti- CD56 (FITC), mixed gently and then incubated for 20 min at 4ºC. Cells were washed twice with FACS staining buffer by centrifugation at 300 x g for 5 min at 4ºC. The cell pellets were re-suspended in 200 µl staining FACS buffer and transferred into 12x75mm flow acquisition tubes containing 200 µl staining FACS buffer. For intracellular staining, cells stained as above were permealized and fixed in one step using 200 µl of PFA and then stained with anti-GzB (PE). The cells were then washed twice and acquired by flow cytometry using Calibur™ (BD Biosciences, San Jose, CA) and analyzed using flowJo software (BD Biosciences).

Table 1. Demographics and clinical parameters in SCLC patients and control groups

Parameter	Controls		NSCLCs Patients	
	No.	%	No.	%
Sex				
Male	4	90.0	4	90.0
Female	1	10.0	1	10.0
p$_{Control}$			>0.05	
Age				
Min. – Max.	35.0 – 75.0		23.0 – 30.0	
Mean ± SD.	55.52 ± 4.81		26.32 ± 2.43	
Smoker			>0.05	
Nil	0	0.0	5	100.0
Ve-	1	10	0	0.0
Ve+	4	90	0	0.0
			<0.001*	
Performance				
0	0	0.0	1	20.0
1	0	0.0	2	40.0
2	0	0.0	2	40.0
3	0	0.0	0	0.0
Healthy	5	100.0	0	0.0
p$_{Control}$			[FE]p=0.007*	

Table 2. CBC analysis of blood samples from SCLC patients and controls

Parameter	Control	NSCLCs Patients
WBCs/mm3		
Min. – Max.	4750.0 – 10750.0	4.000 – 14750.0
Mean ± SD.	7254 ± 2245.0	9062.0 ± 2349.0
p$_{Control}$		<0.05*
PL/mm3		
Min. – Max.	150000.0–420000.0	120000.0 – 138000.0
Mean ± SD.	2770833 ±8730.9	128000.0 ± 1073.0
p$_{Control}$		0.034*
PBMCs count		
Min. – Max.	1.27 – 1.37	0.53 – 1.50
Mean ± SD.	1.31 ± 0.05	1.01 ± 0.44
p$_{Control}$		0.310
Lymph %	31.5	69.36
Lymph (10³)	2.6	2.84
Mono %	3	8.4
Mono (10³)	0.255	0.344
Gran %	60	22.64
Gran (10³)	5.1	0.92

Measurements of plasma cytokines and chemokine levels

Plasma samples were evaluated with a total of 104 assays (consisting of 102 unique analytes), performed using Luminex immunobead assays as indicated below. All primary data points were collected on a Luminex FLEXMAP 3D® system. Analyte concentrations were calculated from a 7-point curve using a five-parametric fit algorithm (xPONENT® v4.0.3 Luminex Corp., Austin, TX). All data met minimum quality control thresholds defined by the kit manufacturer with a percent coefficient of variation (%CV) values ≤10%, all as previously defined (Fidler, Frankenberger et al. 2017).

Statistical analysis

Data were analyzed using SPSS software version 22 and Mann–Whitney or one-way analysis of variance (ANOVA) with post-hoc for non-parametric analyses. Correlations were performed using Spearman's rank test.. P values < 0·05 were considered significant.

RESULTS

Phenotypic analysis of CD8+ T cells, NK and NKT cells

The gating strategy of T, NK and NKT cells using flow cytometry analysis of PBMCs is shown in Figure 1. This represented analyses showed the total T cells (CD3+ CD56-) as well as CD4 (CD56-

CD3$^+$ CD4$^+$) and CD8$^+$ T cells (CD56$^-$ CD3$^+$ CD4$^-$). It also showed NK cells (CD56$^+$CD3$^-$) and NKT cells (CD56$^+$ CD3$^+$-cells). Based on this gating strategy, we then analyzed the % (Figure 2A) and absolute (Figure 2B) numbers of these cells in from healthy controls and lung cancer patients. Flow cytometry analysis of CD8, NK and NKT cell frequencies were identified for healthy controls and early diagnosed lung cancer patients. The absolute numbers of all studied cell populations were significantly decreased with CD8 and NK.

cells showing the most evident decrease in lung cancer patients compared to healthy controls. However, CD8$^+$ T cells were recorded to have the highest absolute number in all populations (Figure 2).
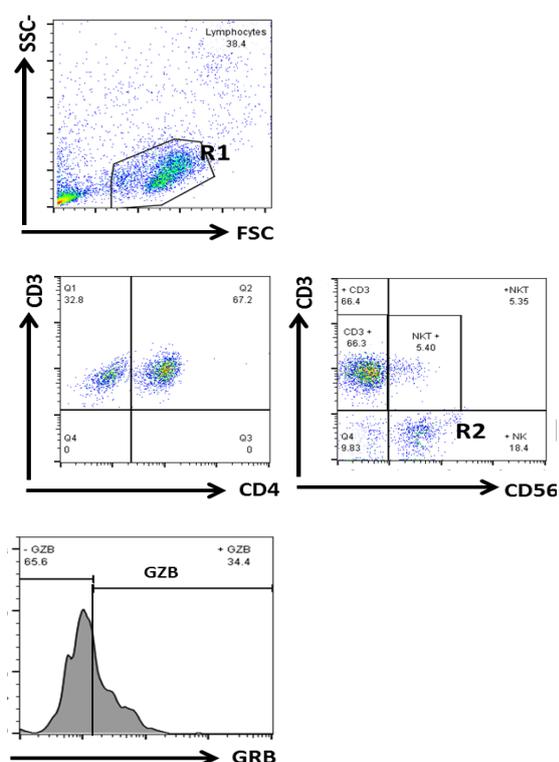
Figure 1. Gating strategy of Flow cytometry representing analysis of PBMCs. (A) Shows the events gated (R1) on the total cells, mostly lymphocytes, from the forward and side scatter. (B) Dot blot analysis gated from R1 that shows CD3$^+$CD4$^-$ population (mostly CD8$^+$T cells; left upper quadrant) and CD3$^+$CD4$^-$ (helper) T cells (right upper quadrant). (C) Dot blot analysis gated from R1 that shows CD3$^+$CD56$^-$ population (mostly total T cells; left upper quadrant), CD3$^+$CD56$^+$ (NKT) cells (right upper quadrant) and CD3$^+$CD56$^+$ (NK; R2) cells (lower right panel). (D) A histogram that shows expression of granzyme B (GZB) on NK cells in R2.
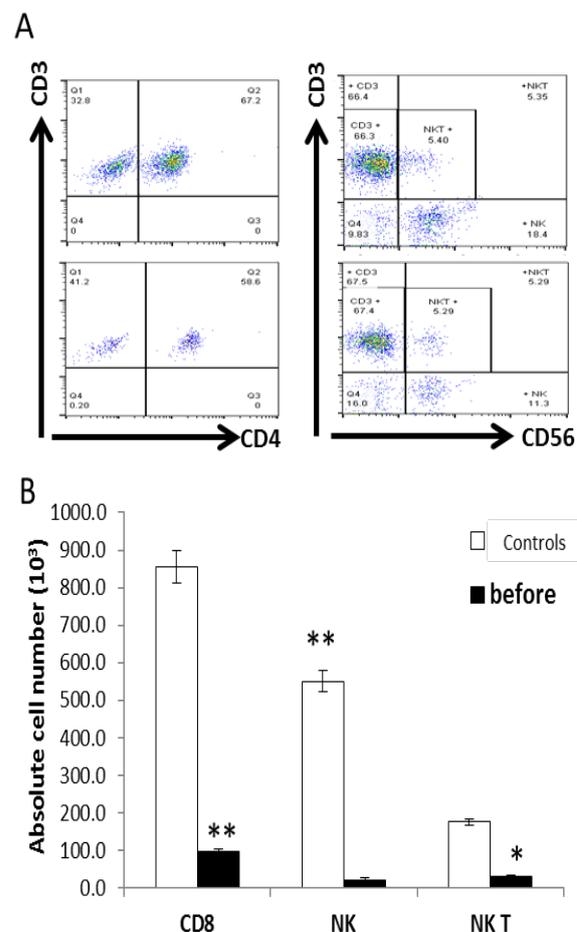

Figure 2. Numbers of T, NK and NKT cells in the peripheral blood of NSCLC patients and controls. (A) Representative flow cytometry analysis of CD8$^+$, NK and NK CD4$^+$ T cell populations. (B) Statistical analysis of the numbers of CD8+, NK and NKT cells from NSCLCs patients (black bars) and controls (white bars). *, $p \leq 0.05$ and **, $p \leq 0.01$.

Production of granzyme B by CD8$^+$, NK and NKT cellsy in lung cancer patients

GzB expression on CD8, NK and NKT frequencies was identified for healthy controls and lung cancer patients. Results demonstrated a notable decrease in the expression levels of granzyme B in all populations of cells of lung cancer patients. Furthermore, this decrease was shown to be prominent in the NK cell population of lung cancer patients when compared to healthy controls (Figure 3).
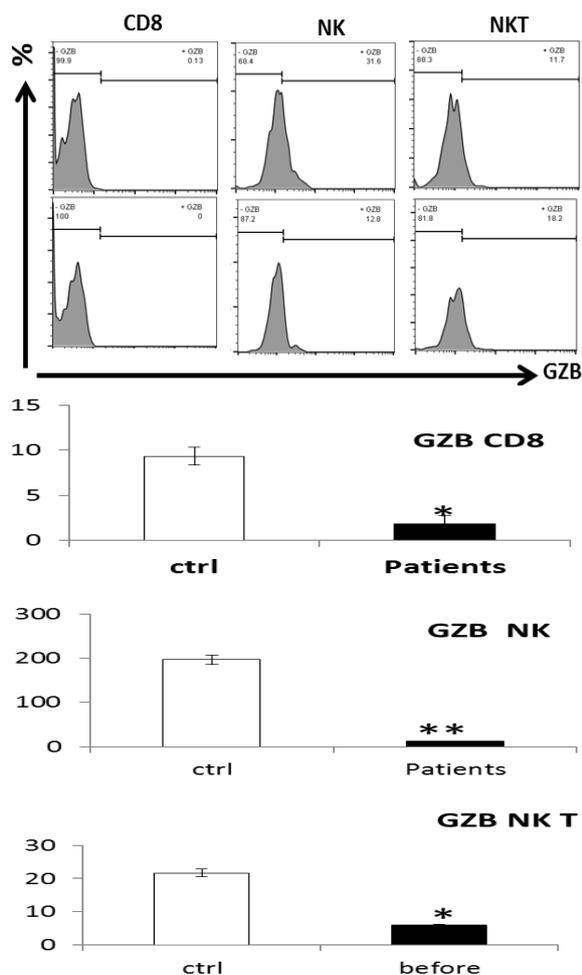
Figure 3: Expression of GZB on CD8+ T cells and NK cells. (A) A representative GzB expression on CD8+, NK and NKT cell populations of controls (upper panel) and NSCLCs patients (lower panel). **(B)** Statistical analysis of GzB expression on CD8+ cells, NK and NKT cells from NSCLCs patients (black bars), compared to CTRL (white bars). *p ≤ 0.05; **, p ≤ 0.01.

High plasma levels of IL-1 and CCL2 in SCLC patient

Plasma IL-1 and CCL2 were measured from healthy controls and lung cancer patients. The results showed that IL-1 and CCL2 demonstrated a marked increase in the lung cancer group of patients. Strikingly, the expression levels of IL-1 were increased by 14000 fold over the levels expressed by healthy controls. On the other hand, CCL2 showed a notable increase in lung cancer patients represented in a 100 fold increase when compared to healthy control (Figure 4). This increase indicates the potential of using the expression levels of these cytokines especially IL-1 as a novel biomarker for the diagnosis of lung cancer patients.
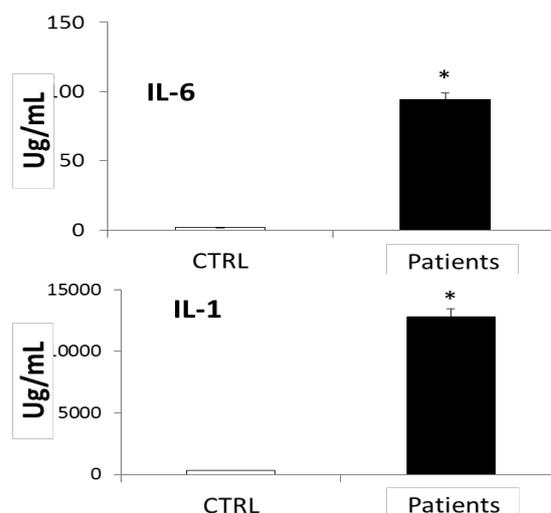
Figure 4. Plasma levels of CCL2 and IL-1 in NSCLC patients. Plasma was collected from the patients and controls and the levels of the chemokine CCL2 and the inflammatory cytokine IL-1 were analyzed by proteomic assay as described in the Materials and Methods. Plasma levels of CCL2 and IL-1 from NSCLCs patients (black bars) and CTRL (white bars). *, p ≤ 0.01.

DISCUSSION

Reports have shown that only 15%–30% of patients respond to treatment which may be due to the production of tumor mediators from cancer cells which enhance tumor surveillance and promoting tumor angiogenesis(Alghamri et al. 2020). Therefore, a broader understanding of different immunosuppressive strategies is important to overcome immune evasion that prevent host immune cells from mounting effective anti-tumor immune is needed.

This pilot study was conducted on a group of 5 early diagnosed patients with lung cancer before induction of chemotherapy and compared to another group of 5 healthy donors. All patients were in an age group ranging between 35 to 65 years which is consistent previous reports that stated that the age of peak prevalence of lung cancer was between 30-70 years (Dehle et al. 2013). Male predominance was manifested in our present study with male to female ratio of 80:20. Also, all males were found to be positive smokers with a long-term history of practicing this habit. This is consisted with the hypothesis that tobacco smoking is the main reason for the development of lung cancer as Tockman et al. reported that more than 80% of lung cancer cases occur in smokers or ex-smokers (Houghton 2018).

In our study, lung cancer patients showed no significant change in hemoglobin levels which is compatible with the results of Phan et al. (Phan et al. 2018). Meanwhile, platelet counts in lung cancer patients were found to be less than 100.000/mm^3 demonstrating a significant decrease compared to healthy controls which are also consistent with the previous results of Omar et al. (Omar et al. 2018). As for the values of WBCs, a decrease was found in lung cancer patients who were revealed to have apparent leukopenia which is in line with the previous finding of Urata et al (Yasuda et al. 2018).

This work was conducted to determine the percentage and functionality of CD8, NK and NKT cells in lung cancer patients before induction of chemotherapy. Our results showed that CD8, NK and NKT count in lung cancer patients was decreased in comparison with non-lung cancer individuals. This finding suggests a combined deficiency in the count of the various lymphocytic components of the anti-tumour response. This decrease in count may be due to myeloid-derived suppressor cells (MDSCs) and regulatory T cells (T$_{reg}$) which is linked with different cancer stages and progression reflecting the tumors microenvironment, which is consistent with our previous work (Diaz-Montero et al. 2009). Also, this decrease can be through the effect of the soluble factors which include prostaglandin E2 (PGE2), transforming growth factor (TGF)-β which take part in this immune evasion mechanism and assist in the tumor cell anti-apoptotic properties and chemotherapeutic resistance (Dehle et al. 2013, Lin and Zhao 2015).

The decrease of NKT and NKCD8 cells in some types of cancers may have a different explanation. In a study by Wang et al. the cytotoxic function of NK T-like cells was assessed by isolating them from human ovarian and prostate cancer patients. The study concluded that the cytotoxic function of these NK T-like cells was compromised in these patients, due to the release of soluble major histocompatibility complex (MHC) class I chain-related molecules by cancer cells impairing the lytic activity via down-regulation of the NKG2D receptor (Peng et al. 2016).

Expression of GzB from CD8, NK and NKT cells was recorded to be decreased in patients before induction of chemotherapy when compared to healthy control. This result is also consistent with the findings of Mukaro et al (Soriano, Mukaro et al. 2012) who suggested that the previously mentioned lung cancer mediators are responsible for inhibition of the CTL and NK function as secretors of GzB.

In healthy controls, expression levels of GzB were recorded to be significantly increased in NK cells when compared to the other subsets of cells. This finding reveals that NK cells are the main secretors of GzB in normal conditions and have the main role in the body defence mechanism against cancer. Moreover, these cells have the ability to attack and decompose the foreign bodies and can be used in developing a new treatment based on immunotherapy as reported by Liu Jin et al. (Liu et al. 2018)

As for cytokines, expression levels of IL-1 were increased 14000 fold more than the healthy control in the plasma. This is consistent with recent studies that analyzed the expression of IL-1 in the tumor microenvironment and reported that IL-1 has a role in tumor progression. It functions by many mechanisms which include driving chronic non-resolving inflammation, tumor angiogenesis, activation of the IL-17 pathway, induction of MDSC and macrophage recruitment, invasion and metastasis (Mantovani et al. 2018) Therefore these previous findings were confirmed in our study by showing the effect of cytokines on inhibiting the function of the cytotoxic T cells and natural killer cells. This inhibition comes as a result of the induction of the MDSC cell population by IL-1 which results in the production of MDSC-induced tumor mediators ultimately affecting the response of the immune system against the lung cancer cells.

As for the CCL2 profile, it exhibited similar results with IL-1 but with a different scale as it showed a 100 fold increase. This is consistent with the findings of Wang et al. which reported that CCL2 functions as monocyte chemo attractant protein secreted to recruit immune cells to the sites of inflammation and may also stimulate angiogenesis through a direct effect

on endothelial cells. In addition, chemo-resistance may also be mediated by cell stress responses involving the over expression of CCL2 (Wang et al. 2018). These large scales of the increase may be used for developing new tools for the serological detection of cancer which can benefit in the evolvement of new treatment strategies.

Collectively, these results confirm that the functionality of CTL and NK cells are impaired in early diagnosed lung cancer patients due to mediators and secretions produced by lung cancer cells. This impaired functionality may be improved by finding a way to activate the immune cells against these lung cancer mediators but this topic needs further research.

Conflict of interest
The authors claim no conflict of interest.

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Egyptian Association for Cancer Research (EACR)
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EACR is an NGO society that was declared by the Ministry of Social Solidarity (Egypt) No. 1938 in 19/11/2014 based on the initiative of Prof. Mohamed Labib Salem, the current Chairman of EACR. EACR aims primarily to assist researchers, in particular young researchers in the field of cancer research through workshops, seminars and conferences. Its first international annual conference entitled "Anti-Cancer Drug Discovery" was successfully organized in April 2019 (http://acdd.tanta.edu.eg). Additionally, EACR aims to raise the awareness of the society about the importance of scientific research in the field of cancer research in prediction, early diagnosis and treatment of cancer. EACR is also keen to outreach the scientific community with periodicals and news on cancer research including peer-reviewed scientific journals for the publication of cutting-edge research. The official scientific journal of EACR is "International Journal of Cancer and biomedical Research (IJCBR: https://jcbr.journals.ekb.eg) was successfully issued in 2017 and has been sponsored by the Egyptian Knowledge Bank (EKB: www.ekb.eg).

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