Original Article

Expression of PD1 and BTLA on the CD8⁺ T Cell and $\gamma\delta$ T Cell Subsets in Peripheral Blood of Non-Small Cell Lung Cancer Patients

Yi Bao^{1,2*}, Juanfen Mo¹, Jiayuan Wu¹, Chenxi Cao²

1. Central Laboratory; 2. Department of Oncology

The Second Hospital of Jiaxing City, Jiaxing, Zhejiang 314000, China

Key words: CD8⁺ T cell; $\gamma\delta$ T cell; programmed cell death protein 1; B and T lymphocyte attenuator; non-small cell lung cancer

Objective To investigate the expression and regulation of programmed cell death protein 1 (PD1), B lymphocyte and T lymphocyte attenuator (BTLA) in peripheral blood of patients with non-small cell lung cancer (NSCLC); to examine the correlation of the mRNA levels between PD and BTLA in NSCLC.

Methods Flow cytometry was used to detect the expression of PD1 and BTLA on the surfaces of CD8⁺ T cells and $\gamma\delta$ ⁺ T cells in the peripheral blood samples collected from 32 in-patients with stage IV NSCLC and 30 healthy individuals. We compared the expression of PD1 and BTLA on the surfaces of $\gamma\delta$ ⁺ T cells in the NSCLC patients with bone metastasis before and after the treatment of zoledronic acid. The correlations of PD1 and BTLA, as well as their ligands were analyzed using Pearson correlation analysis with the cBioPortal data platform.

Results The frequency of PD1 on the surfaces of CD3⁺CD8⁺ T cells was significantly higher than that of the $\gamma\delta$ T cells in both healthy controls ($t=2.324$, $P=0.024$) and NSCLC patients ($t=2.498$, $P=0.015$). The frequency of PD1 on CD3⁺CD8⁺T cells, rather than on CD3⁺ $\gamma\delta$ T cells, was significantly upregulated in advanced NSCLC patients compared with that in healthy controls ($t=4.829$, $P<0.001$). The PD1⁺BTLA⁺ $\gamma\delta$ T cells of the healthy individuals were significantly lower than in the NSCLC patients ($t=2.422$, $P=0.0185$). No difference in percentage of PD1⁺ $\gamma\delta$ ⁺ and BTLA⁺ $\gamma\delta$ ⁺ cells were observed in 7 NSCLC patients with bone metastasis before and after zoledronic acid treatment. PD1 was positively correlated with BTLA in both lung adenocarcinoma ($r=0.54$; $P<0.05$) and lung squamous-cell carcinoma ($r=0.78$; $P<0.05$).

Conclusions The upregulation of co-inhibitory molecules occurs on the surfaces of both CD8⁺ T cells and $\gamma\delta$ T cells in advanced NSCLC, suggesting that these molecules were involved in regulating the inactivation of CD8⁺T cells and $\gamma\delta$ ⁺ T cells, immune escape and tumor invasion.

Lung cancer is the most common malignant tumor in China, with the top ranked morbidity and

Received August 28, 2018; Accepted October 22, 2018; Published online December 6, 2019

* Corresponding E-mail: ybao2011@gmail.com; phone: 86-0573-82073185

Fund supported by the Healthcare Technology Plan of Zhejiang Provincial Health Bureau (No. 2016KYB292); Technology Plan of Science and Technology Bureau of Jiaxing, Zhejiang province (No. 2016AY23054).

mortality rates.¹ Non-small cell lung cancer (NSCLC) accounts for about 85% of lung cancers in total. In recent years, adenocarcinoma has gradually taken over squamous cell carcinoma becoming the most common subtypes of NSCLC. The clinical application of immune checkpoint inhibitors such as programmed death protein 1 (PD1) and its ligand (PDL1) blockades has gradually changed the traditional strategies of the treatment for advanced NSCLC.² In June 2018, the National Medical Product Administration of China officially approved Nivolumab (OPDIVO®) for the treatment of locally advanced or metastatic NSCLC. This approval has shed light on the immunotherapy in cancer treatment.

According to differences in expression of T cell receptors (TCRs), human T lymphocytes are classified into $\alpha\beta$ T cells and $\gamma\delta$ T cells subtypes.³ $\alpha\beta$ T cells are considered to be the dominant subtype of immune T cells and have the capacity of specifically recognizing antigens mediated by a major histocompatibility complex (MHC).⁴ Unlike $\alpha\beta$ T cells, $\gamma\delta$ T cells represent a minor subset that does not require self- MHC-restricted priming, and are functionally similar to innate-like immune cells.⁵ T cell activation is considered to be stimulated by double signals: the TCR recognition of the antigen-MHC molecular complex, followed by a second signal produced by the activation of co-stimulatory molecules.⁶ However, to prevent the overactivation of T cells and the occurrence of autoimmune responses, co-inhibitory signals, such as cytotoxic T-lymphocyte antigen-4 (CTLA4), PD1 and the newly discovered signal B lymphocytes and T lymphocyte attenuator (BTLA), are also present on the surfaces of T cells.⁷ The stimulation of these signal pathways provides negative regulatory signals to suppress over-activation of T cells.⁸

In this study, we examined the expression of PD1 and BTLA on the surfaces of CD8⁺ T cells and $\gamma\delta$ ⁺ T cells in patients with advanced NSCLC and healthy individuals, and explored whether zoledronic acid regulates these co-inhibitory molecules in NSCLC patients with bone metastasis; additionally, the corrections of gene expression profiles including PD1 and BTLA, as well as their ligands were studied using the 全称(TCGA) NSCLC datasets. This study provides preliminary data for understanding the effectiveness of using adoptive T immunotherapy and checkpoint inhibitors against PD1/PDL1 pathway in the treatment of lung cancer.

MATERIALS AND METHODS

Subject recruitment and sample preparation

The study was approved by the ethics committee of the second hospital of Jiaxing

City(20181102h01), and informed consents were obtained by patients and healthy volunteers who provided the blood samples. Blood specimens were collected from 32 in-patients with stage IV NSCLC from January 2016 to June 2017. There were 24 (75%) males and 8 (25%) females, with mean age of 60.6 ± 1.6 years old. The diagnosis of NSCLC was confirmed by pathology, including adenocarcinoma ($n=20$) and squamous cell carcinoma ($n=12$). The TNM staging was based on the criteria of the American Joint Committee on Cancer (AJCC) staging manual (8th edition).⁹ Among these 32 patients, there were 7 patients with bone metastasis, and we collected their blood specimen before and 3 weeks after zoledronic acid treatment (a single 4 mg dose intravenous infusion). We recruited 30 healthy volunteers for controls, including 17 males and 13 females, with mean age of 57.5 ± 1.8 years old.

Blood specimen collection

We collected 2 mL peripheral blood from each fasting subject using sodium citrate dipotassium anticoagulant tubes, and all samples were examined for analysis within 24 hours.

Flow cytometry staining

To determine the frequency of co-inhibitory biomarkers on the surface of $\gamma\delta$ T cells, we conducted multicolored immunofluorescence staining using freshly collected blood specimens from all subjects. The antibodies were conjugated to fluorescent markers as follows: CD3-PE-Cy5.5, TCR $\gamma\delta$ -APC, PD1-FITC, BTLA-PE, and CD8-APC-CY7. The mouse IgG1 with fluorescent markers, including PE-Cy5.5, APC, FITC and PE, were used as isotype controls (BD Biosciences). For extracellular staining, 50 μ L blood sample was incubated with different combinations of fluorochrome-coupled antibodies (10 μ L of each antibody). After 20-min incubation at room temperature, cells were washed twice with 1X PBS and flow cytometry was performed using a flow cytometer (BD FACSCanto II, BD Biosciences, USA). Data were collected and analyzed with DIVA software (version 6.1.3; BD Biosciences, San Jose, CA, USA).

Datasets extraction

The NSCLC mRNA expression datasets were obtained from <http://cbiportal.org>, including 586 lung adenocarcinoma samples and 511 lung squamous cell carcinoma samples derived from The Cancer Genome Atlas (TCGA). The cBioportal data analysis platform⁶ was used to study correlations of the expressions between PD1 and BTLA, PD1 and its receptor PDL1, BTLA and its receptor herpes virus entry mediator (HVEM).

Statistical analysis

Data were presented as mean $\pm$ standard deviation. Unpaired Student's *t*-test or paired *t*-test was used for comparisons between groups. Pearson correlation analyses were performed to analyze correlations of mRNA levels of genes expression using the TCGA database. The Pearson correlation coefficient ≥ 0.4 was defined as medium-degree correlation, and ≤ 0.2 was defined as no correlation. GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA, USA) was used for all statistical calculation and figure generation. *P*-value < 0.05 indicates statistical significance.

RESULTS

Expression of PD1 and BTLA on the surfaces of CD8⁺ and $\gamma\delta$ ⁺ T lymphocytes in the peripheral blood of healthy controls

To assess the frequency of PD1 and BTLA on the surface of CD8⁺ and $\gamma\delta$ ⁺ T cells in peripheral blood of healthy individuals, lymphocytes were first gated on the basis of a forward scatter/side scatter (FSC/SSC) profile, followed by CD8⁺ and $\gamma\delta$ ⁺ T cell analyses in a gated population with CD3⁺ staining. PD1 and BTLA antibodies were used to determine the percentage of PD1 and BTLA positive CD8⁺ and $\gamma\delta$ ⁺ T cells (**Figure 1A**). The results showed that the percentages of PD1⁺CD8⁺ T cells and PD1⁺ $\gamma\delta$ ⁺ T cells were 10.97% $\pm$ 0.91% and 7.70% $\pm$ 1.07%, respectively, with a statistically significant difference ($t=2.324$, $P=0.024$)(**Figure 1B**). The percentages of BTLA⁺CD8⁺ T cells and $\gamma\delta$ ⁺ T cells were 54.89% $\pm$ 3.05% and 57.16% $\pm$ 4.02%, respectively, and there was no significant difference between the two groups ($t=0.449$, $P=0.67$) (**Figure 1C**).

Figure 1. The expression of PD1 and BTLA on the surface of CD8⁺ T cells and γδ⁺ T cells in peripheral blood of healthy controls.

A. The gating strategy showing cells analyses using flow cytometry. B. Higher percentage of PD1⁺ cells was observed in CD3⁺CD8⁺ T cells compared with CD3⁺γδTCR⁺ T cells. C. The percentage of BTLA⁺ cells was not significant different between CD3⁺CD8⁺ and CD3⁺γδTCR⁺ T cells. FSC, forward scatter; SSC, side scatter; PD1, programmed cell death protein 1; TCR, T cell receptor; BTLA, B lymphocyte and T lymphocyte attenuator.

Expression of PD1 and BTLA on the surfaces of CD8⁺ and γδ⁺ T lymphocytes in patients with advanced NSCLC

The gating strategy was the same as that in Figure 1A. In the peripheral blood of advanced NSCLC, the percentages of PD1⁺CD8⁺ T cells and PD1⁺γδ⁺ T cells were 19.85±1.58% and 12.45%±2.51%, respectively, with statistically significant differences ($t=2.498$, $P=0.015$) (**Figure 2A**), and the percentages of BTLA⁺CD8⁺ and BTLA⁺γδ⁺ T cells were 56.71%±3.64% and 56.68%±4.41% ($n=32$), respectively, with no significant difference ($t=0.004$, $P=0.997$) (**Figure 2B**). The frequency of PD1⁺CD8⁺ T cells between patients with NSCLC and healthy controls were 19.85%±1.58% and 10.97%±0.91%, respectively, with significant difference observed between the two groups ($t=4.829$, $P<0.001$) (**Figure 2C**). NSCLC patients tend to have higher percentage of PD1⁺γδ⁺ T cells than the healthy controls [12.45±2.51% ($n=32$) vs. 7.70 ± 1.07% ($n=30$)], but the statistics failed to show a significant difference ($t=1.709$, $P=0.09$) (**Figure 2D**). We did not observe any difference in the BTLA⁺CD8⁺ and BTLA⁺γδ⁺ T lymphocytes between healthy controls and patients with advanced NSCLC (**Figure 2E-F**).

Figure 2. PD1 and BTLA expression on the surface of CD8⁺ T cells and γδ⁺ T cells in peripheral blood of advanced NSCLC and healthy controls.

A. Higher percentage of PD1⁺ cells was observed in CD3⁺CD8⁺ T cells than CD3⁺γδTCR⁺ T cells. B. The percentage of BTLA⁺ cells in CD3⁺CD8⁺ T cell was not significant different from CD3⁺γδTCR⁺ T cells. C. The percentage of PD1⁺ cells in CD3⁺CD8⁺ T cells of NSCLC was higher than that of healthy controls. D. The percentage of PD1⁺ cells in CD3⁺γδ⁺ T cells of NSCLC patients appeared to be higher than that of healthy controls with no significant difference. E and F. The percentage of BTLA⁺ cells in CD3⁺CD8⁺ T cell and in CD3⁺γδ⁺ T cells was not significant different between NSCLC patients and healthy individuals.

Proportion of PD1⁺BTLA⁺ γδ⁺ T cells in the peripheral blood of NSCLC patients

Using the gating strategy shown in Fig 1A, the double positive expression of PD1 and BTLA on the surfaces of γδ⁺ T cells in the peripheral blood of the study subjects was analyzed. The percentages of PD1⁺BTLA⁺γδ⁺ T cells in healthy controls and NSCLC patients were 5.05%±1.08% and 12.08%±2.55% respectively, with a statistically significant difference ($t = 2.422$, $P = 0.0185$) (Figure 3).

Figure 3. The positive expression of PD1 and BTLA on the surface of CD3⁺γδ⁺ T cells between healthy controls and patients with NSCLC.

Changes in γδ⁺T cells and co-inhibitory molecules after zoledronic acid treatment in NSCLC patients with bone metastases

In 7 NSCLC patients with bone metastases treated with zoledronic acid, the frequency of the γδ⁺ TCR cell population was 3.57%±0.69% before the treatment and 10.83%±3.80% 3 weeks after the treatment, with an increasing trend, but the difference was not statistically significant ($t=2.010$, $P=0.064$). Moreover, we did not observe any differences in the percentages of PD1+γδ⁺ and BTLA+γδ⁺ cell before and after zoledronic acid treatment (**Figure 4**).

Figure 4. The frequency changes of γδ⁺TCR cells before and after zoledronic acid treatment in the NSCLC patients with bone metastasis. A. CD3⁺γδ⁺TCR cells. B. PD1⁺γδ⁺TCR cells. C. BTLA⁺γδ⁺TCR cells.

Correlation of mRNA levels of PD1, BTLA and their ligands in NSCLC

Using the gene expression profiles of lung adenocarcinoma and lung squamous cell carcinoma from the TCGA database, we further analyzed the correlations of mRNA levels between PD1 and PD-L1, PD1 and BTLA, BTLA and HVEM in tumor tissues (**Figure 5**). In lung adenocarcinoma (AC), PD1 and its ligand PD-L1 showed a slightly positive correlation ($r=0.33$); a significant positive correlation between PD1 and BTLA was observed in the lung AC dataset ($r=0.54$, $P<0.05$); BTLA and its ligand HVEM were slightly correlated ($r=0.23$). In lung squamous cell carcinoma (SCC),

PD1 and its ligand PD-L1 was barely correlated ($r = 0.18$); a strong positive correlation was observed between PD1 and BTLA ($r = 0.78$, $P < 0.05$); BTLA and its ligand HVEM also showed a positive correlation ($r = 0.48$, $P < 0.05$).

Figure 5. Scatter plots of correlations between PD1 and BTLA gene expressions, and correlations with their corresponding ligands.

A. Between PD1 and PDL1 in lung AC; B. between PD1 and BTLA in lung AC; C. between BTLA and HVEM in lung AC; D. Between PD1 and PDL1 in lung SC; E. Between PD1 and BTLA lung SC; F. between BTLA and HVEM in lung SC. AC, adenocarcinoma; SC, squamous cell carcinoma.

DISCUSSION

This study suggested that the co-inhibitory molecules PD1 and BTLA were both present on the surfaces of CD8⁺ T cells and $\gamma\delta$ ⁺ T cells. In both NSCLC patients and healthy individuals, the PD1 expression on circulating CD8⁺ T cells were significantly higher than those on $\gamma\delta$ ⁺ T cells, but there was no significant difference in the expression of BTLA between CD8⁺ T cells and $\gamma\delta$ ⁺ T cells. In addition, the percentage of PD1⁺CD8⁺ in the peripheral blood in NSCLC patients was higher than in healthy individuals; a similar trend was also observed in PD1⁺ $\gamma\delta$ ⁺ T cells, but no statistically significant difference was observed. The proportions of neither BTLA⁺CD8⁺ T cells nor BTLA⁺ $\gamma\delta$ ⁺ T cells in peripheral T cell was different between healthy individuals and patients with advanced NSCLC; however, the frequency of PD1⁺BTLA⁺ $\gamma\delta$ ⁺ T cells was higher in NSCLC patients than that in healthy individuals.

PD1 and BTLA are two co-inhibitory molecules that have been extensively studied in recent

years, especially PD1. PD1 is widely expressed on the surfaces of T cells, B cells, and nature killer cells (NK cells).¹⁰ The expression of PD1 on the resting T cells is very low in healthy individuals.¹¹ After the activation of T cells, PD1 is transiently upregulated on the surface of the cells; but the expression of PD1 will be downregulated when these T cells are ready to exert cytotoxic effects.¹² The continuously higher expression of PD1 is present on the surface of exhausted T cells.¹³ During the upregulation of PD1 expression, this molecule can bind to its ligand PDL1 on the surfaces of tumor cells. The activation of the PD1/PDL1 pathway leads to T cell inactivation and exerts immunosuppressive effects.¹² Currently, the effectiveness of preclinical trials of monoclonal antibodies targeting PD1/PDL1 molecules is mainly reported in the treatment malignant melanoma and NSCLC,¹³ and these antibodies have been approved for clinical use in several countries, including China. PD1 can also be rapidly upregulated on the surfaces of $\gamma\delta^+$ T cells after activation in the peripheral blood of healthy individuals, and mainly expressed on the terminal effector memory T cells (CD45RA⁺CD27⁻) $\gamma\delta^+$ T subpopulation.¹⁴ However, the expression pattern of PD1 on $\gamma\delta^+$ T cells in peripheral blood of the patients with advanced lung cancer is unclear. Our results showed that the expression of PD1 on the surfaces of CD8⁺ T cells and $\gamma\delta^+$ T cells was upregulated in patients with advanced NSCLC, and more obviously on the CD8⁺ T cells, suggesting that the presence of higher percentages of exhausted CD8⁺ T and $\gamma\delta^+$ T cells in the circulation during the progress of NSCLC leads to a loss of the ability to eliminate cancer and may be associated with the occurrence of immune escape and failure of immunotherapy.¹⁵ Indeed, blocking the PD1/PDL1 signaling pathway has been approved as a therapeutic strategy in the treatment of NSCLC, and our data suggest that in addition to CD8⁺ T cells, the downregulation of PD1 on the surfaces of $\gamma\delta^+$ T cells may also be associated with enhanced antitumor responses and tumor regression.

BTLA is another negative costimulatory molecule discovered recently. It is a type I transmembrane glycoprotein distributed on the surfaces of cell membranes.¹⁶ BTLA is highly expressed on T cells and has also been detected on the surfaces of B cells, NK cells, macrophages and dendritic cells at different expression levels.¹⁷ BTLA is expressed both on the mature $\alpha\beta$ T cells and $\gamma\delta$ T lymphocytes in the peripheral blood, but it is not expressed on regulatory T cells.¹⁸ BTLA ligand, HVEM, belongs to the family of tumor necrosis factor receptors. HVEM is widely expressed in the peripheral blood, including T cells and B cells, and can produce inhibitory signals by binding to BTLA through its specific domain.¹⁹

In a recent study using adoptive tumor infiltrating lymphocytes (TIL) immunotherapy in patients with advanced malignant melanoma, after infusing *in vitro* expanded T lymphocytes isolated from tumor tissues, the researchers found that the patients who responded well to the treatment had a higher percentage of BTLA⁺CD8⁺ T cells in the expanded TIL cells, but it was unclear whether these exogenously large amounts of BTLA⁺CD8⁺ T cells play any role.²⁰ In our study, there was no significant difference in the expression of BTLA on either CD8⁺ T cells or $\gamma\delta$ ⁺ T cells between healthy controls and lung cancer patients, however, we found a high proportion of PD1⁺BTLA⁺ $\gamma\delta$ ⁺T in advanced NSCLC patients. These results in our study may be essential to further clarify the roles of BTLA⁺ /HVEM in the progression of NSCLC.

By analyzing gene expression profiles from the NSCLC TCGA data sets, we found a positive correlation between PD1 and BTLA in the tumor tissues of NSCLC patients. Since we did not obtain tumor tissues from all the NSCLC patients in this study, it may be hard to get a directive conclusion regarding the correlation of PD1 and BTLA by using TCGA data sets. Nevertheless, the current data provide clues for future studies. Interestingly, in a recent study which characterized lung cancer into epithelial subtype and mesenchymal subtype, both upregulated gene and protein expressions of PD1 and BTLA were observed in tumor tissues derived from the mesenchymal subtype of lung cancer rather than the epithelial subtype. This finding indicated the two inhibitory molecules and their associated signaling pathways may be involved in epithelial-mesenchymal transition (EMT), drug resistance and poor prognosis in lung cancer.²¹

We compared the proportional changes of $\gamma\delta$ ⁺ T cells, PD1⁺ $\gamma\delta$ ⁺ and BTLA⁺ $\gamma\delta$ ⁺ T cells in lung cancer patients with bone metastases before and after zoledronic acid treatment. zoledronic acid is a commonly used medicine which slows down bone resorption and allows bone remodeling. It has shown that zoledronic acid has a capacity *in vitro* to promote a large scale of $\gamma\delta$ T cells expansion, which is used as a candidate of the adoptive T cell immunotherapy. We found that after three weeks of zoledronic acid administration, there appeared an increasing trend of $\gamma\delta$ ⁺ T cells in peripheral blood of advanced NSCLC patients, especially in patients younger than 50 years old. However, the difference was not statistically significant, which might be due to the small number of cases in this study. We did not find any change of PD1 or BTLA expression on the surfaces of $\gamma\delta$ ⁺ T cells before and after zoledronic acid treatment. Further studies with large samples are needed to draw a clear conclusion. Previous studies have shown that V γ 9V δ 2, the most dominant $\gamma\delta$ T cell in peripheral

blood, can be largely expanded *in vitro* by small phosphorylated non-peptide antigens, including isopentenyl pyrophosphate and zoledronic acid.^{22,23} A retrospective study has shown that zoledronic acid is associated with a prolonged overall survival (OS) in the patients with breast cancer.²⁴ However, *in vivo* proliferation of $\gamma\delta$ T cells by zoledronic acid may be affected by the age of patient as well as the treatments patient received such as chemotherapy.

Overall, the PD1/PDL1 and BTLA/HVEM inhibitory signaling pathways are involved in tumor cell escape. By studying the expression and regulation of PD1 and BTLA on T cells, we provide important preliminary data for understanding the effectiveness of immunotherapy in the treatment of lung cancer.

Conflict of interest statement

All authors declared no conflicting interests in this study.

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; 68(6):394-424. doi: 10.3322/caac.21492.
2. Moon EK, Langer CJ, Albelda SM. The era of checkpoint blockade in lung cancer: taking the brakes off the immune system. *Ann Am Thorac Soc* 2017; 14(8):1248-60. doi: 10.1513/AnnalsATS.201702-152FR.
3. Cruz MS, Diamond A, Russell A, et al. Human alphabeta and gammadelta T cells in skin immunity and disease. *Front Immunol* 2018; 9:1304. doi: 10.3389/fimmu.2018.01304.
4. Chien YH, Meyer C, Bonneville M. gammadelta T cells: first line of defense and beyond. *Annu Rev Immunol* 2014; 32:121-55. doi: 10.1146/annurev-immunol-032713-120216.
5. Urban EM, Chapoval AI, Pauza CD. Repertoire development and the control of cytotoxic/effector function in human gammadelta T cells. *Clin Dev Immunol* 2010; 2010: 732893. doi: 10.1155/2010/732893.
6. Rudd CE, Taylor A, Schneider H. CD28 and CTLA-4 coreceptor expression and signal transduction. *Immunol Rev* 2009; 229(1):12-26. doi: 10.1111/j.1600-065X.2009.00770.x.
7. Chikuma S. CTLA-4, an essential immune-checkpoint for T-cell activation. *Curr Top Microbiol Immunol* 2017; 410:99-126. doi: 10.1007/82_2017_61.
8. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat Rev Immunol* 2013; 13(4):227-42. doi: 10.1038/nri3405.
9. Edge SB, Compton CC. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. *Ann Surg Oncol* 2010; 17(6):1471-4. doi: 10.3322/caac.21388.
10. Chen L. Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. *Nat Rev Immunol* 2004; 4(5):336-47. doi: 10.1038/nri1349.
11. Riley JL. PD-1 signaling in primary T cells. *Immunol Rev* 2009; 229(1):114-25. doi:

- 10.1111/j.1600-065X.2009.00767.x.
12. Kulpa DA, Lawani M, Cooper A, et al. PD-1 coinhibitory signals: the link between pathogenesis and protection. *Semin Immunol* 2013; 25(3):219-27. doi: 10.1016/j.smim.2013.02.002.
 13. Hall RD, Gray JE, Chiappori AA. Beyond the standard of care: a review of novel immunotherapy trials for the treatment of lung cancer. *Cancer Control* 2013; 20(1):22-31. doi: 10.1177/107327481302000105.
 14. Pasero C, Speiser DE, Derre L, et al. The HVEM network: new directions in targeting novel costimulatory/co-inhibitory molecules for cancer therapy. *Curr Opin Pharmacol* 2012; 12(4):478-85. doi: 10.1016/j.coph.2012.03.001.
 15. Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. *Nat Rev Immunol* 2015; 15(8):486-99. doi: 10.1038/nri3862.
 16. Gertner-Dardenne J, Fauriat C, Orlanducci F, et al. The co-receptor BTLA negatively regulates human Vgamma9Vdelta2 T-cell proliferation: a potential way of immune escape for lymphoma cells. *Blood* 2013; 122(6):922-31. doi: 10.1182/blood-2012-11-464685.
 17. Watanabe N, Gavrieli M, Sedy JR, et al. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. *Nat Immunol* 2003; 4(7):670-9. doi: 10.1038/ni944.
 18. Pasero C, Olive D. Interfering with coinhibitory molecules: BTLA/HVEM as new targets to enhance anti-tumor immunity. *Immunol Lett* 2013; 151(1-2):71-5. doi: 10.1016/j.imlet.2013.01.008.
 19. Ware CF, Sedy JR. TNF superfamily networks: bidirectional and interference pathways of the herpesvirus entry mediator (TNFSF14). *Curr Opin Immunol* 2011; 23(5):627-31. doi: 10.1016/j.coi.2011.08.008.
 20. Radvanyi LG, Bernatchez C, Zhang M, et al. Specific lymphocyte subsets predict response to adoptive cell therapy using expanded autologous tumor-infiltrating lymphocytes in metastatic melanoma patients. *Clin Cancer Res* 2012; 18(24):6758-70. doi: 10.1158/1078-0432.CCR-12-1177.
 21. Lou Y, Diao L, Cuentas ER, et al. Epithelial-mesenchymal transition is associated with a distinct tumor microenvironment including elevation of inflammatory signals and multiple immune checkpoints in lung adenocarcinoma. *Clin Cancer Res* 2016; 22(14):3630-42. doi: 10.1158/1078-0432.CCR-15-1434.
 22. Beetz S, Wesch D, Marischen L, et al. Innate immune functions of human gammadelta T cells. *Immunobiology* 2008; 213(3-4):173-82. doi: 10.1016/j.imbio.2007.10.006.
 23. Marten A, Lilienfeld-Toal M, Buchler MW, et al. Zoledronic acid has direct antiproliferative and antimetastatic effect on pancreatic carcinoma cells and acts as an antigen for delta2 gamma/delta T cells. *J Immunother* 2007; 30(4):370-7. doi: 10.1097/CJI.0b013e31802bfff16.
 24. Lipton A, Cook RJ, Major P, et al. Zoledronic acid and survival in breast cancer patients with bone metastases and elevated markers of osteoclast activity. *Oncologist* 2007; 12(9):1035-43. doi: 10.1634/theoncologist.12-9-1035.