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Original Article

Peripheral circulating Ts (CD8+CD28-)/CD8+ is elevated in non small cell lung cancer patients

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Abstract: Antitumor immune response is usually inhibited by immunosuppressive tumor microenvironment. CD8+CD28- T lymphocyte (Ts; or CD8+ Treg) is another regulatory T cell apart from CD4+CD25highCD127low Treg, and it has been detected infiltrating in tumor tissue of various types as well. The aim of this study is to investigate possible role of both Tregs in peripheral circulation of non small cell lung cancer (NSCLC) patients. Twenty-eight eligible NSCLC patients pariticipated this study, and 17 healthy volunteers plus 1 patient of pulmonary bulla and 2 patients of congenital chest wall deformity were also included in this study as control group. Peripheral circulating CTL (CD8+CD28+)/CD8+, Ts or CD8+ Treg (CD8+CD28-)/CD8+, CD4+ Treg (CD4+CD25highCD127low/CD4+ were assayed by Flow Cytometry. We observed that Ts (CD8+CD28-)/CD8+ in NSCLC group is significantly higher than that in control group (P=0.0056). CTL (CD8+CD28+)/CD8+ in NSCLC group is significantly lower than that in control group (P=0.0013). CD4+ Treg (CD4+CD25highCD127low)/CD4+ in NSCLC group is not significantly different from that in control group (P=0.8689). In conclusion, Ts (CD8+ Treg) instead of CD4+ Treg might be an immunosuppressive factor in peripheral circulation of NSCLC patients.

Keywords: Ts, CD8+ Treg, tumor immune, peripheral circulation

Introduction

Malignant tumor consists of genetically heterogeneous cells. These neoplastic cells have been observed capable of eliciting host immune response [1-3]. However, although rare cases of tumor spontaneous regression caused by host antitumor immune attack have been reported [4, 5], in most cases tumor could naturally progress in spite of elicited immune response. During recent decades, secrets of tumor evasion from immune attack is partly uncovered in that immunosuppressive microenvironment within tumor tissue is dominant so that immune effector cells are disabled or functionally altered [6]. Among various immunosuppressive factors with tumor microenvironment (TME), regulatory T cells (Treg) have been proved playing crucial role, and their recruitment and function within tumor were well studied [7]. In healthy people, Treg is responsible for maintaining host immune homeostasis, preventing excessive immune response. It has been established that Treg plays important role in autoimmune disease, organ transplantation, inflammatory diseases, etc [8, 9]. CD8+CD28- T cell is another T cell subset with similar immune regulatory function with CD4+ Treg, which is termed as T suppressor (Ts) or CD8+ regulatory T cell (CD8+ Treg). Although CD8+CD28- T cell is less focused on compared with its counterpart-CD4+ Treg, its immunosuppressive function has been emphasized during recent decade, and its role in organ transplantation [10], autoimmune diseases [11], inflammatory diseases [12] and cancer has been reported.

Evidences suggest Ts might be another important immunoregulatory cell type suppressing antitumor response. Ts cells were detected infiltrating within tumor tissue in various tumor type; purified CD8+CD28- T cell from tumor-

infiltrating lymphocyte could inhibit T cell proliferation and cytotoxic T cell activity in vitro [13]. Currently studies on Ts cell's role in tumor is very few. Considering tumor could exert systemic effect to facilitate its progression and metastasis, in this study, we focus on peripheral circulating immunosuppressive factors by investigating proportional changes of CD8+ CD28-/CD8+ T cell in peripheral circulation of non small cell lung cancer (NSCLC) patients, aiming to evaluate its systemic influence in tumor, and provide more collaborative evidence to establish its role in tumor.

Materials and methods

This study was approved by Research Ethics Committee of both General Hospital of PLA and General Hospital of Bei Jing Millitary Command, and carried out in accordance with The Code of Ethics of the World Medical Association. Informed consent were obtained from all participants enrolled in this study, and individual informations were protected. From November 2015 to February 2016, eligible NSCLC patients who were admitted to either General Hospital of Bei Jing Command or General Hospital of PLA were included as NSCLC group in this study. The inclusion criteria were as follows: 1. being clinically diagnosed as lung cancer; 2. being newly diagnosed without receiving any antitumor therapy previously; 3. without acute or chronic inflammatory disease during study; 4. without suffering from immunodeficiency condition; 5. without suffering from immune-related disease; 6. without history of long-term receiving immunity influencing drug therapy. The exclusion criteria were as follows: 1. being pathologically diagnosed as benign disease or small cell lung cancer (SCLC); 2. without pathologic diagnosis; 3. receiving incomplete examinations; 4. being with other concomitant malignancy.

Besides, 20 persons were also included in this study as healthy control group. Among them, 1 person was diagnosed as pulmonary bulla, 2 persons were diagnosed as congenital chest deformity. They were all admitted in General Hospital of Bei Jing Command, while other members of control group were healthy volunteers. The inclusion criteria for control group were as follows: 1. without any type of malignant tumor; 2. without acute or chronic inflammatory disease during study; 3. without suffering from immunodeficiency condition; 4. without suffering from immune-related disease; 5. without history of long-term receiving immunity influencing drug therapy.

Peripheral blood sample

Peripheral venous blood were obtained from patients at morning and stored in heparin-coated tube, then blood samples were transfered immediately to laboratory.

Flow cytometry assay of proportion of Ts, CTL, CD4+ Treg

The reagents we used for immunostaining were as follows: FITC conjugated anti-CD4, PE-conjugated anti-CD25, ECD-conjugated anti-CD45, PC5-conjugated anti-CD127, PC7-conjugated anti-CD3, FITC-conjugated anti-CD8, PE-conjugated anti-CD28. (all reagents were commercially bought from Beckman Coulter Corporation, USA).

Cell staining

Blood samples were stained according to manufacturer's instruction, the following panel were designed as staining combinations: 1: CD4-FITC/CD25-PE/CD45-ECD/CD127-PC5/CD3-PC7; 2: CD8-FITC/CD28-PE/CD45-ECD/CD3-PC7.

Quantification by flow cytometry

After staining, blood samples were assayed by a 5-colored uni-laser Flow Cytometer (Beckman Coulter FC500). Data analysis were processed with Kaluza software (Beckman Coulter Corporation).

The combinations of antibodies represent as follows: CD3+CD8+CD28- for T suppressor (Ts) or CD8+ Treg, CD3+CD8+CD28+ for Cytotoxic T lymphocyte (CTL), CD3+CD4+CD25highCD127-low for CD4+ regulatoty T lymphocyte (CD4+ Treg).

Statistical analysis

The data are presented as mean or mean ± SD. To compare respective proportion of Ts, CD4 Treg, CTL between 2 groups, student t test was used. *P* values less than 0.05 is considered sig-

Table 1. Basic information of participants

		NSCLC group	Control group※
Age		60.82 ± 12.46	25.21 ± 8.17
Gender	Male	14	4
	Female	14	16
Staging	I	10	
	II	6	
	III	4	
	IV	8	
Pathology	Adenocarcinoma	23	
	Squamous carcinoma	4	
	Adenosquamous carcinoma	1	

※Mean age of participants in NSCLC group is significantly higher than that in control group (P=0.001).

nificant. Statistical analysis is performed using SPSS software (version 13 for Windows, SPSS, Inc.).

Results

General characteristics of participants

There were 28 eligible NSCLC patients participating this study. Among them, 14 cases were male, 14 cases were female; 22 cases were adenocarcinoma, 4 cases were squamous cell carcinoma, 2 cases were adenosquamous carcinoma. The average age for NSCLC group and control group are respectively 60.82 ± 12.46 VS 25.21 ± 8.17 (P=0.001) (see **Table 1**).

*Flow cytometry assessment of Ts, CTL, CD4+ Treg: (**Figure 1**)*

Ts or CD8+ Treg (CD8+CD28-): Proportion of Ts among CD8+ T lymphocyte in NSCLC group is significantly higher than that in control group (46.31% ± 16.11% VS 32.83% ± 14.73%, P= 0.0056); (**Figure 2**).

CTL (CD8+CD28+): Proportion of CTL among CD8+ T lymphocyte in NSCLC group is significantly lower than that in control group (50.84% ± 16.83% VS 67.17% ± 14.73%, P=0.0013); (**Figure 3**).

CD4+ Treg (CD4+CD25highCD127-): Proportion of CD4+ Treg among CD4+ T lymphocyte in NSCLC group is not significantly different from that in control group (6.95% ± 2.21% VS 7.06% ± 1.19%, P=0.8689); (**Figure 4**).

Discussion

Malignant tumor remains one of the biggest threats to human health and enormous economic burden to societies. According to latest published cancer statistics, cancer-related death worldwide in 2012 amount to 8.2 million, and newly diagnosed cases amount to 14.1 million [14]. Molecular and biologic studies reveal that neoplastic cells are genetically unstable and heterogeneous, which theoretically become target of host immune attack. Many evidences demonstrate the existence of host immune response targeting malignant tumor cells in human and animal models [1-3]. However, in most cases tumor grows wildly in host in spite of detected existence of antitumor immune response. Moreover, various types of immunotherapies aiming to augment immune function failed in most patients. During recent decade, concept of tumor microenvironment (TME) was proposed to explain the failure of antitumor immune attack against tumor cells. According to cancer immunoediting theory [15], majority of neoplastic cells were eliminated by antitumor immune response, while a genetically heterogeneous subset survived and could exploit various means to form suppressive immune milieu at tumor site so as to escape immune attack. One important pathway is to recruit regulatory T cells (Treg) from peripheral circulation (natural Treg, nTreg) and incuce topical naïve CD4+ T cell converting into Treg (induced Treg, iTreg).

Treg's role in tumor microenvironment has been well studied, and some newly developed monoclonal agents targeting to block some of its inhibitory effect achieved inspiring clinical benefit [16, 17]. In contrast to Treg, CD8+CD28- T cell termed as T suppressor (Ts) or CD8+ Treg is far less emphasized. During recent decade, Ts or CD8+ Treg, was proved capable of exerting similar immunoregulatory function with Treg. Ciubotariu etc provided direct evidence that CD8+CD28- T cell could suppress human CD4+ Th cell activation by interfering with antigen presenting cell providing necessary co-stimulating signal when bearing antigen [18]. Later,

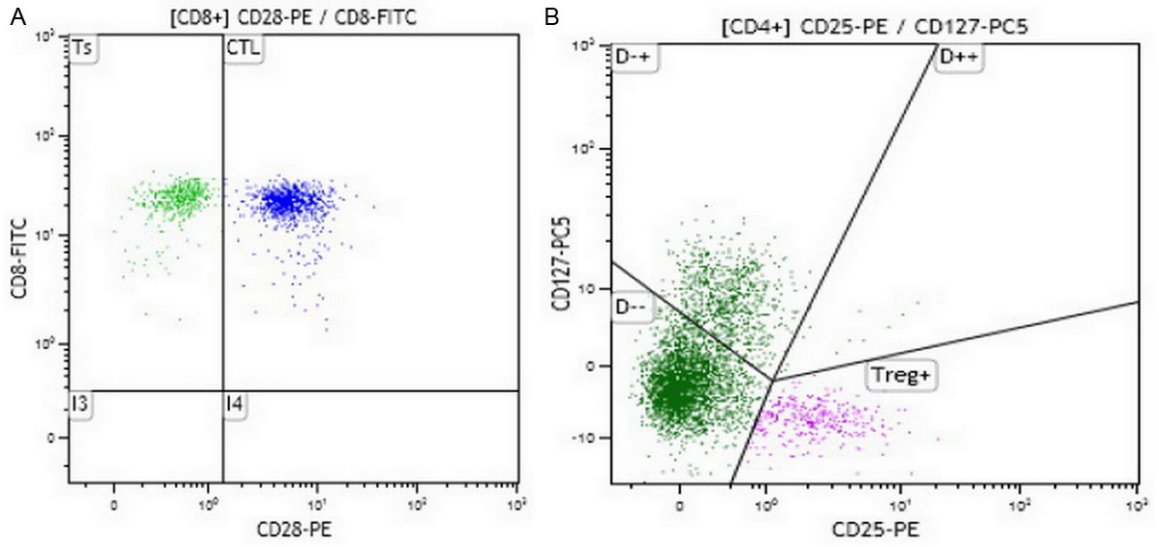

Figure 1. Flow Cytometry distribution of Ts, CTL, CD4 Treg (A, B). (A) Coexpression of CD8 and CD28, Ts is indicated as CD8+CD28-, CTL is indicated as CD8+CD28+; (B) Coexpression of CD25 and CD127, CD4+ Treg is indicated as CD25highCD127low.

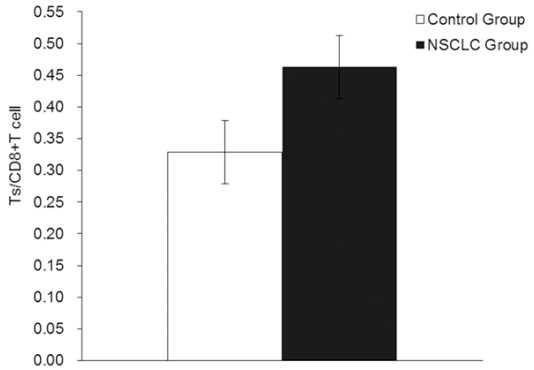

Figure 2. Comparison of Ts. In NSCLC group, peripheral circulating Ts (CD8+CD28-)/CD8+ is significantly higher than those in control group (46.305 ± 16.116 VS 32.833 ± 14.732, P=0.0056).

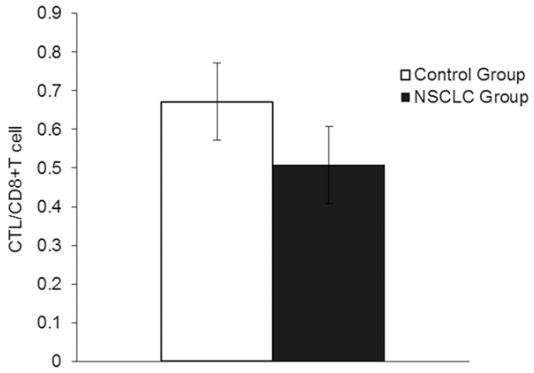

Figure 3. Comparison of CTL. In NSCLC group, peripheral circulating CTL (CD8+CD28+)/CD8+ is significantly lower than those in control group (50.837 ± 16.834 VS 67.172 ± 14.729, P=0.0013).

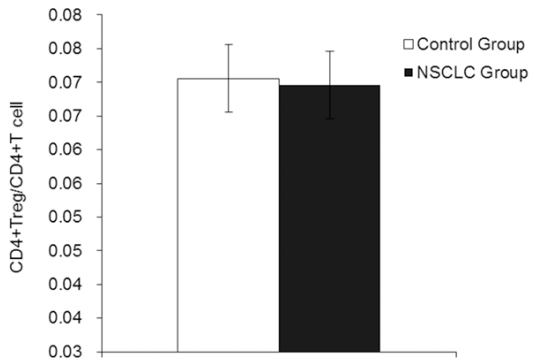

Figure 4. Comparison of CD4+ Treg peripheral circulating CD4+ Treg (CD4+CD25highCD127low)/CD4+ is not significantly different between NSCLC group and control group (6.954 ± 2.208 VS 7.058 ± 1.194, P=0.8689).

Chang etc found that CD8+CD28- Ts could induce upregulation of inhibitory receptor ILT3 and ILT4 in monocyte and dendritic cell, thus reduce their expression of co-stimulatory molecules, finally cause corresponding antigen-specific Th cell into unresponsiveness [19]. Manavalan etc reported Ts could also work on endothelial cell in a similar way [20]. Recently Liu etc reported that human mesenchymal stromal cells could enhance CD8+ Treg's immunoregulatory function. More importantly, they proposed that CD8+ Treg's function included inhibiting naive CD4+ T cell proliferation and activation, reducing production of IFN-γ by activated CD4+ T cell and inducing apoptosis of activated

CD4+ T cell. They also found that CD8+ Treg's working manner might be IL-10 and FasL dependent [21].

Presently Ts or CD8+ Treg has been proved to play a role in various diseases including organ transplantation, autoimmune disease, inflammatory disease and cancer. However, relevant studies are just beginning, especially for studies on CD8+ Treg's role in tumor which are few. Pages etc reported that CD8+CD28- T cells infiltrated in human colorectal cancer tissue, nevertheless, these cells seemed to be cytotoxic rather than immunosuppressive, and correlated with better prognosis [22]. Filaci etc made comprehensive study on tumor-infiltrating CD8+CD28- T cell function. They found that tumor-infiltrating CD8+CD28- T cell were observed in various tumor types, and purified tumor-infiltrating CD8+CD28- T cell exhibited suppressive function on T cell proliferation and cytotoxity [13]. Parodi etc recently reported that both CD4+ Treg and CD8+ Treg were found infiltrating in human bladder and renal cancer tissues, while constitution of both Tregs within tumor mainly by CD4+ Treg or CD8+ Treg varied in different cases. They also reported that more Treg than effector T cell within tumor is correlated with tumor relapse [23]. All these findings collectively establish that Ts or CD8+ Treg cell play immunosuppressive role in tumor microenvironment.

In this study, we detected that CD8+CD28-/CD8+ T cell in peripheral circulation of NSCLC patients was significantly higher than that of healthy people, while CD4+ Treg/CD4+ T cell in peripheral circulation did not differ between NSCLC patients and healthy people. This finding indicates that Ts or CD8+ Treg instead of CD4+ Treg is an immunosuppressive factor in peripheral circulation, which has two-fold meanings: firstly, since local immune status is closely associated with host peripheral circulation immune status, such as immune cells recirculation or recruitment, theoretically effector T cells infiltrating tumor tissue could be impaired or even inactivated before their arriving at tumor by Ts or CD8+ Treg. If it were proved, then elevated Ts in peripheral circulation would undoubtedly be an important contributing factor for tumor immunosuppressive milieu and also potential target for immunotherapy. Presently relevant study is lacking, further investigations on peripheral circulation effector T cell's function in tumor patient would provide direct evidences; secondly, considering hematogenous metastatic tumor cells transport through blood vessels to arrive at distant organ, Ts cell might facilitate metastasis by impairing circulating CTL or Th cell function so that tumor cells would have more chances safely arriving in stead of being captured and killed. of course it also need experimental evidences to support. If true, it would be new target for clinical prevention and control of metastasis.

There are two possible flaws in this study. The first is that when setting control group, we did not take age-matching into consideration. According to Simone and colleagues' study, frequency of CD8+CD28- T cell in peripheral circulation increases with aging [24]. Till now, no other studies report similar result. If it is true, the influence of aging should be excluded as a disturbing factor. As it is impossible to discriminate influence of aging in this study, if necessary, setting an age-matched control group and making comparison again could solve this problem. The second flaw is that some studies proposed that tumor-infiltrating CD8+CD28- T cells are functionally heterogeneous, being cytotoxic or immunoregulatory, moreover, there is no known distinguishing marker to discriminate them [25, 26]. So a question rises naturally: is increased CD8+CD28- T cells in peripheral circulation of NSCLC patients mainly cytotoxic or immunoregulatory? Further studies assaying circulating CD8+CD28- T cell's function could ascertain its character.

In conclusion, we observed that CD8+CD28-/CD8+ T cell in peripheral circulation of NSCLC patients is significantly elevated compared with healthy people, which indicates that Ts or CD8+ Treg might be peripheral immunosuppressive factor.

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Disclosure of conflict of interest

None.

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References

[1] Gross L. Intradermal immunization of C3H mice against a sarcoma that originated in an animal of the same line. Cancer Res 1942; 3: 326-333.

[2] Hewitt HB, Blake ER and Walder AS. A critique of the evidence for active host defense against cancer, based on personal studies of 27 murine tumors of spontaneous origin. Br J Cancer 1976; 33: 241-259.

[3] Klein G, Klein E. Immune surveillance against virus-induced tumors and nonrejectability of spontaneous tumors: contrasting consequences of host versus tumor evolution. Proc Natl Acad Sci U S A 1977; 74: 2121-2125.

[4] Alam MA, Das D. Spontaneous regression of hepatocellular carcinoma-a case report. J Gastrointest Cancer 2016; [Epub ahead of print].

[5] Lopez-Pastorini A, Plönes T,Brockmann M, Ludwig C, Beckers F and Stoelben E. Spontanesous regression of non-small cell lung cancer after biopsy of a mediastinal lymph node metastasis: a case report. J Med Case Rep 2015; 9: 217-221.

[6] Chen F, Zhuang X, Lin L, Yu P, Wang Y, Shi Y, Hu G, Sun Y. New horizons in tumor microenvironment biology. challenges and opportunities. BMC Med 2015; 13: 45.

[7] Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B,Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen L and Zou W. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 2004; 10: 942-949.

[8] Mauro DI, Franca F, Alessandra C, Terenzi A, Castellino F, Bonifacio E, Del Papa B, Zei T, Ostini RI, Cecchini D, Aloisi T, Perruccio K, Ruggeri L, Balucani C, Pierini A, Sportoletti P, Aristei C, Falini B, Reisner Y, Velardi A, Aversa F and Martelli MF. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. Blood 2011; 117: 3921-3928.

[9] Yamada A, Arakaki R, Saito M, Tsunematsu T, Kudo Y and Ishimaru N. Role of regulatory T cell in the pathogenesis of inflammatory bowel disease. World J Gastroenterol 2016; 22: 2195-2205.

[10] Baeten D, Louis S, Braud C, Braudeau C, Ballet C, Moizant F, Pallier A, Giral M, Brouard S and Soulillou JP. Phenotypically and functionally distinct CD8+ lymphocyte populations in long-term drug-free tolerance and chronic rejection in human kidney graft recipients. J Am Soc Nephrol 2006; 17: 294-304.

[11] Scarsi M, Ziglioli T, Airò P. Decreased circulating CD28-negative T cells in patients with rheumatoid arthritis treated with abatacept are correlated with clinical response. J Rheumatol 2010; 37: 911-916.

[12] Marcq IM, Pomié C, Romagnoli P and van Meerwijk JP. CD8+CD28- regulatory T lymphocytes prevent experimental inflammatory bowel disease in mice. Gastroenterology 2006; 131: 1775-85.

[13] Filacin G, Fenoglio D,Fravega M, Ansaldo G, Borgonovo G, Traverso P, Villaggio B, Ferrera A, Kunkl A, Rizzi M, Ferrera F, Balestra P, Ghio M, Contini P, Setti M, Olive D, Azzarone B, Carmignani G, Ravetti JL, Torre G and Indiveri F. $CD8^+CD28^-$ T regulatory lymphocytes inhibiting T cell proliferative and cytotoxic functions infiltrate human cancers. J Immunol 2007; 179: 4323-4334.

[14] Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015; 65: 87-108.

[15] Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science 2011; 331: 1565-1570.

[16] Ott PA, Hodi FS, Robert C. CTLA-4 and PD-1/PD-L1blockade: new immunotherapeutic modalities with durable clinical benefit in melanoma patients. Clin Cancer Res 2013; 19: 5300-5309.

[17] Romano E, Romero P. The therapeutic promise of disrupting the PD-1/PD-L1 immune checkpoint in cancer: unleashing the CD8 Tcell mediated anti-tumor activity results in significant, unprecedented clinical efficacy in various solid tumors. J Immunother Cancer 2015; 3: 15.

[18] Ciubotariu R, Coloval AI, Pennesi G, Liu Z, Smith D, Berlocco P, Cortesini R and Suciu-Foca N. Specific suppression of human CD4+ Th cell response to pig MHC antigens by CD8+CD28- regulatory T cells. J Immunol 1998; 161: 5193-5202.

[19] Chang CC, Ciubotariu R, Manavalan JS, Yuan J, Colovai AI, Piazza F, Lederman S, Colonna M, Cortesini R, Dalla-Favera R and Suciu-Foca N. Tolerization of dendritic cells by T(s) cells: the crucial role of inhibitory receptors ILT3 and ILT4. Nat Immuno 2002; 3: 237-43.

[20] Manavalan JS, Kim-Schulze Scotto SL, Naiyer AJ, Vlad G, Colombo PC, Marboe C, Mancini D, Cortesini R and Suciu-Foca N. Alloantigen spe-

cific CD8+CD28- FOXP3+ T suppressor cells induce ILT3+ ILT4+ tolerogenic endothelial cells, inhibiting alloreactivity. Int Immunol 2004; 16: 1055-1068.

[21] Liu Q, Zheng H, Chen X, Peng Y, Huang W, Li X, Li G, Xia W, Sun Q and Xiang AP. Human mesenchymal stromal cells enhance the immunomodulatory function of CD8+CD28-regulatory T cells. Cell Mol Immunlol 2015; 12: 708-718.

[22] Pagès F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molidor R, Mlecnik B, Kirilovsky A, Nilsson M, Damotte D, Meatchi T, Bruneval P, Cugnenc PH,Trajanoski Z, Fridman WH and Galon J. Effector memory T cells, early metastasis, and survival in colorectal cancer. N Engl J Med 2005; 353: 2654-2666.

[23] Parodi A, Traverso P, Kalli F, Conteduca G, Tardito S, Curto M, Grillo F, Mastracci L, Bernardi C, Nasi G, Minaglia F, Simonato A, Carmignani G, Ferrera F, Fenoglio D and Filaci G. Residual tumor micro-foci and overwhelming regulatory T lymphocyte infiltration are the causes of bladder cancer recurrence. Oncotarget 2016; 7: 6424-6435.

[24] Simone R, Zicca A, Saverino D. The frequency of regulatory CD3+CD8+CD28-CD25+ T lymphocytes in human peripheral blood increases with age. J Leukoc Biol 2008; 84: 1454-1461.

[25] Korecka-Polak A, Bocian K, Pachówka M, Jałbrzykowska A and Korczak-Kowalska G. Suppressor properties of human CD8+CD28- T cells in mixed leukocyte reaction are not affected by CsA and RAPA. Arch Immunol Ther Exp (Warsz) 2016; 64: 409-16.

[26] Baeten D, Louis S, Braud C, Braudeau C, Ballet C, Moizant F, Pallier A, Giral M, Brouard S and Soulillou JP. Phenotypically and functionally distinct CD8+ lymphocyte populations in long-term drug-free tolerance and chronic rejection in human kidney graft recipients. J Am Soc Nephrol 2006; 17: 294-304.