Pathology & Oncology Research (2019) 25:647–652
https://doi.org/10.1007/s12253-018-0512-7

ORIGINAL ARTICLE

The Effect of CD86 Expression on the Proliferation and the Survival of CLL Cells

Ferenc Takács[1] · Csilla Tolnai-Kriston[1] · Márk Hernádfői[1] · Orsolya Szabó[1] · Gábor Szalóki[1] · Ágota Szepesi[1] · Ágnes Czeti[1] · András Matolcsy[1] · Gábor Barna[1]

Received: 27 September 2018 / Accepted: 15 October 2018 / Published online: 8 November 2018

Abstract
Micro-environment plays important role in the pathogenesis of CLL by providing protective niche for CLL cells. Several molecules play important role in communication between CLL cells and immune cells like CD86.Some of the data suggest that CLL patients with high CD86 level need earlier treatments and cells with higher CD86 expression has higher proliferation rate but the role of CD86 in the survival and proliferation of CLL cells is unclear. We investigated the effect of CD86 expression to CLL cells in 50 peripheral blood and 15 lymph node biopsy samples from CLL patients. Our results showed that the expressions of CD86 increased significantly after 7 day culturing in medium, or in the presence of bone marrow stromal cells (BMSCs). We found positive correlation between CD86 and CD23 expression ($p < 0.05$), but no correlation with other markers. Furthermore, no correlation were found between the CD86 expression and the proliferation of CLL cells. Analysis of clinical data showed that cases with high CD86 expression had lower level of serum lymphocyte count ($p < 0.04$) at the time of the diagnosis. CD86 shows multiple appearances in the lymph nodes containing pseudofollicules, but no correlation was found between CD86 positivity, and Ki67 positivity. Our results suggest that the use of CD86 molecule as a proliferation marker for CLL is highly questionable. However, the CD86 molecule may interfere with the immune system of patients with CLL by activating and depleting immune functions. That can be the reason why CD86 positivity may mean worse prognosis.

Keywords CLL · CD86 · Micro-environment · Proliferation marker

Introduction

The chronic lymphocytic leukemia (CLL) is the most common leukemia in adults in the western world. The CLL cells have heterogeneous biological attribution so the clinical appearance of the disease largely depends on biological behaviour of CLL cells [1]. Based on clinical progression two groups of disease can be distinguished: there are patients who usually does not need treatment for several years, while patients with poor prognosis need an early treatment, and have less survival prospects [2]. Several prognostic factors (e.g. p53 mutation, 11q deletion) [3] have been found that can be used to foreshow the course of the disease and help to plan the therapy.

✉ Gábor Barna
barna.gabor@med.semmelweis-univ.hu

[1] 1st Department of Pathology and Experimental Cancer Research, Semmelweis University, Üllői út 26, Budapest H-1085, Hungary

The micro-environment plays an important role in pathogenesis of CLL, provide protective niche for the surviving and proliferating CLL cells [4, 5]. Not only the microenvironment has effect on the CLL cells, but CLL cells are also form their environment to get more survival signal from it [6], and cause immunsuppression as produce IL-10 or anti-inflamatory cytokines [7, 8]. Several molecules play important role in communication between CLL cells and immune cells like CD86.

The CD86 molecule (B7–2) is a monomer immunoglobulin-like protein belonging to the B-7 family that is expressed on antigen presenting cells (APCs, like dendritic cells, macrophages, B cells) together with the CD80 molecule (B7–1). CD86 plays a role as a co-stimulator molecule to make and regulate immunological synapses (IS) [9]. Dai et al. showed that the expression of CD86 is lower in CLL compared to normal B cells, however there are only few data available about its role in the survival of CLL cells [10].

Huemer et al. showed that CLL cells with $CD86^{high}$ $CXCR4^{low}$ expression had a greater expression of Ki67 than the $CD86^{low}$ population, and was overlapped with $CD5^{high}$

$CXCR4^{low}$ cells. In addition, patients with $CD86^{high}$ expression needed earlier therapeutic intervention compared to ones with $CD86^{low}$ expression [11].

To understand the importance of CD86 in the survival and the proliferation of CLL cells, we investigated the relationship of CD86 expression with other surface markers, some clinical parameters and its expression pattern in lymph node. Our results showed that CD86 expression correlated positively with the expression of CD23, but had no effect on the proliferation rate of the CLL cells neither in peripheral blood nor in lymph node samples.

Materials and Methods

CLL Samples

50 peripheral blood samples (male female ratio was 1.27 with median of age 67 years [40–87]) and 15 lymph node biopsy of patients with CLL (male female ratio was 1.5 with the median age 53.5 [42–90]) were examined. For the analysis of clinical parameters (e.g. count of lymphocytes, LDH, time to treatment) we had data from 18 patients (7 from the $CD86^{low}$ group and 11 from the $CD86^{high}$ group). The diagnosis of CLL was based on the World Health Organization (WHO) classification of tumors of lymphoid tissue [12]. Only those blood samples were used in which the ratio of CLL cells among lymphocytes exceeded 85%. The study was conducted in accordance with the Declaration of Helsinki and has been approved by the local Ethics Committee in the Semmelweis University.

Isolation and Culturing of CLL Cells

CLL cells were isolated by density gradient centrifugation using Ficoll-Histopaque-1077 (Sigma Aldrich, San Louis, MO, USA). Isolated CLL cells were cultured at a concentration of 2×10^6 cells/ml in RPMI-1640 [Sigma Aldrich] supplemented with 10% Fetal Calf Serum [FCS] [Pan Biotech], 40 mg/L Gentamycin [Sandoz, Boucherville, QC Canada] and 2 mM L-Glutamine [Gibco Carbbad CA USA] final concentration. CLL cells were cultured in medium alone or in co-culture with bone marrow stromal cells (BMSC). BMSCs were isolated from bone marrow aspirates of normal or ITP patients with no abnormal cells determined by flow cytometry, and prepared as described previously. BMSC cultures were maintained in 24-well plates at a concentration of 2×10^4 cells/ml in Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich) supplemented with 20% FBS (Sigma Aldrich), gentamycin (Sandoz) and L-glutamine (Gibco). Co-cultures contained 10^6 CLL cells and 2×10^4 BMSC in 24 well plate.

Immunophenotype of CLL Cells by Flow Cytometry

The immunophenotype of CLL cells were determined by flow cytometry. Measurements were performed using FACSCalibur (Becton-Dickinson Biosciences, (BD)) and Navios (Beckman Coulter, (BC)) flow cytometers and analyzed by CellQuest Pro (BD), or Kaluza 1.5. (BC) software. Data of 20,000 cells were collected from every samples. The antibodies used in the measurements are listed in Table 1. and their concentrations were titrated to obtain the best staining index. The results were based on geometric mean fluorescence intensity (MFI). The samples were divided into high and low CD86 expression groups, based on the median fluorescence of the samples. The cut off value was determined as the median value of the samples (7.9 MFI).

Proliferation Assay

For the proliferation assay, 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) dye was used at final concentration of 5 μM. For proliferation assay, 10^6 cells were cultured in 1 ml medium (RPMI-1640 supplemented with 10% FBS, gentamicin and L-glutamine). We measured the autofluorescence of the unlabeled cells on the 0th day of the experiment. The measurement was performed by a FACS Calibur (BD) flow cytometer and the result was evaluated using the CellQuest Pro (BD) software. 20,000 cells were measured and geometric mean fluorescence intensity (MFI) was used for the results.

Analysis of Apoptosis by Annexin V/PI Staining

After 7 days of culturing CLL cells, they were stained with Annexin V-Alexa Fluor 647 (Biolegend) and propidium-iodide (PI) (Sigma-Aldrich) according to the method described by Kriston Cs et al. [13]. Samples were measured by

Table 1 List of the antibodies that were used in the flow cytometric experiments

Antibodies	Manufacturer
CD86-PE	BD Bioscienses
CD86-APC	BioLegend
CD23-PE	Beckman-Coulter
CD5-FITC	DAKO
CD184-PE	BD Bioscienses
CD49d-PE	BD Bioscienses
CD19-PC5.5.	Beckman-Coulter
CD38-ECD	Beckman-Coulter
CD29-PE	BD Bioscienses
ROR1-FITC	Milteny Biotec
CD126-APC	BioLegend

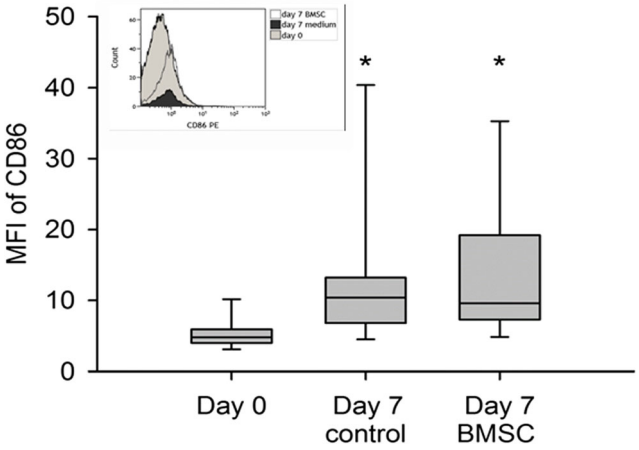

Fig. 1 The elevating of CD86 expression after 7 days cultivation. CLL cells were cultured in different conditions for 7 days. Mean MFI values of CD86 expression of the CLL cells were increased after seven days of cultivation in medium or in co-culture with BMSCs. In the small picture we compared the distribution of the CD86 expression. Only the geometric mean fluorescence were increased during the seven days culturing, the maximums of the distributions remained unchanged. The significantly different populations were labeled with *. Statistical significance was set as $p < 0.05$

flow cytometry. Living cells were defined as Annexin V/PI negative population.

Immunohistochemistry

Paraffin embedded lymph node sections were used for the immunohistochemical stainings. CD86 antibody (Abcam) and Polymer Kit (Post Primary Block + Polymer)(Novocastra Novolink) were used for staining, and DAB chromogen (Diamino-benzidine [Dako]) for detection. Immunohistochemistry of Ki67 (DAKO Mib1) was performed with Leica Bond max immunoassay using the polymer-based Bond KIT. For the development, DAB (Diamino-benzidine [Dako]) was used. Positive (tonsil) and negative controls (unstained) were used in our experiments to control the stainings.

Immunohistochemical samples were scanned by 3DHistech intersection scanner and were evaluated by Panoramic Viewer software in addition to light microscopy. The images were acquired with the same magnification (0.32).

Statistical Analysis

All variables were tested for normal distribution to select the appropriate parametric or non-parametric statistical procedure. Wilcoxon, Mann–Whitney U-tests, Spearman correlation coefficient was used for statistical evaluation using the SPSS statistical package version 20.0 (SPSS, Chicago, IL, USA). Statistical significance was set at $p < 0.05$.

Results

Correlation between CD86 and Other Cell Surface Markers

In our study CLL cells were cultured in different conditions for 7 days. The expression of CD86 were significantly increased after 7 days cultivation in medium (mean MFI values of CD86 expression changed from 6.33 to 15.85, $p = 0.001$, $n = 31$, Fig. 1) and also in co-culture with BMSCs (mean MFI values changed from 6.33 to 19.24, $p = 0.001$, $n = 31$, Fig. 1.). We compared the distribution of the CD86 expression of the CLL cells and we found that only the geometric mean fluorescence were increased during the seven days culturing, the maximums of the distributions remained unchanged. It means that the number of cells with lower level of CD86 were decreased.

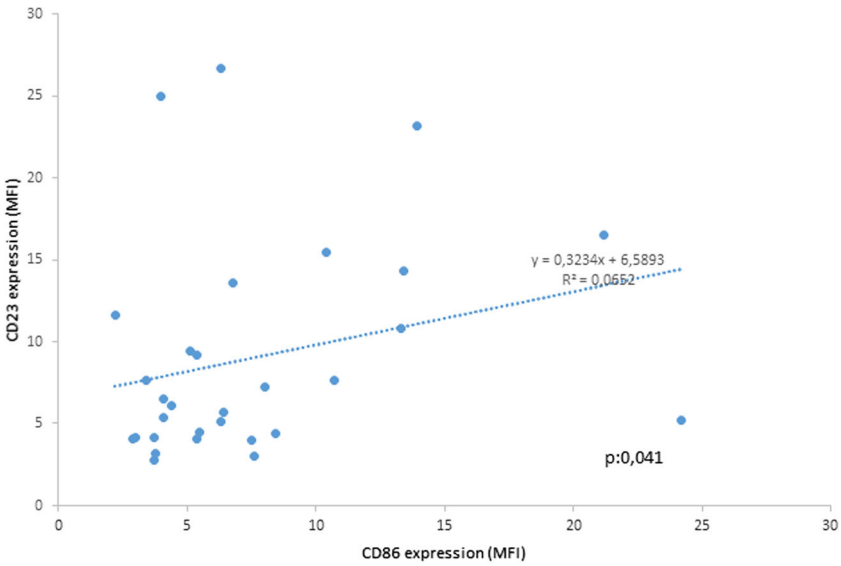

Fig. 2 Correlation between initial CD86 and CD23 expression in CLL cells. We compared the correlation between the expression of CD86 and several other surface markers. CD86 and CD23 expression showed significant positive correlation among the investigated ones. Statistical significance was set as $p < 0.05$

Table 2 Clinical parameters of blood samples

Clinical parameters	CD86-	CD86+	*p* value
LDH (U/l)	362 ± 202	371.69 ± 121	0.34
Count of lymphocytes (G/l)	62.68 ± 52	41,92 ± 58	0.04
TTT (day)	1325 ± 1422	714 ± 730	0.37

We marked with bold the significant results

We investigated the correlation between the expression of CD86 and several other surface markers (Table 1.) but significant correlation were detected only with CD23 (Fig. 2.) ($p < 0.05$). In the literature positive correlation of CD86 with CD5 and negative correlation with CXCR4 had been reported [11]. Among our samples 16 cases were found with $CD5^{high}$ $CXCR4^{low}$ expression but only 6 of them had high CD86 expression.

Analysis of Clinical Data

We analysed LDH levels and lymphocyte counts at the time of the diagnosis and time to treatment (TTT) values. There was no significant difference neither in the level of LDH (371.69 ± 121.91 U/l vs. 362 ± 202.36 U/l, $p = 0.34$) nor TTT values (714 ± 730.71 days vs. 1325 ± 1422.11 days $p = 0.37$) between CLL cases with high or low CD86 expression: Lymphocyte count was significantly lower in patients with high CD86 expression (41.92 ± 58.45 G/l vs. 62.68 ± 52.31 G/l, $p = 0.04$) (Table 2.).

Effect of CD86 to Apoptosis and Proliferation

We examined the apoptosis and proliferation of CLL cells with various CD86 expression. We did not found any correlation between the CD86 level and the apoptosis ratio of CLL cells after 7 days culturing (in medium $CD86^{low}$ 33.38 ± 27%, $CD86^{high}$ 32.8 ± 21% $p = 0.85$, in stroma $CD86^{low}$ 48.85 ± 23%, $CD86^{high}$ 52.4 ± 25%, $p = 0.6$). We performed CFSE staining for detection of proliferation, but CFSE assay showed no proliferation neither in the case of $CD86^{low}$ nor among $CD86^{high}$ CLL cells regardless they were alone in medium or in co-culture with BMSCs. (Figs. 2 and 3).

Expression Pattern of CD86 in Lymph Nodes

We examined the expression pattern of CD86 in lymph nodes, which contains proliferation centers, pseudofollicules. CD86 expression showed various pattern in the lymph nodes: 8 samples were negative, in 4 samples CLL cells had intracellular CD86 positivity, in 1 sample intracellular and membrane CD86 positivity were detected, and in 2 samples only membrane positivity were detected in CLL cells. The CD86 positive cells were not linked to the pseudofollicules (Fig. 4.). We also performed a Ki67 staining. Ki67 positive samples showed various CD86 staining from negative to membrane positivity. There was no correlation between CD86 and Ki67 expression (Fig. 4.).

Discussion

The micro-environment plays an important role in CLL pathogenesis, and CD86 has a prominent role in the communication of B-cells with the surrounding cells [5]. Their are few data about the role of CD86 in the survival of CLL cells.

In our studies, we compared the expression of CD86 with several surface markers and found postive correlation only between CD86 and CD23 expression. The correlation between these two molecules can be originated from IL-4 secretion, which cytokine enhances the expression of CD23 on B-cells [14] and the expression of CD86 on monocytes and macrophages as were previously described [15]. We detected

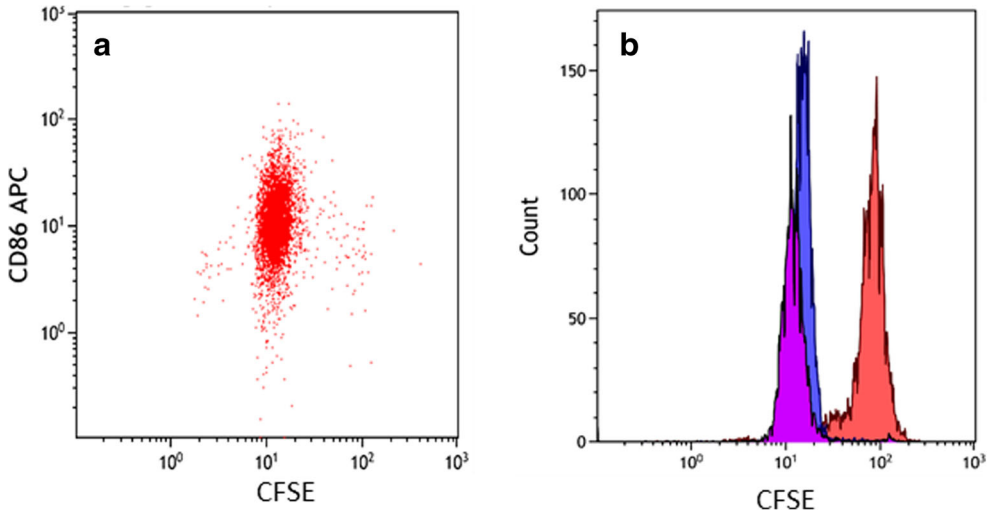

Fig. 3 Correlation between proliferation and CD86 expression. We performed CFSE staining for detection of proliferation on CLL cells cultured for 6 days. Correlation could not be detected between CD86 expression and CFSE staining on the 2nd day (**a**) or after 6 day culturing (**b**). The first spike showed the 0th day, the second the 5th and the third 6th day of culturing

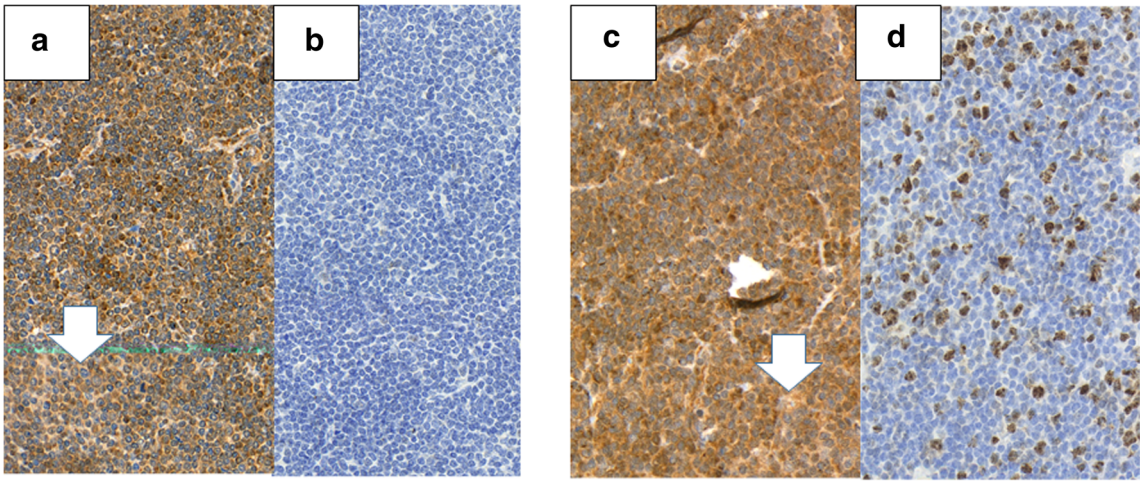

Fig. 4 Pattern of CD86 expression in lymph nodes. CD86 expression showed various pattern in the lymph nodes and there was no correlation with Ki67 staining: CD86 intracellular and membrane positivity (**a**) and Ki67 negative (**b**) lymph nodes. CD86 membrane positivity (**c**) and Ki67 positive (**d**) lymph node. (A pseudofollicule were marked with white arrow

that the expression of CD86 was increasing during different culturing conditions. The effect of BMSCs were more remarkable on CLL cells than the medium alone but there were no significant differences between the different culturing conditions. Hock et al. showed that CD86 expression of CLL cells were increased by culturing them with activating T-cells after 72 h, but they did not analysed the CD86 expression before and after culturing [16]. We found that the during the culturing the number of cells with lower CD86 expression were decreased that caused the increasing of the average of the CD86 expression. One theory is that these cells can be more sensitive to spontaneous apoptosis than cells with higher CD86 expression, however we did not found any correlation between the CD86 level and the apoptosis ratio after 7 days culturing. The other explanation is that during culturing stress cells can increase their CD86 level but its upper level is determined individually.

We investigated whether there was any correlation between CD86 and $CD5^{high}$ $CXCR4^{low}$ phenotype to prove the results of Huemer et al. [11]. In our study only just a part of the CLL cases with high CD86 expression showed this correlation. The reason for the difference in the obtained results can originated from the different analysis method. We examined the CD86 intensity of the entire CLL population while they examined just the part of the populations.

Our results from the analysis of clinical datas showed that in samples with high CD86 expression patients had lower level of serum lymphocyte count ($p < 0.04$) at the time of the diagnosis. We found that these patients had higher level of serum LDH, and needed therapeutic intervention earlier than patients with low CD86 expressing CLL cells, however later results were not significant due to the low number of samples. They supported the observations of Huemer and his team [11].

In our proliferation studies we could not detected higher proliferation activity in CLL cells with high CD86 expression either in peripheral blood samples, which opposed to the results of Huemer et al. [11]. It can be explained by that they focused on only a small portion of the $CD86^{high}$ CLL cells and assessed proliferation rate by Ki67 expression, which indicates that the cells were not in G0 phase. In contrast with it in our expreiments CFSE staining were used, which directly detects proliferating cells [17]. We did not find any correlation between the CD86 expression and the proliferation of CLL cells.

We studied the expression pattern of CD86 of CLL cells in lymph nodes. The lymph nodes containing pseudofollicules showed various CD86 staining, and we could not find any correlation between CD86 positivity and Ki67 positivity.

Conclusion

Our results suggest, that the use of the CD86 molecule as a proliferation marker in CLL is highly questionable. However, the CD86 molecule may play a role in CLL pathogenesis, as clinical data indicate: cases with enhanced CD86 expression needed prior therapeutic intervention. The CD86 molecule may interfere in the immune system of CLL by activating, and depleting the immune functions. That can be the reason why CD86 positivity may mean worse prognosis. Further investigations are needed to uncover this connection.

Acknowledgments This work was supported by the NVKP_16-1-2016-0004 grant of the Hungarian National Research, Development and Innovation Office (NFKIH).

Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

References

1. Zhang S, Kipps TJ (2014) The pathogenesis of chronic lymphocytic leukemia. Annu Rev Pathol 9:103–118
2. Hallek M et al (2008) Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. Blood 111(12):5446–5456
3. Gorczyca W (2010) Flow Cytometry in Neoplastic Hematology. Informa Healthcare London, London
4. Burger JA et al (2000) Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1. Blood 96(8):2655–2663
5. Panayiotidis P et al (1996) Human bone marrow stromal cells prevent apoptosis and support the survival of chronic lymphocytic leukaemia cells in vitro. Br J Haematol 92(1):97–103
6. Plander M et al (2011) Chronic lymphocytic leukemia cells induce anti-apoptotic effects of bone marrow stroma. Ann Hematol 90(12): 1381–1390
7. Alhakeem SS et al (2018) Chronic Lymphocytic Leukemia-Derived IL-10 Suppresses Antitumor Immunity. J Immunol 200(12):4180–4189
8. Joss A et al (2000) IL-10 directly acts on T cells by specifically altering the CD28 co-stimulation pathway. Eur J Immunol 30(6): 1683–1690
9. Brzostek J, Gascoigne NR, Rybakin V (2016) Cell Type-Specific Regulation of Immunological Synapse Dynamics by B7 Ligand Recognition. Front Immunol 7:24
10. Dai ZS et al (2009) Defective expression and modulation of B7–2/CD86 on B cells in B cell chronic lymphocytic leukemia. Int J Hematol 89(5):656–663
11. Huemer M et al (2014) AID induces intraclonal diversity and genomic damage in CD86(+) chronic lymphocytic leukemia cells. Eur J Immunol 44(12):3747–3757
12. Swerdlow SH et al (2016) The 2016 revision of the World Health Organization classification of lymphoid neoplasms. Blood 127(20): 2375–2390
13. Kriston C et al (2018) In contrast to high CD49d, low CXCR4 expression indicates the dependency of chronic lymphocytic leukemia (CLL) cells on the microenvironment. Ann Hematol 97(11): 2145–2152
14. Kim SH, Lee CE (2011) Counter-regulation mechanism of IL-4 and IFN-α signal transduction through cytosolic retention of the pY-STAT6:pY-STAT2:p48 complex. Eur J Immunol 41(2):461–472
15. Deszo EL et al (2004) IL-4-dependent CD86 expression requires JAK/STAT6 activation and is negatively regulated by PKCdelta. Cell Signal 16(2):271–280
16. Hock BD, MacPherson SA, McKenzie JL (2017) Idelalisib and caffeine reduce suppression of T cell responses mediated by activated chronic lymphocytic leukemia cells. PLoS One 12(3): e0172858
17. Gerdes J et al (1983) Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 31(1):13–20