Archives of Advances in Biosciences 2021:12(3) doi.org/10.22037/aab.v12i3.35823

Original Article

T cell therapy of B-CLL lymphoma against CD20 alternative splice variant: in vitro experiments

Hamid Chegni[1*], Zuhair M.Hassan[2], Hadise Babaii [3]

[1] Department of Immunology , School of Paramedical Science ,Shahid Beheshti University of Medical Science,Tehran, Iran.

[2] Department of Immunology, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran.

[3] School of Paramedical Science ,Shahid Beheshti University of Medical Science,Tehran, Iran.

Article Information
Received:2021-08-15
Revised: 2021-08-21
Accepted: 2021-09-11

Correspondence
Hamid Chegni
Email:h.chegini2010@yahoo.com

Cite this article as:
Chegni H, Hassan Z. M., Babaii H.T cell therapy of B-CELL lymphoma against CD20 alternative splice variant: in vitro experments. Archives of Advances in Biosvience 2021: 12(3).

Abstract

Introduction: Cancer immunotherapy has become a major player in modern oncology. In immunotherapy, immune cells selectively recognize and kill cancerous cells; this way, many side effects of chemotherapy and radiotherapy are reduced. One of the immunotherapy methods is the T cell therapy, which is based on the use of T cells with determined antigenic specificity for cancer cells. The search for unique tumor antigens which are specific to malignant cells and have the ability to stimulate cellular immunity is one of the main targets of malignancies treatment.

Materials and methods: D393-CD20 is an alternative splicing form of the CD20 surface receptor, which lacks 168 nucleotides in exons 3 to 7 of the CD20 sequence. D393-CD20 peptide is merely expressed on cancerous B cells, such as Burkitt's lymphoma (BL), DLBCL and B-CLL. In this study we isolated and expanded a CD8+ T lymphocyte specific clone for a D393-CD20 antigen to examine the effect on B-CLL cell line. To this end, we evaluated the impact of cytotoxic T cells upon D393-CD20 antigen, expressed on malignant B cells. We also evaluated the ratio of apoptosis using flow cytometry and MTT.

Results: Results suggest that targeting D393-CD20 antigen with specific CD8+ T lymphocytes is very effective in preventing tumor cells growth.

Conclusion: Targeting D393-CD20 can be a proper choice for B-cell lymphoma immunotherapy.

Keywords: T cell therapy, B-CLL lymphoma CD20, Alternative splice variant, In vitro.

1. Introduction

B cell malignancies include a large and heterogeneous group of lymphoproliferative disorders from non-Hodgkin lymphomas (NHLs) such as follicular lymphoma (FL) and chronic lymphocytic leukemia (CLL) to more aggressive subtypes like diffuse large B-cell lymphoma (DLBCL) [1, 2]. B cell disorders are responsible for more than 85% of all NHL cases [3]. CLL is the most prevalent blood malignancy with slow progression. On the other hand, B cell lymphoma is the most prevalent among NHLs which can be successfully treated in 2/3 of all cases. High-dose chemotherapy and hematopoietic stem cell (HSC) transplantation increase the life expectancy of patients whose immunochemotherapy has failed as their first-line therapy [4-6]. However, only 50% of the patients volunteer for these treatments. Besides, roughly 20% of the patients who are treated with rituximab have a three-year survival subsequent to HSC transplantation. [7] Hence, it is necessary to develop new strategies for B-cell lymphoma treatment. In recent decades, traditional concepts related to organic compounds as safe drugs have been challenged by use of cell therapy. Despite their complexity, cell therapies show great potential for combating human diseases. [8] Cancer immunotherapies have become the main element of modern oncology: Car T – cell therapy, PDL /PDL1 therapy and immune CTL are successfully used. Trained immune system components isolated from patient's body to target cancerous cells are used in immunotherapeutic methods and avoiding toxic compounds results in reduction of many side effects related to conventional treatments.

In general, immune cells can target cancerous cells in two ways: making unique molecules in cancerous cells (specific antigens or specific mutations) and/or recognizing molecules that have higher expression in cancerous cells than normal cells (antigens related to tumor) [9]. Immunotherapy is one of the most effective strategies for cancer treatment and it is associated with increased survival and higher tolerance due to its long term effect. [10] Treatment with genetic engineered T cells can completely cure acute lymphoblastic leukemia (ALL) and extensive lymphoma of B lymphocyte (DLBCL) in individuals experiencing resistance to chemotherapy [8]. Like other medicines, recognition of T cells treatment pharmacology for effective use of clinical environment is crucial. Via genetic and cellular tools application and with the purpose of producing T cells by determined antigenic specificity, T cell therapies have been used to cure cancer [11]. D393-CD20 antigen is one of the neo-antigens produced from alternative splicing of the CD20 surface receptor. D393-CD20, in comparison with CD20, lacks 168 nucleotides of exons from 3 to 7. This antigen exists only in the surface of malignant B cells (expressed by in vitro EBV-transformed B-cell lines though) and it doesn't express on the surface of normal lymphocytes. Interestingly, the expression of D393-CD20 increases on the surface of tumor cells that have resistance to the treatment by Rituximab [12], and due to its immunogenicity, D393-CD20 seems to be an ideal candidate for T cell therapy of B cell lymphomas [13-16]. In this study, we isolated and expanded a D393-CD20 specific CD8+ T cell clone and tested the capacity of this clone against D393-CD20 to be effective in B-CLL cell line.

2. Materials and Methods

Materials: 1mM L-glutamine ,1mM sodium pyruvate ,1%non-essential amino acids ,1%Kanamycin, 1% heat-inactivated fetal bovine serum (FBS 1%) or heat-inactivated human serum 5% (Sigma Aldrich ,USA).D393-CD20 peptide was synthesized (Bio Basic Company, Canada), Ficoll (Sigma Aldrich ,USA), IL-2(Rosh ,Germany), FITC anti-human CD4 / PE anti-

human CD8 monoclonal antibodies, etc. The B-CLL cell line was first established in 1979 from Epstein-Barr virus (EBV), leading to immortalized neoplastic lymphocytes and chronic lymphocytic leukemia in a 47-year-old woman (Raii Stage II) diagnosed). ACC-733 was established in Pasteur Institute of Iran containing 20% FBS, 50 U/ml Penicillin, 50mg/ml Streptomycin, 2mM L-glutamine, 1mM sodium pyruvate, and essential amino acids at 37 centigrade degree humidified 5% CO2 atmosphere [17].

Isolation and expansion of a T cell clone specific to a D393-CD20 peptide

The present study was carried out on B-CLL cell line. Blood donor specimens were just required for D393-Cd20 peptide-specific T lymphocyte clones. This study was conducted according to the rules and regulations passed by the Local Ethics Committee. In this regard, blood cells were collected from MHC-class I A2+ and healthy donors in Shariati Hospital, Tehran, Iran. D393-CD20- Ag-specific T lymphocyte clones were yielded as described earlier. The isolated clones were joined with specific peptides in order to select D393-CD20-specific peptides. Subsequently, the level of IFN was measured to assure the selection of peptide-specific clones[18].

The D393-CD20 peptide was synthesized with the following amino acid sequence: KPLFRRMSSLELVIAGIVEN, as that of the published CD20 alternative splicing sequence (13). Cells were incubated at 37°C and 5%CO2. After 5 and 10 days of culture, IL2 was respectively added at 10 and 100 UI to all of the wells that were further incubated up to day 13.Cells were then counted and cloned by limiting dilution to isolate D393-CD20 specific T lymphocyte clones according to the previously described methods [16-19]. The specificity and phenotype of isolated clones were determined as previously described [20] and a single CD8+ T cell clone was selected for further experiments. The B-CLL cell line was purchased from the Pasteur Institute of Tehran cell bank and was cultured for further studies. To evaluate the effect of D393-CD20 antigen-specific T lymphocyte clones on B-CLL cell line, we co-cultured them, and the rate of apoptosis was determined using flow cytometry. Cytotoxicity was assessed utilizing MTT technique.

3. Rusults

The level of IFN was measured to assure the selection of peptide-specific clones

Clone 15 had the most significant difference in IFN production level in the presence of a peptide compared to the negative control group. In addition, clones 11, 7, and 6 also had significant differences in IFN production (Figure1)

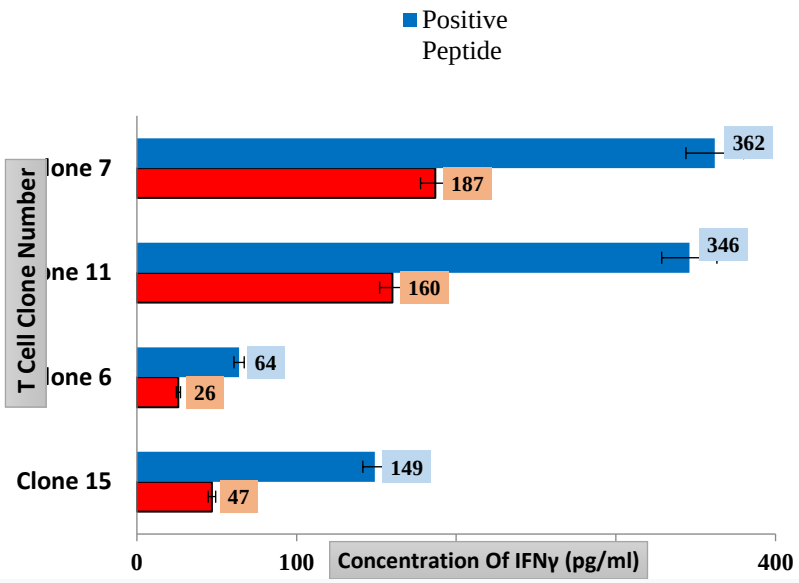

Figure 1: Comparison of IFNγ secretion in clones 6, 7, 11, 15 with D393-CD20 peptide (positive peptide) and without D393-CD20 peptide (negative peptide)

Analysis of flow cytometry

After staining D393-CD20 antigen-specific T lymphocyte clones with FITC-binding Anti-CD4 and PE-bound Anti-CD8, using flow cytometry, it was found that 86.6% of the population were CD8 + and CD4-.

B-CLL apoptosis using flow cytometry

We cultured the selected specific clones (clones 15, 11, 7, 6) with a B-CLL cell line with a ratio of 1: 10 in 96-plate U-shaped wells. Regarding the negative control, we used B-CLL co-culture with non-specific D393-CD20 antigen-specific T lymphocyte clones. The results were obtained using flow cytometry. It was found that clone 15 stimulates the most apoptosis ratio in the B-CLL cell line.

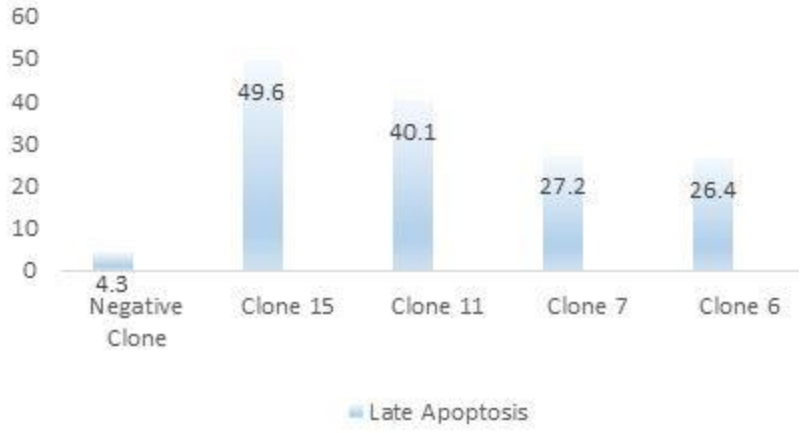

Diagram 1. The results from percentage of B-CLL cell line apoptosis in co-culture with specialized T lymphocyte clones of D393-CD20 antigen and its comparison with unspecialized T lymphocyte clones of D393-CD20 antigen as negative control with apoptosis technique

MTT results

We placed the plate in the ELISA reader and measured the absorption at 450 nm. For each T lymphocyte clone, three types of wells were selected as follows: One well containing T lymphocyte clone without B-CLL cell line, a well containing B-CLL cell line without T lymphocyte clone, and a well with co-culture of T lymphocytes with B-CLL cell line. MTT test results were calculated with this formula:

The percentage of immolated cells= $\frac{(B-CLL\ cell\ line\ OD + T\ lymphocyte)OD - CO\ culture\ OD}{(B-CLL\ cell\ line\ OD + T\ lymphocyte\ OD)}$

After comparing the percentage of B cells and cytotoxic T lymphocytes against D393-CD20 antigen, it was found that the apoptosis rate induced by specific T lymphocytes by clones 5 and 15 was 64.45 and 51.1 percent, respectively. These results are consistent with flow cytometry results.

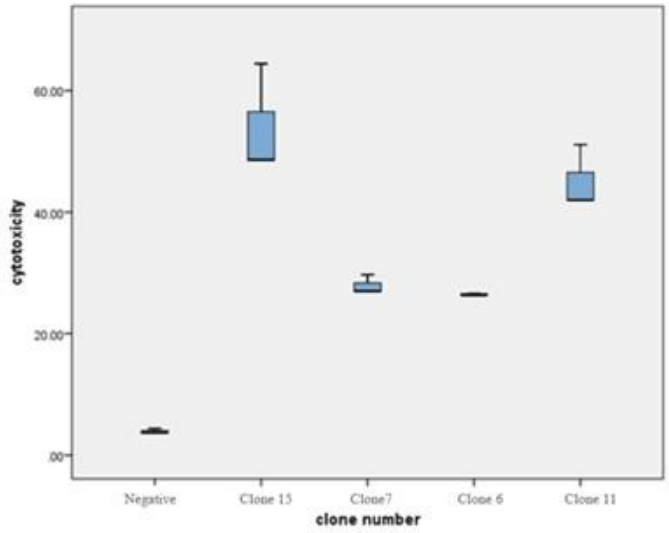

Figure 2. Culture of B lymphocytes with D393-CD20 antigen-specific T lymphocytes and nonspecific T lymphocytes as a negative group (n=10) Means±SDs, *p < 0.05

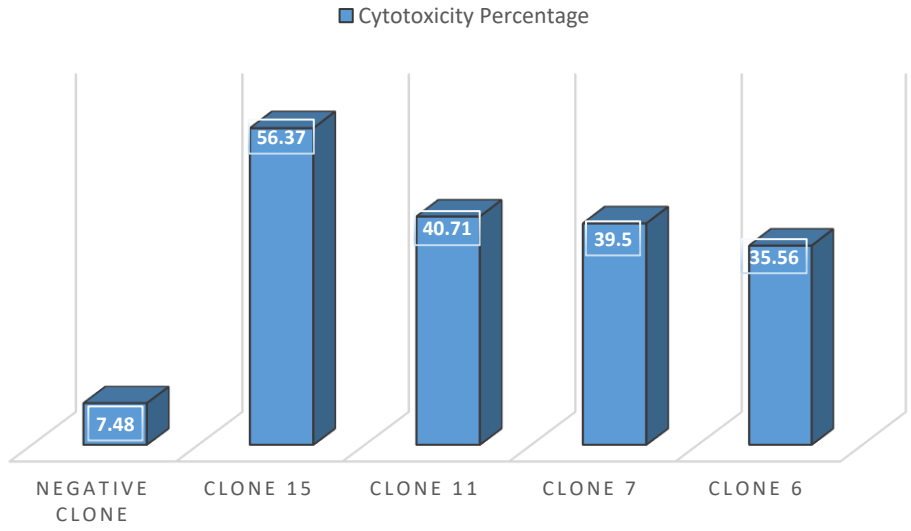

Diagram 2. Results of B-CLL cell line cytotoxicity in culture with D393-CD20 antigen-specific T lymphocyte clones and comparison with non-specialized T lymphocytes Clone D393-CD20 antigen as a negative control by MTT technique.

4. Discussion

In 1960, a serious attempt was made for tumor treatments by passive immunization using T lymphocyte and it was demonstrated that cellular immunity was responsible for tumor tissue rejection [21]. Since it was determined that human tumor infiltrating lymphocytes (TILs) could be expanded in vitro and injected to patients, it was concluded that a translational approach of tumor immunology was possible [22]. A study by Forget showed that separation of TIL from tumor environment followed by TIL in vitro propagation and reinjection in a patient suffering from metastatic melanoma can be effective for the patient's treatment. [23] In another study, Owens separated TIL from biopsy samples of patients with ovarian cancer and propagated them in vitro. When TIL was challenged with tumor cells, an increase in anti-tumor cytokine was evident, suggesting the effectiveness in these cells for inactivation of tumor cells [24]. In addition, there was an association between controlling tumor progression and the rate of CD8 + T lymphocyte infiltration into the tumor site. The discovery of tumor antigens plays a crucial role in developing new diagnostic tools and anti-cancer immunotherapies. Recent studies have shown that alternative splicing produces different types of tumor antigens .Alternative splicing can alter mRNA structure by adding or removing exons and can affect the function, stability, and binding properties of proteins. The various types of antigens produced by the tumor can affect tumor pathogenesis and can be used as new markers for diagnostic and therapeutic approaches. A study by Celay and colleagues targeted AE2 antigen (a membrane sheath related to PH regulation inside cells) in B lymphoma cells. Their results in *in vitro* showed that immunotherapy against this antigen has a twofold effect on treatment of B cell malignancy, because it induces apoptosis in tumor B cells and also increases the anti-tumor immune responses by decreasing the number of Treg cells [25-28].

Kobayashi conducted a study on four patients with oral cancer in Japan. In vitro proximity of blood lymphocytes of the patients with Survivin peptides demonstrated that Survivin-B80-88 peptides have the highest immunogenicity for CTL. These may be good candidates for immunotherapy against Survivin in gastrointestinal, colon, breast, lung, bladder, and oral cancers [29]. CD20-induced mRNA transcription (named D393-CD20) has recently been identified in malignant B cells of patients with leukemia and lymphoma. D393-CD20 is formed due to the lack of 168 nucleotides from exons 3 to 7 compared to the normal type of CD20.D393 - CD20 mRNA is not present in normal B cells but is present in malignant or deformed B cells [13-15]. Another study by Vauchy found that D393-CD20 antigen was present on the surface of malignant B cells and that D393-CD20-specific CD4 + T cells were detectable in patients with B-cell lymphoma and could produce IFN.

In addition, in this study, vaccination with D393-CD20 peptides in human HLA transgenic mice revealed that D393-CD20 antigen peptides could bind to both MHC class I and class II MHC molecules so that they can multiply both TCD8 + and TCD4 + cells. This study suggests that targeting the D393-CD20 antigen could provide more effective immunotherapy in treating B cell malignancy [30]. Gamonet in another study found that the D393-CD20 antigen was present on the surface of B tumor cells and that the effects of the EBV virus on B cells could increase D393-CD20 membrane expression. Also, in patients with CLL, it was found that the expression of D393-CD20 at the level of tumor cells is higher in patients who are in stages B and C. Bonavida found

that anti-CD20 monoclonal antibody (Rituximab) could be effective in treating B-NHL tumors. Using this therapeutic approach in combination with chemotherapy, radiation therapy and proteasome inhibitors can lead to greater effectiveness in treatment [31].It has been observed that some patients are still tolerant to Rituximab for unknown reasons; however, D393-CD20 expression levels are also increased in tolerant patients [32]. Recent findings indicate that D393 -CD20 can be a good candidate for immunotherapy due to its high immunogenicity and expression in malignant B lymphocytes. The function of D393-CD20 is ambiguous, and there are very few studies on the D393-CD20 peptide [14]. This study found that CD8 + T clones specific to the D393-CD20 peptide could directly detect tumor B cells and induce apoptosis in them. The direct effect of CD8 + T cells on malignant cells is confirmed by previous research showing that D393-CD20 is a natural antigen processed in tumor B cells that may appear on the surface of tumor B cells with MHC class I molecules.

In summary, in this study, we isolated a series of D393-CD20-specific CD8 + T lymphocyte clones from the PBMC of a normal individual. These clones were amplified in vitro in the presence of the synthetic peptide D393-CD20 adjacent to the B-CLL cell line. Using flow cytometry and MTT methods, it was observed that specific clones D393-CD20 induced apoptosis in B-CLL cell line.

5. Conclusion

D393-CD20 promotes the proliferation of CD8 + T lymphocytes in vitro, and these cells can kill cancer cells that have this antigen on their surface, thus a good candidate for immunotherapy.

It seems that the possible role of D393-CD20 junction peptides as antigens is a relatively recent observation and few studies are available on their possible role as candidates for B-CLL immunotherapy. Although this study demonstrates a preclinical approach to T cell transfer therapy and in vivo studies are needed in animal models, it shows that D393-CD20 is highly immunogenic for T + CD8 cells and could be used for future therapeutic approaches.

In addition, since D393-CD20 junction peptides not expressed in normal lymphocytes, , this antigen could be exploited in future studies for the vaccination of individuals at higher risk for B cell lymphoma or normal individuals for the prevention of B cell related malignancies.

Acknowledgments

This study has been funded by Tarbiat Modares University.

The authors would like to thank Sabrina Mariotti (Istituto Superiore di Sanità, Rome) for cell culture advice.

Conflict of interest

The authors declare no conflict of interest in this study.

Refrences

1. Boffetta P. Epidemiology of adult non-Hodgkin lymphoma. Ann Oncol. 2011; 22(4): 27–31.
2. Hallek M. Chronic lymphocytic leukemia: 2015 Update on diagnosis, risk stratification, and treatment. Am J Hematol. 2015; 90:447–460.
3. Gilles Salles, Martin Barrett, Robin Foà and et al. Rituximab in B-Cell Hematologic Malignancies: A Review of

20 Years of Clinical Experience. Adv Ther. 2017; 34(10): 2232–2273.

4. Hallek M. Chronic lymphocytic leukemia: 2013 update on diagnosis, risk stratification and treatment. Am J Hematol. 2013;88(9):803–816.

5. Feugier P, Van Hoof A, Sebban C, et al. Long-term results of the R-CHOP study in the treatment of elderly patients with diffuse large B-cell lymphoma: a study by the Groupe d'Etude des Lymphomes de l'Adulte. J Clin Oncol. 2005; 23(18):4117–26.

6. Pfreundschuh M, Trümper L, Osterborg A, et al. CHOP-like chemotherapy plus rituximab versus CHOP-like chemotherapy alone in young patients with good-prognosis diffuse large-B-cell lymphoma: a randomised controlled trial by the MabThera International Trial (MInT) Group. Lancet Oncol.2006 May; 7(5):379-91.

7. Gisselbrecht C, Glass B, Mounier N, et al. Salvage regimens with autologous transplantation for relapsed large B-cell lymphoma in the rituximab era. J Clin Oncol. 2010; 28(27):4184–90.

8. Michael C. Milone1, Vijay G. Bhoj. The Pharmacology of T Cell Therapies. Mol Ther Methods Clin Dev. 2018 16; 8: 210–221.

9. Finn O. J. Cancer immunology. New England Journal of Medicine. 2008; 358(25):2704–2715.

10. Gabriele D'Errico, Heather L. Machado. A current perspective on cancer immune therapy: step-by-step approach to constructing the magic bullet. Clinical and Translational Medicine. 2017; 6(1): p. 3.

11. Topalian S.L., Muul L.M., Solomon D., Rosenberg S.A. Expansion of human tumor infiltrating lymphocytes for use in immunotherapy trials. J. Immunol. Methods. 1987; 102(1):127–141.

12. Sitaru C,Thiel J. The need for markers and predictors of Rituximab treatment resistance. Exp Dermatol. 2014 Apr; 23(4):236-7.

13. Henry C, Deschamps M, Rohrlich P-S, et al**.** Identification of an alternative CD20 transcriptvariant in B-cell malignancies coding for a novelprotein associated to rituximab resistance. Blood. 2010 Mar 25; 115(12):2420-9.

14. Gamonet C, Ferrand C, Colliou N, et al. Lack of expression of an alternative CD20 transcript variant in circulating B cells from patients with Pemphigus. Exp Dermatol. 2014; 23 (1):66–7**.**

15. Small GW, McLeod HL, Richards KL. Analysis of innate and acquired resistance to anti-CD20 antibodies in malignant and nonmalignant B cells**.** Peer J .2013; 1(10): e31.

16. Godet Y, Fabre E, Dosset M, Lamuraglia M, et al**.** Analysis of spontaneous tumor-specific CD4 Tcell immunity in lung cancer using promiscuous HLA-DR telomerase-derived epitopes: potential synergistic effect with chemotherapy response **.**Clin Cancer Res Off J Am Assoc Cancer Res. 2012**;** 18(10):2943–53.

17. Chegni H, Hassan ZM, Nisini R, Ebrahimi M, Sabouni F. Preliminary in vitro effects of CD8+ T lymphocyte specific for the CD20 alternative splicing D393-CD20 peptide expressed on Burkitt lymphoma cells. Asian Pacific journal of cancer prevention: APJCP. 2019;20(8):2563.

18. Chegni, H., Hassan, Z., Nisini, R., Ebrahimi, M., & Sabouni, F. (2018). T Cell Therapy of Burkitt's Lymphoma in Nude Mice Model Tumorized with Ramos Cell Line. *Journal of clinical* & cellular immunology, 09.

19. Mariotti S, Nisini R.Generation of Human T cell Clones.Methods in Molecular Biology, 2009;514(6):65-93.

20. Nikolova, M, Echchakir, H, Wechsler J, Boumsell L, Bensussan A. Bagot, M. Isolation of a CD8alphaalpha+ CD4- tumour T-cell clone with cytotoxic activity from a CD4+ CD8- cutaneous T-cell lymphoma. Br J Dermatol 2003; 148(1):24-9.

21. Mitchison NA. Studies on the immunological response to foreign tumor transplants in the mouse. I. The role of lymph node cells in conferring immunity by adoptive transfer. J Exp Med. 1955; 102(2):157-177.

22. Rosenberg SA, Packard BS, Aebersold PM, et al. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. N Engl J Med. 1988; 319(25):1676-1680.

23. Forget MA, Haymaker C, Hess KR, Meng YJ,et al . Prospective Analysis of Adoptive TIL Therapy in Patients with Metastatic Melanoma: Response, Impact of Anti-CTLA4, and Biomarkers to Predict Clinical Outcome.Clin Cancer Res. 2018; 24(18):4416-4428.

24. Owens GL, Price MJ, Cheadle EJ, Hawkins RE, Gilham DE, Edmondson RJ. Ex vivo expanded tumour-infiltrating lymphocytes from ovarian cancer patients release anti-tumour cytokines in response to autologous primary ovarian cancer cells. Cancer Immunol Immunother. 2018; 67(10):1519-1531.

25. Zhang L, Vlad A, Milcarek C, Finn OJ. Human mucin MUC1 RNA undergoes different types of alternative splicing resulting in multiple isoforms.Cancer Immunol Immunother CII . 2013;62:423–35.

26. Lin J, Lin L, Thomas DG, et al. Melanoma-associated antigens in esophageal adenocarcinoma: identification of novel MAGE-A10 splice variants. Clin Cancer Res off J Am Assoc Cancer Res 2004; 10(17):5708–16.

27. Faustino NA, Cooper TA. Pre-mRNA splicing and human disease. Genes Dev 2003; 17(4):419–37.

28. Celay J, Lozano T, Concepcion AR, Beltrán E, Rudilla F, García-Barchino MJ ,et al: Targeting the anion exchanger 2 with specific peptides as a new therapeutic approach in B lymphoid neoplasms. Haematologica. 2018 Jun; 103(6):1065-1072.

29. Jun-ichi Kobayashi, Toshihiko Torigoe, Yoshihiko Hirohashi,Satomi Idenoue, Akihiro Miyazaki,Akira Yamaguc:Comparative study on the immunogenicity between an HLA-A24-restricted cytotoxic T-cellepitope derived from survivin and that from its splice variant survivin-2B in oral cancer patients. Journal of Translational Medicine 2009; 7:1

30. Charline Vauchy, Clementine Gamonet, Christophe Ferrand, Etienne Daguindau, et al: CD20 alternative splicing isoform generates immunogenic CD4helper T epitopes. Int. J. Cancer. 2014: 137(1):116–126.

31. Clémentine Gamonet, Elodie Bole‐Richard, Aurélia Delherme, et al. New CD20 alternative splice variants: molecular identification and differential expression within hematological B cell malignancies. .Bio Med Central .2016; 5:7

32. Benjamin B,et al : Postulated Mechanisms of Resistance of B-Cell Non-Hodgkin Lymphoma to Rituximab Treatment Regimens: Strategies to Overcome Resistance. Seminoncol. 2014;41(5):667-77.

Clone number	B-CLL cell line OD	T lymphocyte clone OD	Co-culture OD	Cytotoxicity Percentage
Negative CLONE	0.219	0.195	0.383	**7.48**
CLONE 15	0.299	0.148	0.195	**56.37**
CLONE 11	0.285	0.108	0.233	**40.71**
CLONE 7	0.275	0.173	0.271	**39.50**
CLONE 6	0.275	0.158	0.279	**35.56**

Table 1. MTT test results for B-CLL cell culture with D393-CD20 antigen-specific T lymphocyte clones and non-specialized clone for negative control. The data were represented as the average ± SD (n = 3).