Investigating B Cell Function and Immune Checkpoint Expression in Patients with Chronic Lymphocytic Leukemia

By

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Preface

The study described in this thesis was carried out by Aviwe Ntsethe at the University of KwaZulu-Natal, Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, Durban, South Africa under the supervision of Professor Bongani Nkambule and co-supervision of Professor Phiwayinkosi Dludla. The contents of this work have not been submitted in any form for any degree to another tertiary institution, and the use of other people's work has been duly acknowledged in the text.

Chapter 1 covers the background, aims and objectives of the project. Chapter 2 is a comprehensive examination of the existing literature and elucidation of the knowledge gap. This chapter is structured into three parts, general literature review, systematic review protocol and systematic review. The systematic review protocol has been published in peer reviewed journal. Chapters 3, 4, and 5 consist of experimental manuscripts. Chapter 3 experimental paper has been published in an accredited peer reviewed journal.

Aviwe Ntsethe: [redacted] Date: 18 July 2024

As a candidate's Supervisor I agree to the submission of this thesis.

Professor BB. Nkambule: [redacted] Date: 18 July 2024

Professor PV. Dludla: [redacted] Date: 18 July 2024

Declaration

I, Aviwe Ntsethe declare that:

i. The research reported in this thesis, except where otherwise indicated, is my own original work.
ii. This thesis has not been submitted for any degree or examination at any other university.
iii. This thesis does not contain other person's data, pictures, graphs, or other information, unless specifically acknowledged as being sourced from other persons.
iv. This thesis does not contain other person's writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
    a) Their words have been re-written, but the general information attributed to them has been referenced.
    b) Where their exact words have been used, then it has been properly referenced in the reference section.

Signed:  Date: 18 July 2024

Publications directly related to this thesis

Published manuscripts

1. **Aviwe Ntsethe**, Phiwayinkosi V. Dludla, Tawanda M. Nyambuya, Siphamandla R. Ngcobo, Bongani B. Nkambule. The impact of immune checkpoint inhibitors in patients with chronic lymphocytic leukemia (CLL): A protocol for a systematic review and meta-analysis of randomized controlled trials. Medicine (Baltimore). 2020;99(28):e21167.
2. **Aviwe Ntsethe**, Zekhethelo A. Mkhwanazi, Phiwayinkosi V. Dludla, Bongani B. Nkambule. B Cell Subsets and Immune Checkpoint Expression in Patients with Chronic Lymphocytic Leukemia. Curr. Issues Mol. Biol. 2024, 46(3), 1731-1740.

Manuscripts under review

1. **Aviwe Ntsethe,** Phiwayinkosi V. Dludla, Bongani B. Nkambule. Effectiveness of chemoimmunotherapy and the associated major severe adverse events in adult patients with chronic lymphocytic leukemia: A systematic review of randomized controlled trials.
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2. **Aviwe Ntsethe,** Phiwayinkosi V. Dludla, Bongani B. Nkambule. The B-cell function in patients with chronic lymphocytic leukemia.
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3. **Aviwe Ntsethe,** Phiwayinkosi V. Dludla, Bongani B. Nkambule. Evaluating soluble immune checkpoint profiles in patient with chronic lymphocytic leukemia.

List of other publications

1. Siphamandla R. Ngcobo, Bongani B. Nkambule, Tawanda M. Nyambuya, Kabelo Mokgalaboni, **Aviwe Ntsethe**, Vuyolwethu Mxinwa. Activated monocytes as a therapeutic target to attenuate vascular inflammation and lower cardiovascular disease-risk in patients with type 2 diabetes: A systematic review of preclinical and clinical studies. Biomed Pharmacother. 2022;146:112579.

Dedication
To all patients with chronic lymphocytic leukemia

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List of abbreviations

APC: Allophycocyanin

APC/Cy7: Allophycocyanin-cyanine 7

BV421: Brilliant violet 421

CD: Cluster differentiation

CIT: Chemoimmunotherapy

CLL: Chronic lymphocytic leukemia

CTLA-4: Cytotoxic T-lymphocyte-associated protein 4

FBC: Full blood count

FITC: Flourescein isothiocynate

FSC: Forward side scatter

ICs: Immune checkpoints

ICIs: Immune checkpoint inhibitors

IFN-γ: interferon gamma

IGHV: immunoglobulin heavy chain variable region gene

IL: interleukin

LAG-3: Lymphocyte activation gene 3

MeSH: Medical subject headings

OS: overall survival

PD-1: Programmed death-1

PD-L1: Programmed death ligand 1

PE: Phycoerythrin

PE/Cy7: Phycoerythrin-cyanine 7

PerCP: Peridinin chlorophyll protein

PFS: Progression-free survival

PKC: Protein kinase c

SSC: Side scatter

TIM-3: T-cell immunoglobulin-3

TNF-α: Tumour necrosis factor alpha

WCC: white cell count

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Abstract

Introduction

Abnormal accumulation of functionally incompetent B cell and dysregulated immune checkpoint expression are hallmark of chronic lymphocytic leukemia (CLL). In fact, the increased levels of immune checkpoint expression and CD38 positive B cells are consistent with the progression of CLL. Malignant B cell produce immune checkpoints and cytokines that inhibit B cell function and exacerbate the disease progression. In this study, we aimed to investigate the B cell function and immune checkpoint expression in patients with CLL.

Methods

A systematic review evaluating the effectiveness of chemoimmunotherapy and the associated major severe adverse events in adult patients with CLL was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 guidelines. Peripheral blood from a total of 21 patients with CLL and 12 controls were collected. Complete blood count was measured and B cells were isolated using BD IMag isolation system. The baseline levels of B cell subsets, immune checkpoints (cytotoxic T-lymphocyte-associated protein-4, programmed death-ligand-2 and programmed death-1) expression on various B cell subsets and soluble immune checkpoints (soluble interleukin-2 receptor alpha, T cell immunoglobulin and mucin domain-containing protein 3, galectin-9, programmed cell death-1, programmed death-ligand-1 and cytotoxic T-lymphocyte associated protein-4) expression were measured and correlated with prognostic markers and Rai staging. Furthermore, the levels of B cell subsets and immune checkpoint expression on various B cells were measured post protein kinase C activation and immune checkpoint blockage.

Results

Cumulative evidence from the systematic review that included 14 studies showed that targeted therapy combined with immunotherapy is more effective than chemoimmunotherapy in treatment-naïve and high-risk CLL patients. However, these treatments are associated with some major severe adverse events. In the experimental studies, there was increased levels of activated B cells ($P < 0.0001$) in patients with CLL. The immune checkpoints PD-1 and CTLA-4 were elevated on total B cells, activated B cells and memory B cells ($P < 0.05$). However, the increased immune checkpoint expression was not correlated with a prognostic marker, beta-2 microglobulin (B2M) levels. It was demonstrated that the levels of memory B cells and activated memory B cells increased following anti-CTLA-4, anti-PD1 and anti-PD-L1 treatment while levels of activated B cells were significantly decreased ($P < 0.01$). Moreover, the immune checkpoints CTLA-4, PD-1, PD-L1 and PD-L2 expression levels were increased in B cell subsets following B cell stimulation. However, the levels of CTLA-4, PD-1 and PD-L1 were downregulated on total B cells following anti-PD1, PD-L1 and CTL-4 treatment ($P < 0.05$). The

baseline levels of soluble immune checkpoint CD25, TIM-3, galectin-9, PD-1 and PD-L1 were elevated in patients with CLL ($P < 0.001$). However, there were no associations between the soluble immune checkpoints and B2M levels, Rai stage, fluorescence in situ hybridization (FISH) status such as del17p and international prognostic index for chronic lymphocytic leukemia (CLL-IPI) score.

Conclusion

In this study we showed that in patients with CLL there is an increased expression of immune checkpoints on various B cell subsets. However, there is no correlation between these immune checkpoint expressions and prognostic markers or Rai staging. Our study further showed that soluble immune checkpoints associated with more aggressive disease characteristics were also elevated on these patients with CLL. The use of immune checkpoint blockage could benefit patients with CLL. However, the predictive value of the immune checkpoints and the use of immune checkpoint blockage require further study in larger cohorts of patients.

CHAPTER 1: INTRODUCTION

1.1 Research background

The number of deaths attributed to cancer have increased over the year with 10 million recorded in 2020, making it the leading cause of death worldwide (1, 2). Chronic lymphocytic leukemia is one of the common types of blood cancer, accounting 37% of all leukemia cases (3, 4). In the year 2021, chronic lymphocytic leukemia (CLL) was responsible for approximately 4,320 deaths, representing about 0.7% of all cancer-related deaths (5). The median age of diagnosis with CLL among patients is 75 years and it affects more males than females (4). CLL is a type of leukemia characterized by the uncontrolled accumulation of abnormal matured B cells in the bone marrow, blood, and lymphoid tissues (6). It is a relatively common form of leukemia, with varying clinical courses and outcomes among affected individuals.

Despite advancements in treatment, CLL remains a significant cause of morbidity and mortality globally (7). The exact causes of CLL are not fully understood, but genetic and environmental factors such as exposure to certain chemicals or occupational hazards are believed to play a role in its development (8, 9). Treatment strategies for CLL are tailored based on various factors, including disease stage, genomic characteristics, and patient fitness. Watchful waiting is often employed for asymptomatic patients with low-risk CLL, and treatment options include chemotherapy, immunotherapy, and in some cases, stem cell transplantation or combination of these therapies (10, 11). Ongoing research efforts aim to further improve outcomes and develop novel therapies for CLL, with the goal of reducing the burden of this disease and improving overall survival rates.

1.2 Significance of the study

Extensive efforts have been devoted to comprehending the mechanisms employed by malignant cells to elude immune-mediated destruction (12). Over the years, considerable research has been conducted to gain insights into the ways in which cancer cells manage to evade attacks from the immune system (10, 12, 13). The malignant cells often employ various strategies to avoid detection by immune cells, such as downregulating the expression of specific molecules that are essential for immune recognition (14-16). They can also alter their antigen presentation machinery or release immunosuppressive factors, which inhibit the activity of immune cells or induce a state of immune tolerance (12). By understanding the underlying mechanisms, the aim is to enhance the immune system's ability to recognize and eliminate malignant cells, ultimately leading to the development of more effective therapeutic approaches to counteract the ability of the cancer to escape immune attack.

A major factor contributing to the resistance of B cell apoptosis in CLL is the dysregulation of the B-cell receptor (BCR) signaling pathway (17, 18). In CLL, B cells often express a clonal BCR that functions independently of antigens, resulting in the activation of multiple signaling pathways, including the phosphoinositide 3-kinase (PI3K)/Akt and Nuclear factor kappa B (NF-κB) pathways (19,

20). These signaling cascades play a crucial role in promoting the survival and proliferation of malignant B cells (21). The BCR signaling pathway involves the activation of kinases such as Lyn Src family tyrosine kinase (Lyn), spleen tyrosine kinase (Syk), and Bruton's tyrosine kinase (Btk) (22). These kinases play an important role in transmitting signals from the BCR resulting in B cell activation and proliferation in CLL (23). In fact, patients with CLL experience a lower occurrence of adverse events and improved progression-free survival when treated with zanubrutinib, an inhibitor targeting Bruton's tyrosine kinase (24, 25).

Patients with CLL have an increased expression of immune checkpoints such as T-cell immunoglobulin-3 (TIM-3), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), lymphocyte activation gene 3 (LAG-3), and programmed cell death protein 1 (PD-1) (26, 27). Immune checkpoints are proteins that regulate immune function and play a vital role in preventing autoimmunity (28, 29). The overexpression of these checkpoints in patients with CLL is associated with T-cell dysfunction, impaired antitumor activity, and reduced immune surveillance (26, 27). Targeting these immune checkpoints with specific inhibitors, known as immune checkpoint inhibitors, has shown promising results in restoring T-cell function and enhancing the immune response against malignant cells in various types of cancer (30, 31). In fact, PD-1-targeting agent Pembrolizumab has shown a selective efficacy in patients with CLL with Richter transformation (RT) with elevated expression of programmed death-ligand 1 (PD-L1) and PD-1 (32). In order to better stratify patients with CLL into appropriate risk and treatment groups and improve patient outcomes, more accurate prognostic markers are required. Moreover, the precise role of these immune checkpoints in the context of B cells, which are the main cell type affected in CLL, remains poorly understood.

PD-1 protein is one of the regulators of B cell activation (33). The activated B cells express two PD-1 ligands, PD-L1 and programmed death-ligand 2 (PD-L2) (34-36). PD-L2 modulate antibody production by inhibiting interleukin 5 (IL-5) production in T cells (37). Therefore, the expression levels of immune checkpoints, including PD-1 and PD-L2, on different B cell subsets in patients with CLL may contribute to the regulation of antibody production. Despite significant advancements in CLL pathogenesis, the involvement of immune checkpoints on B cells and their impact on B-cell function and survival have not been thoroughly explored. Understanding how these immune checkpoints modulate B-cell function could provide valuable insights into CLL progression and potential therapeutic targets.

Patients diagnosed with CLL show varying responses to CTLA-4 inhibition (38). Interestingly, the effectiveness of this therapy appears to be influenced by the expression levels of CTLA-4 in patients with CLL. Patients with CLL with high CTLA-4 expression may experience unfavorable outcomes, as the therapy can trigger signals that promote cell survival (38). However, those with low CTLA-4 expression may derive potential benefits from CTLA-4 blocking therapy (38). The development of models that simulate immune checkpoint blockade under different conditions of immunological

activation and exhaustion may help to identify patients with CLL who are likely to respond positively to immunotherapy. This tailored approach has the potential to enhance patient outcomes by tailoring immunotherapies to individuals who are more likely to benefit from them.

The chromosomal abnormalities identified through fluorescence in situ hybridization (FISH) and the International Prognostic Index for Chronic Lymphocytic Leukemia (CLL-IPI) offer vital prognostic insights for patients with CLL (39). Patients can be categorized into risk groups based on chromosomal abnormalities identified by FISH, with del(17p) associated with the worst outcomes, especially when treated with combination chemoimmunotherapy (40). it is essential to evaluate how these immune checkpoints correlate with the CLL-IPI score, Rai staging, and specific FISH status, such as 17p13, to enhance disease monitoring and stratification into prognostic subgroups. Despite the utility of the Rai staging systems in categorizing patients based on expected overall survival (41), the lack of validation across diverse populations highlights the necessity of incorporating a range of prognostic markers.

1.3 Aims of the study

1. To evaluate the immune checkpoint expression profile on B cell subsets in patients with CLL and determine their relationship with prognostic markers.
2. To investigate the B cell function in patients with CLL.
3. To evaluate soluble immune checkpoint expression profiles in patients with CLL assess their relationships with prognostic markers.

1.4 Research questions

1. Are the levels of B cell subsets and immune checkpoint expression altered in patients with CLL?
2. Is there any association between immune checkpoints levels and prognostic markers?
3. What is the B cell-mediated immune response in patients with CLL?

1.5 Objectives

1. To enumerate baseline B cell subsets (memory B cells, activated B cells, activated memory B cells) in patients with CLL.
2. To enumurate baseline immune checkpoints (PD-1, PD-L2, CTLA-4) in B cell subsets.
3. To determine the relationship between immune checkpoint expression and an independent prognostic marker for patients with CLL, beta-2 microglobulin (B2M)
4. To stimulate B cells with Phorbol 12-myristate 13-acetate (PMA) and ionomycin, and enumerate the immune checkpoints expression in B cell subsets
5. To evaluate the soluble immune checkpoint profiles in patients with CLL and assess their relationship with B2M and other clinical parameters such as fluorescent in situ hybridization (FISH) status, Rai staging and international prognostic index for chronic lymphocytic leukemia (CLL-IPI) score.

References

1. World Health Organization. Cancer 2022 [Available from: https://www.who.int/news-room/fact-sheets/detail/cancer.
2. Ferlay J, Ervik M, Lam F, Colombet M, Mery L, Piñeros M, et al. Global cancer observatory: cancer today. Lyon: International Agency for Research on Cancer; 2020. Cancer Tomorrow. 2021.
3. DeSantis CE, Miller KD, Goding Sauer A, Jemal A, Siegel RL. Cancer statistics for african americans, 2019. CA: a cancer journal for clinicians. 2019;69(3):211-33.
4. The surveillance e, and end results (SEER) program of the National Cancer Institute. Cancer Stat Facts: Leukemia—Chronic Lymphocytic Leukemia (CLL). 2021 [3755-7]. Available from: https://seer.cancer.gov/statfacts/html/clyl.html;.
5. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer statistics, 2021. Ca Cancer J Clin. 2021;71(1):7-33.
6. Lanasa MCJH, the American Society of Hematology Education Program Book. Novel insights into the biology of CLL. 2010;2010(1):70-6.
7. Ou Y, Long Y, Ji L, Zhan Y, Qiao T, Wang X, et al. Trends in disease burden of chronic lymphocytic leukemia at the global, regional, and national levels from 1990 to 2019, and projections until 2030: a population-based epidemiologic study. Frontiers in oncology. 2022;12:840616.
8. Slager SL, Rabe KG, Achenbach SJ, Vachon CM, Goldin LR, Strom SS, et al. Genome-wide association study identifies a novel susceptibility locus at 6p21. 3 among familial CLL. Blood, The Journal of the American Society of Hematology. 2011;117(6):1911-6.
9. Miranda-Filho A, Piñeros M, Ferlay J, Soerjomataram I, Monnereau A, Bray F. Epidemiological patterns of leukaemia in 184 countries: a population-based study. The Lancet Haematology. 2018;5(1):e14-e24.
10. Hallek M, Shanafelt TD, Eichhorst B. Chronic lymphocytic leukaemia. Lancet. 2018;391(10129):1524-37.
11. (NCCN). NCCN. Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma. NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines). 2021 [Available from: https://www.nccn.org/guidelines/guidelines-detail?category=1&id=1415.
12. Gonzalez H, Hagerling C, Werb Z. Roles of the immune system in cancer: from tumor initiation to metastatic progression. Genes & development. 2018;32(19-20):1267-84.
13. Montillo M, Hamblin T, Hallek M, Montserrat E, Morra E. Chronic lymphocytic leukemia: novel prognostic factors and their relevance for risk-adapted therapeutic strategies. Haematologica. 2005;90(3):391-9.
14. Taghiloo S, Allahmoradi E, Ebadi R, Tehrani M, Hosseini-Khah Z, Janbabaei G, et al. Upregulation of Galectin-9 and PD-L1 immune checkpoints molecules in patients with chronic lymphocytic leukemia. Asian Pacific journal of cancer prevention: APJCP. 2017;18(8):2269.
15. Brusa D, Serra S, Coscia M, Rossi D, D'Arena G, Laurenti L, et al. The PD-1/PD-L1 axis contributes to T-cell dysfunction in chronic lymphocytic leukemia. haematologica. 2013;98(6):953.
16. Gamaleldin M, Ghallab O, Nadwan E, Abo Elwafa R. PD-1 and PD-L1 gene expressions and their association with Epstein-Barr virus infection in chronic lymphocytic leukemia. Clinical and Translational Oncology. 2021;23:2309-22.
17. Chen R, Tsai J, Thompson PA, Chen Y, Xiong P, Liu C, et al. The multi-kinase inhibitor TG02 induces apoptosis and blocks B-cell receptor signaling in chronic lymphocytic leukemia through dual mechanisms of action. Blood cancer journal. 2021;11(3):57.
18. Ondrisova L, Mraz M. Genetic and non-genetic mechanisms of resistance to BCR signaling inhibitors in B cell malignancies. Frontiers in oncology. 2020;10:591577.
19. Chatzigeorgiou A, Lyberi M, Chatzilymperis G, Nezos A, Kamper E. CD40/CD40L signaling and its implication in health and disease. Biofactors. 2009;35(6):474-83.
20. Haselager MV, Kater AP, Eldering E. Proliferative signals in chronic lymphocytic leukemia; what are we missing? Frontiers in Oncology. 2020;10:592205.
21. Pascutti MF, Jak M, Tromp JM, Derks IA, Remmerswaal EB, Thijssen R, et al. IL-21 and CD40L signals from autologous T cells can induce antigen-independent proliferation of CLL cells. Blood, The Journal of the American Society of Hematology. 2013;122(17):3010-9.

22. Wang H, Guo H, Yang J, Liu Y, Liu X, Zhang Q, et al. Bruton tyrosine kinase inhibitors in B-cell lymphoma: beyond the antitumour effect. Experimental Hematology & Oncology. 2022;11(1):1-13.
23. Koehrer S, Burger JA. Chronic lymphocytic leukemia: disease biology. Acta Haematologica. 2023.
24. Molica S, Tam C, Allsup D, Polliack A. Advancements in the Treatment of CLL: The Rise of Zanubrutinib as a Preferred Therapeutic Option. Cancers (Basel). 2023;15(14).
25. Tam CS, Brown JR, Kahl BS, Ghia P, Giannopoulos K, Jurczak W, et al. Zanubrutinib versus bendamustine and rituximab in untreated chronic lymphocytic leukaemia and small lymphocytic lymphoma (SEQUOIA): a randomised, controlled, phase 3 trial. Lancet Oncol. 2022;23(8):1031-43.
26. Palma M, Gentilcore G, Heimersson K, Mozaffari F, Näsman-Glaser B, Young E, et al. T cells in chronic lymphocytic leukemia display dysregulated expression of immune checkpoints and activation markers. Haematologica. 2017;102(3):562-72.
27. Rezazadeh H, Astaneh M, Tehrani M, Hossein-Nataj H, Zaboli E, Shekarriz R, et al. Blockade of PD-1 and TIM-3 immune checkpoints fails to restore the function of exhausted CD8+ T cells in early clinical stages of chronic lymphocytic leukemia. Immunologic Research. 2020;68:269-79.
28. Joller N, Peters A, Anderson AC, Kuchroo VK. Immune checkpoints in central nervous system autoimmunity. Immunological reviews. 2012;248(1):122-39.
29. Dyck L, Mills KH. Immune checkpoints and their inhibition in cancer and infectious diseases. European journal of immunology. 2017;47(5):765-79.
30. Galluzzi L, Humeau J, Buqué A, Zitvogel L, Kroemer G. Immunostimulation with chemotherapy in the era of immune checkpoint inhibitors. Nature reviews Clinical oncology. 2020;17(12):725-41.
31. Carlino MS, Larkin J, Long GV. Immune checkpoint inhibitors in melanoma. The Lancet. 2021;398(10304):1002-14.
32. Ding W, LaPlant BR, Call TG, Parikh SA, Leis JF, He R, et al. Pembrolizumab in patients with CLL and Richter transformation or with relapsed CLL. Blood, The Journal of the American Society of Hematology. 2017;129(26):3419-27.
33. Thibult M-L, Mamessier E, Gertner-Dardenne J, Pastor S, Just-Landi S, Xerri L, et al. PD-1 is a novel regulator of human B-cell activation. International immunology. 2013;25(2):129-37.
34. Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nature immunology. 2001;2(3):261-8.
35. Kaku H, Rothstein TL. Octamer binding protein 2 (Oct2) regulates PD-L2 gene expression in B-1 cells through lineage-specific activity of a unique, intronic promoter. Genes & Immunity. 2010;11(1):55-66.
36. Yamazaki T, Akiba H, Iwai H, Matsuda H, Aoki M, Tanno Y, et al. Expression of programmed death 1 ligands by murine T cells and APC. J Immunol. 2002;169(10):5538-45.
37. McKay JT, Haro MA, Daly CA, Yammani RD, Pang B, Swords WE, et al. PD-L2 Regulates B-1 Cell Antibody Production against Phosphorylcholine through an IL-5-Dependent Mechanism. J Immunol. 2017;199(6):2020-9.
38. Ciszak L, Frydecka I, Wolowiec D, Szteblich A, Kosmaczewska A. Patients with chronic lymphocytic leukaemia (CLL) differ in the pattern of CTLA-4 expression on CLL cells: the possible implications for immunotherapy with CTLA-4 blocking antibody. Tumor Biology. 2016;37:4143-57.
39. Nabhan C, Raca G, Wang YL. Predicting prognosis in chronic lymphocytic leukemia in the contemporary era. JAMA oncology. 2015;1(7):965-74.
40. Chavez JC, Kharfan-Dabaja MA, Kim J, Yue B, Dalia S, Pinilla-Ibarz J, et al. Genomic aberrations deletion 11q and deletion 17p independently predict for worse progression-free and overall survival after allogeneic hematopoietic cell transplantation for chronic lymphocytic leukemia. Leukemia research. 2014;38(10):1165-72.
41. Rai KR, Sawitsky A, Cronkite EP, Chanana AD, Levy RN, Pasternack BS. Clinical staging of chronic lymphocytic leukemia. 1975.

Prologue

This chapter is comprised of three sections. Section 1 is the general literature review which discusses available literature of this research project. Section 2 is the published systematic review and meta-analysis protocol which provide detailed methodology on how available literature was appraised and synthesized. Section 3 is the systematic review which was conducted in accordance to a published protocol relevant to this project.

CHAPTER 2: Review

2.1 Literature review

2.1.1 Introduction

The global prevalance of malignancies is rapidly increasing, with 19.3 million new cancer cases were estimated in 2020. The global cancer mortality in 2020 was estimated to be 10 million and about 65% of the cancer deaths occurred in low and middle-income countries (LMICs) (1, 2). The risk of developing malignancies in adults is about 20% and the risk of cancer-related mortality is about 10% (1). It is estimated that by 2030, LMICs will account for 75% of the global cancer-related deaths (3). The cumulative risk of mortality among females in Eastern Africa (10.74%), Middle Africa (8.31%), Northern Africa (8.24%), Southern Africa (10.43%) and Western Africa (8.85%) while among males is 8.63%, 7.94%, 10.62%, 13.23% and 7.65%, respectively (4). In the whole Africa, malignancies cause 7.8% of mortality (4). Leukaemias are amongst the causes of malignancy-related mortality, accounting for 3.1% of the global malignancy-related deaths (4, 5). The most common leukemia affecting adults is Chronic Lymphocytic Leukemia (CLL) with a median age at diagnosis of 70 years (6). This type of leukemia affects more males than females (6). This review provides perspectives on the treatment of CLL and immune checkpoints as possible biomarkers for the prognosis of CLL. Using medical subject headings (MeSH) such as programmed cell death protein 1 (PD-1), programmed death-ligand 1 (PD-L1), lymphocyte-activation gene 3 (LAG-3), T-cell immunoglobulin-3 (TIM-3), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), MEDLINE and Academic Search Complete databases were searched to identify relevant publications to review the potential use of immune checkpoints as prognostic markers of CLL.

2.1.2 The prevalence of chronic Lymphocytic Leukemia

CLL is a lymphoproliferative malignancy, characterized by the accumulation of mature functionally incompetent B-cells in peripheral blood, bone marrow, lymph nodes, and the spleen (7). CLL is the most common type of leukemia among adults in western countries, accounting for about 37% of leukemia cases (8, 9). It is a sporadic disease in Africa, with limited studies (10-12), therefore, the actual prevalence of the disease in Africa cannot be estimated. The median age of diagnosis among patients with CLL is 70 years and is more predominant in males than females (4). In 2024, CLL is projected to cause 4440 deaths, accounting for 18.8% of all leukemia deaths in United States (13).

2.1.3 Etiology and Pathogenesis of Chronic Lymphocytic Leukemia

The etiology and pathogenesis of CLL are not yet fully understood. However, the risk factors such as the presence of hepatitis C, genetic predisposition for CLL, and lifestyle are major contributing factors to the development of CLL (14-16). The detection of CLL-derived hematopoietic stem cells (HSCs) indicate the possibility of the development of CLL at the stem cell stage (17). The HSCs demonstrate

an increased proportion of polyclonal pro-B cells, progressing to the development of monoclonal $CD5^+$ B cell populations (17). However, the precise mechanism behind this phenomenon remains unclear.

Although, immunoglobulin heavy chain variable region-mutated (IGHV-M) CLLs are believed to originate from $CD5^+CD27^+$ B cells that can be found in the post-germinal center (GC) stage. These cells express transcriptional similarities to memory B cells and are likely derived from $CD5^+$ $CD27^-$ B cells that have undergone the GC reaction (18). Furthermore, unmutated immunoglobulin heavy chain variable region (IGHV-UM) CLL appears to originate from pre-germinal center (pre-GC) $CD5^+CD27^-$ B cells. The Monoclonal B-cell lymphocytosis (MBL), a precursor of CLL, and the subsequent development of frank monoclonal CLL cells are influenced by various factors, including additional genetic and epigenetic abnormalities, activation of BCR, and microenvironmental variables. These factors contribute to the progression from MBL to CLL (19) (Figure 2.1.1).

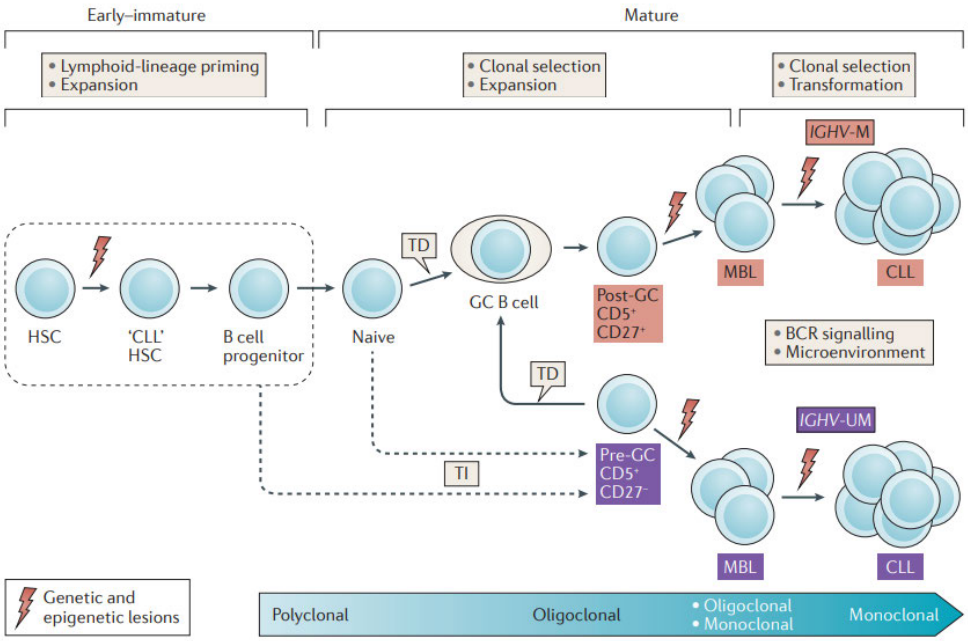

Figure 2.1.1: The pathogenesis of chronic lymphocytic leukemia. T-cell dependent antigen (TD), T-cell independent antigen (TI). Adapted from (19).

Several studies have associated the activation of nuclear factor-κB (NF-κB) with the stimulation of BCR signaling and activation (20). NF-κB is an essential protein complex that regulates cell survival, transcription of DNA and cytokine production (21, 22). NFκB activity has emerged to be involved in the proliferation and survival of malignant cells (23). The immune checkpoints PD-1, its ligands PD-L1 and TIM-3 have been associated with the regulation of NFκB signaling (24). NFκB activity is inhibited by adipose-derived stem cells (ADSCs) through PDL1/PD-1 and Galectin-9 /TIM-3 pathways (24).

2.1.4 The role of B cell and immune checkpoints in the pathogenesis of CLL

Immune checkpoints regulate immune function and thus play an essential role in regulating immune tolerance (25, 26). The signaling of immune checkpoints is dysregulated in patients with CLL, leading to immune dysfunction (27). Several immune checkpoint proteins are dysregulated in patients with CLL these include PD-1, PD-L1, LAG-3, CTLA-4 and TIM-3 (27-30). Notably, PD-1 is expressed on the surface of activated T and B cells, while PD-L1 is expressed on tumor cells, including CLL cells (31, 32). Moreover, PD-L1 is normally expressed on macrophages and can be induced in both activated T and B cells in an inflammatory environment. The expression of PD-L1 downragulates host immune responses in peripheral tissue (33, 34). While the expression of PD-1 may be modified by several factors, including transcription factors and epigenetic changes such as DNA methylation (35). When the PD-1 receptor protein on T cells counteracts with PD-L1 expressed tumor cells, it leads to a wave of inhibitory intracellular signaling that suppresses the activity of T cells (27, 36). This interaction evades CLL cells from immune .

The PD-1/programmed cell death 1 ligand 2 (PD-L2) axis regulates intracellular signaling pathways that may result in T cell exhaustion and immune suppression (37) (Figure 2.2). These signaling pathways activate phosphatases, particularly Src homology 2 domain-containing protein tyrosine phosphatase 2 (SHP2), which dephosphorylates key signaling molecules (38).

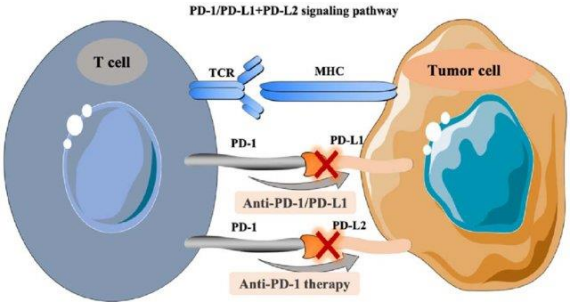

Figure 2.1.2: PD-1/PD-L1 and PD-L2 signaling pathway. PD-1 (Programmed cell death protein-1), PD-L1 (programmed cell death 1 ligand), PD-L2 (programmed cell death 1 ligand 2). Adapted from (39).

PD-L1 and PD-L2 are expressed broadly in peripheral tissue while the cytotoxic T-lymphocyte associated protein 4 (CTLA-4) ligand is presented on the surface of antigen-presenting cell (APC) (31). CTLA-4 is an inhibitory protein expressed on activated T cells and most abundantly expressed on the surface regulatory T cells (Treg) (32). This molecule is also expressed in small quantities on B cells (40) and is highly expressed in patients with CLL (41). When CTLA-4 binds to the B7 ligand, it delivers inhibitory signals that suppress T cell activation and immune responses. In patients with CLL, CTLA-4 signaling contributes to immune evasion by limiting T cell activity against CLL cells (41).

The TIM-3 negatively regulates T cell-mediated immune responses. It exerts its inhibitory effects through interactions with its ligand, Galectin-9, leading to the induction of immune tolerance (42). Elevated levels of Galectin-9 and TIM-3 have been observed in patients with CLL, and their presence is closely linked with disease progression (43, 44). Increased expression of Galectin-9 and TIM-3 in patients with CLL is associated with a more aggressive form of the disease and poorer clinical outcomes (30). The interaction between galectin-9 and TIM-3 promotes immune dysfunction by inhibiting T cell activity and promoting immune tolerance within the CLL microenvironment (30, 44). This immune evasion mechanism allows CLL cells to evade immune surveillance, leading to disease progression and resistance to therapy (44). Consequently, Galectin-9 and TIM-3 have emerged as potential targets for therapeutic interventions aimed at restoring effective anti-tumor immune responses and improving treatment outcomes in patients with CLL.

Exhaustion of B and T cells have been implicated in the pathogenesis of CLL, which contributes to disease progression and immune dysfunction (45). The CLL microenvironment is abundant with B cells expressing various immune checkpoint molecules, such as PD-L1 and TIM-3, which bind with their respective counterreceptors on T cells, including PD-1 and galectin-9 (36, 45, 46). Additionally, CLL-derived B cells can produce cytokines and chemokines that contribute to the formation of an immunosuppressive microenvironment, further promoting T cell exhaustion (47).

An abnormal B cell population in CLL provides chronic antigenic stimulation to T cells, which can result in functional exhaustion. The sustained exposure to CLL antigens results in continuous activation of T cells, eventually leading to T cell dysfunction and exhaustion (48). Additionally, impaired B cell receptor (BCR) signaling is observed in patients with CLL and this may affect antigen presentation, resulting in inadequate co-stimulatory signals to T cells. This deficiency in co-stimulation can contribute to T cell exhaustion and an impaired anti-tumor immune response (36, 49).

2.1.5 Diagnosis and staging of Chronic lymphocytic Leukemia

The peripheral blood count is used to establish the diagnosis of CLL, with the presence of at least 5 x 10^9 /L B lymphocytes in the peripheral blood. In addition, flow cytometry is used to enumarate a clonal B-cell population, which is present for a duration of three months (7). In CLL, blood smears contain distinct smudge cells with characteristic features of fragile lymphocytes associated with CLL (50). These features include small and mature lymphocytes. In peripheral blood, malignant cells express specific surface antigen markers such as CD5, CD19, CD20, and CD23. Notably, the levels of CD20 surface immunoglobulin are lower compared to those observed on normal B cells (7). The recently confirmed immunological panel for CLL diagnosis is CD19, CD5, CD20, CD23, kappa, and lambda (51). In addition, CD43, CD79b, CD81, CD200, CD10 are helpful in refining the diagnosis (51).

2.1.6 Chronic lymphocytic leukemia disease progression and prognosis

Some patients with CLL will not require immediate treatment upon diagnosis, and are often closely monitored without medication for several years (7). The decision to initiate treatment is influenced by the presence of active or symptomatic disease (7). Unless rapid disease progression is evident, asymptomatic individuals in the early stages of CLL in the Rai stage 0 or Binet stage A should be observed without treatment (7). Treatment is not recommended for patients in the low Binet (A or B) or Rai (0-II) stages without symptoms, as culmulative evidence does not support improved survival outcomes with initiating treatment at an early-stage of the disease (7, 52).

2.1.7 Clinical staging of chronic lymphocytic leukemia

The Rai staging system categorizes CLL based on the severity of the illness (53). The Rai staging catogorizes disease severity as Low-risk (stage 0), intermediate-risk (stage I- II), and high-risk (stage III-IV) (Table 2.1.1).

Table 2.1.1: Rai Staging System

Stage	Characteristics
Stage 0	Abnormal increase in the number of lymphocytes in the blood and marrow
Stages I & II	Abnormal increase in the number of lymphocytes in the blood and marrow along with enlarged nodes in any location, splenomegaly (enlarged spleen), and hepatomegaly (enlarged liver)
Stages III & IV	Anemia, defined as a hemoglobin (Hb) level below 11g/dL (stage III), or thrombocytopenia, defined as a platelet count below $100x10^9$/L (stage IV)

The Binet staging system for CLL is based on the number of affected areas, considering the development of swollen lymph nodes larger than 1 cm in diameter or organomegaly (enlarged organs). The specific areas assessed for involvement are: (1) head and neck, including the Waldeyer ring; (2) axillae (both axillae involvement count as one area); (3) groins, including superficial femoral (both groins involvement count as one area); (4) palpable spleen; and (5) palpable liver. These areas are examined clinically to assess enlargement (54). According to the Binet staging system, CLL is divided into three stages: Stage A to C (Table 2.1.2).

Table 2.1.2: Binet Staging System

Stage	Characteristics
Stage A	Fewer than 3 areas of lymphadenopathy; no anemia (hemoglobin ≥ 10 g/dL) and no thrombocytopenia (platelets ≥100x10^9/L)
Stage B	No anemia (hemoglobin ≥10g/dL), no thrombocytopenia (platelets ≥100x10^9/L) and more than 3 areas of lymphadenopathy
Stage C	Anemia (hemoglobin <10 g/dL), thrombocytopenia (platelets <100x10^9/L) and any number of areas of lymphadenopathy

The Binet and Rai staging system is used to distinguish prognostic subgroups (55). The CLL International Prognostic Index (CLL-IPI) is the most recent relevant prognostic score (CLL-IPI) that uses a weighted grading of five independent prognostic factors: TP53 deletion and/or mutation (commonly referred to as TP53 dysfunction or abberations), IGHV mutational status, serum Beta 2-microglobulin (B2M), clinical stage, and age (56).

2.1.8 Treatment of CLL

The wide range of available treatment options in developed countries, allows for the selection of more tailored treatment plan for patients with CLL. This requires the need for expertise in diagnostics, informed clinical judgment, and the appropriate utilization of diagnostic methods and prognostic factors (54). The expanding treatment choices for patients with CLL and the selection of treatment approaches and endpoints in clinical trials may be influenced by the patients' overall health and fitness level (54). Combining chemotherapy and immunotherapy with fludarabine, cyclophosphamide, and rituximab (FCR) is widely considered standard of care for fit patients with CLL (54). However, many patients with CLL are elderly with comorbidities, making them medically-unfit and ineligible for FCR (57).

Accumulating evidence shows that the combination of an anti-CD20 monoclonal antibody with chemotherapy improves outcomes in patients with CLL and coexisting conditions (54, 58-61). Unfit patients may be offered the following combination therapies; venetoclax plus obinutuzumab or ibrutinib monotherapy or chlorambucil plus obinutuzumab (54). Prognostic factors such as IGHV mutation status, B2M, fluorescence in situ hybridization (del13q, trisomy 12, del11q and del17p) and immune checkpoints are still being evaluated to assist with treatment selection (62-66).

2.1.9 Conclusions

The future of treating patients with CLL dependent on the identification of novel prognostic markers and understanding the pathogenesis of CLL, which in turn may lead to tailored treatment and improved patient outcomes. The role of B cell in the pathogenesis and progression of CLL is still not clear and need further investigation. Immune checkpoint molecules such as PD-1, PD-L1, CTLA-4, and TIM-3 are dysregulated in CLL, contributing to immune dysfunction and disease progression. These molecules are potential prognostic markers and therapeutic targets. Treatment varies based on patient fitness, prognostic markers and includes combinations of chemoimmunotherapy (CTI), with newer treatments like venetoclax and ibrutinib showing promise with improved patient outcomes. Studies should focus on prognostic markers that could improve the effectiveness of immunotherapy or combination therapies in patients with CLL. Therefore, prognostic factors are crucial in guiding treatment choices. The review indicates a scarcity of data on CLL in Africa, making it challenging to estimate the actual prevalence and inform treatment strategies tailored to African patients.

References

1. Ferlay J, Colombet M, Soerjomataram I, Parkin DM, Piñeros M, Znaor A, et al. Cancer statistics for the year 2020: An overview. 2021;149(4):778-89.
2. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. 2021;71(3):209-49.
3. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal AJCacjfc. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. 2018;68(6):394-424.
4. Bray F, Laversanne M, Sung H, Ferlay J, Siegel RL, Soerjomataram I, et al. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a cancer journal for clinicians. 2024;74(3):229-63.
5. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a cancer journal for clinicians. 2021;71(3):209-49.
6. Cronin KA, Ries LA, Edwards BK. The surveillance, epidemiology, and end results (SEER) program of the National Cancer Institute. Cancer. 2014;120:3755-7.
7. Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Döhner H, et al. iwCLL guidelines for diagnosis, indications for treatment, response assessment, and supportive management of CLL. 2018;131(25):2745-60.
8. DeSantis CE, Miller KD, Goding Sauer A, Jemal A, Siegel RLJCacjfc. Cancer statistics for african americans, 2019. 2019;69(3):211-33.
9. The surveillance e, and end results (SEER) program of the National Cancer Institute. Cancer Stat Facts: Leukemia—Chronic Lymphocytic Leukemia (CLL). 2021 [3755-7]. Available from: https://seer.cancer.gov/statfacts/html/clyl.html;.
10. Salawu L, Bolarinwa R, Durosinmi MJAhs. Chronic lymphocytic leukaemia: a-twenty-years experience and problems in Ile-Ife, South-Western Nigeria. 2010;10(2).
11. Omoti C, Awodu O, Bazuaye GJIjolh. Chronic lymphoid leukaemia: clinico-haematological correlation and outcome in a single institution in Niger Delta region of Nigeria. 2007;29(6):426-32.
12. Sall A, Touré AO, Sall FB, Ndour M, Fall S, Sène A, et al. Characteristics of chronic lymphocytic leukemia in Senegal. BMC Hematology. 2016;16(1):10.
13. Siegel RL, Giaquinto AN, Jemal A. Cancer statistics, 2024. CA: a cancer journal for clinicians. 2024;74(1).
14. Ghia P, Chiorazzi N, Stamatopoulos KJJoim. Microenvironmental influences in chronic lymphocytic leukaemia: the role of antigen stimulation. 2008;264(6):549-62.
15. Matos DM, Ismael SJ, Scrideli CA, De Oliveira FM, Rego EM, Falcão RPJBjoh. Monoclonal B-cell lymphocytosis in first-degree relatives of patients with sporadic (non-familial) chronic lymphocytic leukaemia. 2009;147(3):339-46.
16. Slager SL, Benavente Y, Blair A, Vermeulen R, Cerhan JR, Costantini AS, et al. Medical history, lifestyle, family history, and occupational risk factors for chronic lymphocytic leukemia/small lymphocytic lymphoma: the InterLymph Non-Hodgkin Lymphoma Subtypes Project. 2014;2014(48):41-51.
17. Kikushige Y, Ishikawa F, Miyamoto T, Shima T, Urata S, Yoshimoto G, et al. Self-renewing hematopoietic stem cell is the primary target in pathogenesis of human chronic lymphocytic leukemia. 2011;20(2):246-59.
18. Seifert M, Sellmann L, Bloehdorn J, Wein F, Stilgenbauer S, Dürig J, et al. Cellular origin and pathophysiology of chronic lymphocytic leukemia. 2012;209(12):2183-98.
19. Fabbri G, Dalla-Favera RJNRC. The molecular pathogenesis of chronic lymphocytic leukaemia. 2016;16(3):145-62.

20. Cui B, Chen L, Zhang S, Mraz M, Fecteau J-F, Yu J, et al. MicroRNA-155 influences B-cell receptor signaling and associates with aggressive disease in chronic lymphocytic leukemia. Blood. 2014;124(4):546-54.
21. Rahman KW, Li Y, Sarkar FH. Inactivation of Akt and NF-κB play important roles during indole-3-carbinol-induced apoptosis in breast cancer cells. Nutrition and cancer. 2004;48(1):84-94.
22. Mansouri L, Papakonstantinou N, Ntoufa S, Stamatopoulos K, Rosenquist R, editors. NF-κB activation in chronic lymphocytic leukemia: a point of convergence of external triggers and intrinsic lesions. Seminars in cancer biology; 2016: Elsevier.
23. Frenzel LP, Claus R, Plume N, Schwamb J, Konermann C, Pallasch CP, et al. Sustained NF-kappaB activity in chronic lymphocytic leukemia is independent of genetic and epigenetic alterations in the TNFAIP3 (A20) locus. International journal of cancer. 2011;128(10):2495-500.
24. Zhou K, Guo S, Tong S, Sun Q, Li F, Zhang X, et al. Immunosuppression of human adipose-derived stem cells on T cell subsets via the reduction of NF-kappaB activation mediated by PD-L1/PD-1 and Gal-9/TIM-3 pathways. Stem cells and development. 2018;27(17):1191-202.
25. Hogg SJ, Vervoort SJ, Deswal S, Ott CJ, Li J, Cluse LA, et al. BET-bromodomain inhibitors engage the host immune system and regulate expression of the immune checkpoint ligand PD-L1. 2017;18(9):2162-74.
26. Dyck L, Mills KHJEjoi. Immune checkpoints and their inhibition in cancer and infectious diseases. 2017;47(5):765-79.
27. Palma M, Gentilcore G, Heimersson K, Mozaffari F, Näsman-Glaser B, Young E, et al. T cells in chronic lymphocytic leukemia display dysregulated expression of immune checkpoints and activation markers. Haematologica. 2017;102(3):562-72.
28. Riches JC, Davies JK, McClanahan F, Fatah R, Iqbal S, Agrawal S, et al. T cells from CLL patients exhibit features of T-cell exhaustion but retain capacity for cytokine production. Blood. 2013;121(9):1612-21.
29. Rezazadeh H, Astaneh M, Tehrani M, Hossein-Nataj H, Zaboli E, Shekarriz R, et al. Blockade of PD-1 and TIM-3 immune checkpoints fails to restore the function of exhausted CD8+ T cells in early clinical stages of chronic lymphocytic leukemia. Immunologic Research. 2020;68:269-79.
30. Taghiloo S, Allahmoradi E, Ebadi R, Tehrani M, Hosseini-Khah Z, Janbabaei G, et al. Upregulation of Galectin-9 and PD-L1 immune checkpoints molecules in patients with chronic lymphocytic leukemia. Asian Pacific journal of cancer prevention: APJCP. 2017;18(8):2269.
31. Ahmadzadeh M, Johnson LA, Heemskerk B, Wunderlich JR, Dudley ME, White DE, et al. Tumor antigen–specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. Blood. 2009;114(8):1537-44.
32. Walker LS. Treg and CTLA-4: two intertwining pathways to immune tolerance. J Autoimmun. 2013;45(100):49-57.
33. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. Nature medicine. 2002;8(8):793-800.
34. Mazanet MM, Hughes CC. B7-H1 is expressed by human endothelial cells and suppresses T cell cytokine synthesis. The Journal of Immunology. 2002;169(7):3581-8.
35. Bally AP, Austin JW, Boss JM. Genetic and epigenetic regulation of PD-1 expression. The Journal of Immunology. 2016;196(6):2431-7.
36. Riches JC, Davies JK, McClanahan F, Fatah R, Iqbal S, Agrawal S, et al. T cells from CLL patients exhibit features of T-cell exhaustion but retain capacity for cytokine production. Blood, The Journal of the American Society of Hematology. 2013;121(9):1612-21.
37. Wykes MN, Lewin SR. Immune checkpoint blockade in infectious diseases. Nature Reviews Immunology. 2018;18(2):91-104.
38. Okazaki T, Honjo T. PD-1 and PD-1 ligands: from discovery to clinical application. International immunology. 2007;19(7):813-24.

39. Zeng Z, Yang B, Liao Z-Y. Current progress and prospect of immune checkpoint inhibitors in hepatocellular carcinoma. Oncology Letters. 2020;20(4):1-.
40. Quandt D, Hoff H, Rudolph M, Fillatreau S, Brunner-Weinzierl MC. A new role of CTLA-4 on B cells in thymus-dependent immune responses in vivo. The Journal of Immunology. 2007;179(11):7316-24.
41. Motta M, Rassenti L, Shelvin BJ, Lerner S, Kipps TJ, Keating MJ, et al. Increased expression of CD152 (CTLA-4) by normal T lymphocytes in untreated patients with B-cell chronic lymphocytic leukemia. Leukemia. 2005;19(10):1788-93.
42. Sheng CC, Han FY. Immunoregulation effects of TIM-3 on tumors. Neoplasma. 2019;66(2):167-75.
43. Pang N, Alimu X, Chen R, Muhashi M, Ma J, Chen G, et al. Activated Galectin-9/Tim3 promotes Treg and suppresses Th1 effector function in chronic lymphocytic leukemia. Faseb j. 2021;35(7):e21556.
44. Xierenguli A, Zeng X, Pang N, Zhagn R, Ma J, Zhao Y, et al. [TIM-3/galectin-9 is involved in negative regulation of T cells in patients with chronic lymphocytic leukemia]. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi. 2020;36(11):1021-5.
45. Scrivener S, Goddard R, Kaminski E, Prentice A. Abnormal T-cell function in B-cell chronic lymphocytic leukaemia. Leukemia & lymphoma. 2003;44(3):383-9.
46. McClanahan F, Hanna B, Miller S, Clear AJ, Lichter P, Gribben JG, et al. PD-L1 checkpoint blockade prevents immune dysfunction and leukemia development in a mouse model of chronic lymphocytic leukemia. Blood, The Journal of the American Society of Hematology. 2015;126(2):203-11.
47. Gassner FJ, Zaborsky N, Catakovic K, Rebhandl S, Huemer M, Egle A, et al. Chronic lymphocytic leukaemia induces an exhausted T cell phenotype in the TCL 1 transgenic mouse model. British journal of haematology. 2015;170(4):515-22.
48. Bichi R, Shinton SA, Martin ES, Koval A, Calin GA, Cesari R, et al. Human chronic lymphocytic leukemia modeled in mouse by targeted TCL1 expression. Proceedings of the National Academy of Sciences. 2002;99(10):6955-60.
49. Ramsay AG, Johnson AJ, Lee AM, Gorgün G, Le Dieu R, Blum W, et al. Chronic lymphocytic leukemia T cells show impaired immunological synapse formation that can be reversed with an immunomodulating drug. The Journal of clinical investigation. 2008;118(7):2427-37.
50. Marionneaux SM, Keohane EM, Lamanna N, King TC, Mehta SR. Smudge cells in chronic lymphocytic leukemia: pathophysiology, laboratory considerations, and clinical significance. Laboratory Medicine. 2021;52(5):426-38.
51. Rawstron AC, Kreuzer KA, Soosapilla A, Spacek M, Stehlikova O, Gambell P, et al. Reproducible diagnosis of chronic lymphocytic leukemia by flow cytometry: An European Research Initiative on CLL (ERIC) & European Society for Clinical Cell Analysis (ESCCA) Harmonisation project. 2018;94(1):121-8.
52. Shustik C, Mick R, Silver R, Sawitsky A, Rai K, Shapiro LJHo. Treatment of early chronic lymphocytic leukemia: intermittent chlorambucil versus observation. 1988;6(1):7-12.
53. Rai KR, Sawitsky A, Cronkite EP, Chanana AD, Levy RN, Pasternack BS. Clinical staging of chronic lymphocytic leukemia. 1975.
54. Hallek M, Al-Sawaf OJAJoH. Chronic lymphocytic leukemia: 2022 update on diagnostic and therapeutic procedures. 2021;96(12):1679-705.
55. Pflug N, Bahlo J, Shanafelt TD, Eichhorst BF, Bergmann MA, Elter T, et al. Development of a comprehensive prognostic index for patients with chronic lymphocytic leukemia. 2014;124(1):49-62.
56. Oncology IC-IWGJTL. An international prognostic index for patients with chronic lymphocytic leukaemia (CLL-IPI): a meta-analysis of individual patient data. 2016;17(6):779-90.
57. Shah N, Tam C, Seymour JF, Rule S. How applicable is fludarabine, cyclophosphamide and rituximab to the elderly? Leukemia & lymphoma. 2015;56(6):1599-610.

58. Goede V, Fischer K, Busch R, Jaeger U, Dilhuydy M, Wickham N, et al. Chemoimmunotherapy with GA101 plus chlorambucil in patients with chronic lymphocytic leukemia and comorbidity: results of the CLL11 (BO21004) safety run-in. 2013;27(5):1172-4.
59. Städler N, Shang A, Bosch F, Briggs A, Goede V, Berthier A, et al. A systematic review and network meta-analysis to evaluate the comparative efficacy of interventions for unfit patients with chronic lymphocytic leukemia. Advances in therapy. 2016;33:1814-30.
60. Eichhorst B, Fink A-M, Bahlo J, Busch R, Kovacs G, Maurer C, et al. First-line chemoimmunotherapy with bendamustine and rituximab versus fludarabine, cyclophosphamide, and rituximab in patients with advanced chronic lymphocytic leukaemia (CLL10): an international, open-label, randomised, phase 3, non-inferiority trial. The lancet oncology. 2016;17(7):928-42.
61. Eichhorst B, Robak T, Montserrat E, Ghia P, Niemann C, Kater A, et al. Chronic lymphocytic leukaemia: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Annals of Oncology. 2021;32(1):23-33.
62. Alimu X, Zhang J, Pang N, Zhang R, Chen R, Zeng X, et al. Galectin-9 and myeloid-derived suppressor cell as prognostic indicators for chronic lymphocytic leukemia. Immunity, Inflammation and Disease. 2023;11(5):e853.
63. Ahmed HA, Nafady A, Ahmed EH, Hassan EEN, Soliman WGM, Elbadry MI, et al. CXC chemokine ligand 13 and galectin-9 plasma levels collaboratively provide prediction of disease activity and progression-free survival in chronic lymphocytic leukemia. Annals of Hematology. 2024;103(3):781-92.
64. Hallek M, Fischer K, Fingerle-Rowson G, Fink AM, Busch R, Mayer J, et al. Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. The Lancet. 2010;376(9747):1164-74.
65. Byrd JC, Furman RR, Coutre SE, Burger JA, Blum KA, Coleman M, et al. Three-year follow-up of treatment-naive and previously treated patients with CLL and SLL receiving single-agent ibrutinib. Blood, The Journal of the American Society of Hematology. 2015;125(16):2497-506.
66. Fidan K. Chronic lymphocytic leukemia. Journal of Current Hematology & Oncology Research. 2023;1(3):59-67.

Study Protocol Systematic Review

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The impact of immune checkpoint inhibitors in patients with chronic lymphocytic leukemia (CLL)

A protocol for a systematic review and meta-analysis of randomized controlled trials

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Abstract

Introduction: The global burden of chronic lymphocytic leukemia (CLL) has constantly increased over the years, with a current incidence of 3.5 cases per 100,000 people. Although the conventional drugs used to treat CLL patients have been effective treatment failure rate in some of the patients is alarming. Therefore, as a result, novel treatment strategies with improved outcomes such as the blockade of immune checkpoints have emerged. However, consensus on the risk-benefit effects of the using these drugs in patients with CLL is controversial and has not been comprehensively evaluated. This systemic review and meta-analysis provide a comprehensive synthesis of available data assessing adverse events associated with the use of immune checkpoint inhibitors in patients with CLL as well as their influence on the overall survival rate.

Methods: This protocol for a systematic review and meta-analysis has been prepared in accordance with Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols 2015 guidelines. A search strategy will be developed using medical subject headings words in PubMed search engine with MEDLINE database. The search terms will also be adapted for gray literature, Embase, and Cochrane Central Register of Controlled Trials electronic databases. Two reviewers (AN and SRN) will independently screen studies, with a third reviewer consulted in cases of disagreements using a defined inclusion and exclusion criteria. Data items will be extracted using a predefined data extraction sheet. Moreover, the risk of bias and quality of the included studies will be appraised using the Downs and Black checklist and the quality and strengths of evidence across selected studies will be assessed using the Grading of Recommendations Assessment Development and Evaluation approach. The Cochran's Q statistic and the I^2 statistics will be used to analyze statistical heterogeneity across studies. If the included studies show substantial level of statistical heterogeneity ($I^2 > 50\%$), a random-effects meta-analysis will be performed using R statistical software.

Ethics and dissemination: The review and meta-analysis will not require ethical approval and the findings will be published in peer-reviewed journals and presented at local and international conferences. This review may help provide clarity on the risk-benefit effects of using immune checkpoint inhibitors in patients with CLL.

Systematic review registration: International prospective Register of Systematic Reviews (PROSERO) number: CRD42020156926.

Abbreviations: CLL = chronic lymphocytic leukemia, CTLA-4 = cytotoxic T-lymphocyte-associated protein 4, LAG-3 = lymphocyte-activation gene 3, PD-1 = programmed death-1, PD-L1 = programmed death-ligand 1, PRISMA-P = Preferred

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2.2 Systematic Review and Meta-analysis Protocol

The impact of immune checkpoint inhibitors in patients with chronic lymphocytic leukemia (CLL): A protocol for a Systematic Review and Meta-Analysis of Randomised Controlled Trials

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Author Contributions

Conceptualization, A.N., B.B.N., P.V.D.; Methodology, A.N., B.B.N., T.M.N., S.R.N.; Investigation, A.N., B.B.N., P.V.D.; Data Curation, A.N., B.B.N.; Writing – Original Draft Preparation, A.N.; Writing – Review & Editing, A.N., B.B.N., P.V.D., T.M.N.; Supervision, B.B.N., P.V.D.; Project Administration, A.N.

Abstract

Background

The global burden of chronic lymphocytic leukemia (CLL) has constantly increased over the years, with a current incidence of 3.5 cases per 100 000 people. Although the conventional drugs used to treat CLL patients have been effective treatment failure rate in some of the patients is alarming. Therefore, as a result, novel treatment strategies with improved outcomes such as the blockade of immune checkpoints have emerged. However, consensus on the risk-benefit effects of the using these drugs in patients with CLL is controversial and has not been comprehensively evaluated. This systemic review and meta-analysis provide a comprehensive synthesis of available data assessing adverse events associated with the use of immune checkpoint inhibitors in patients with CLL as well as their influence on the overall survival rate.

Methods

This protocol for a systematic review and meta-analysis has been prepared in accordance with Preferred Reporting

Items for Systematic Review and Meta-Analysis Protocols 2015 guidelines. A search strategy will be developed using medical subject headings words in PubMed search engine with MEDLINE database. The search terms will also be adapted for gray literature, Embase, and Cochrane Central Register of Controlled Trials electronic databases. Two reviewers (AN and SRN) will independently screen studies, with a third reviewer consulted in cases of disagreements using a defined inclusion and exclusion criteria. Data items will be extracted using a predefined data extraction sheet. Moreover, the risk of bias and quality of the included studies will be appraised using the Downs and Black checklist and the quality and strengths of evidence across selected studies will be assessed using the Grading of Recommendations Assessment Development and Evaluation approach. The Cochran's Q statistic and the I^2 statistics will be used to analyze statistical heterogeneity across studies. If the included studies show substantial level of statistical heterogeneity (I^2> 50%), a random-effects meta-analysis will be performed using R statistical software.

Ethics and dissemination

The review and meta-analysis will not require ethical approval and the findings will be published in peer-reviewed journals and presented at local and international conferences. This review may help provide clarity on the risk-benefit effects of using immune checkpoint inhibitors use in patients with CLL.

Keywords: Adverse events; chronic lymphocytic leukemia, immune checkpoint inhibitors

Introduction

The global incidence of leukemia has significantly increased over the years, with chronic lymphocytic leukemia (CLL) cases outpacing all myeloid and other lymphoid malignancies (1). Although the exact etiology is still not known, age, lifestyle and environmental factors have been identified as some of the major consequences implicated in the development of CLL (2, 3). To date, it is well-established that CLL is the most common type of leukemia, accounting for approximately 37% of all cases of blood malignancies (4), with an average global prevalence of about 3.5 cases per 100 000 people (5). In Africa, statistics on the incidence of CLL is very limited with isolated studies reporting on this form of leukemia (6-11). Nonetheless, various therapeutic drugs including those that modulate the function of immune checkpoints receptors are continuously being developed and their effectiveness tested in the management of patients with CLL worldwide (12, 13).

Immune checkpoints regulate immune function and play a crucial role in preventing autoimmunity.(14-16). However, in CLL, the signaling of immune checkpoints receptor is dysregulated which results in immune dysfunction (17, 18). Briefly, CLL is a monoclonal disorder that is characterized by the accumulation of functionally incompetent B-cells with a distinctive $CD19^{+}$, $CD20^{+}$, $CD5^{+}$, $CD23^{+}$ lymphocyte surface markers and surface immunoglobulin-positive phenotype in the peripheral blood, bone marrow and lymph nodes (19, 20). Hence, anti-CD20 monoclonal based drugs such as rituximab and ofatumumab are used as standard treatment for CLL (12, 21). However, these drugs are associated with severe adverse events such as neutropenia and thrombocytopenia (22-24), with others reporting on their ineffective use as monotherapy (25). Thus, the need to urgently broaden our understanding of the pathophysiological mechanisms implicated in the aggravation of CLL.

Although CLL is a B-cell malignancy, recent studies have also described the involvement of T-cells in the pathogenesis and progression of the disease (26-28). In fact, T-cell exhaustion mediated by an upregulation of co-inhibitory receptors such as programmed death-1 (PD-1), lymphocyte-activation gene 3 (LAG-3), T-cell immunoglobulin-3 (TIM-3) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) has been reported (17, 29). Consequently, this has led to the advancement of immune checkpoint inhibitors that targets both B and T-cell function as a treatment strategy for CLL (30). However, contradictory findings on the effects of using immune checkpoint inhibitors in CLL patients have been reported (13, 30-34). Thus, the exact effect of immune checkpoint inhibitors in CLL is contradictory and needs to be investigated further. As a result, due to high quality of evidence reported in randomized controlled trials (RCTs), this review will target such studies to assess and update available literature on the impact immune checkpoint inhibitors in CLL.

Research question

What are common adverse events associated with the use of immune checkpoint inhibitors in patients with CLL?

Objectives

1. To assess the adverse events associated with the use of immune checkpoint inhibitors in patients with CLL.
2. To estimate the overall survival rate of patient with CLL on immune checkpoints inhibitors.

Methods

This protocol was prepared in accordance with the Preferred Reporting Items for Systematic Review and Meta-analysis Protocols (PRISMA-P) 2015 guidelines (35). In addition, the protocol has been registered on PROSPERO, registration number: CRD42020156926.

Eligibility

Study design

This systematic review and meta-analysis will include RCTs with a clearly defined population and interventions used. While, observational studies, reviews, case studies, and animal studies will be excluded in this study.

Participants

Studies evaluating the use of immune checkpoint inhibitors as a treatment method in adult patients (≥18 years) with CLL, will be included.

Intervention

We will include studies reporting on the use of immune checkpoint inhibitors targeting PD-1, CTLA-4, LAG-3, and TIM-3 signaling as a therapeutic strategy for CLL.

Comparators

CLL patients on immune checkpoint inhibitor drugs that did not develop any associated adverse events.

Outcomes

The primary endpoints will include the following;

1. Adverse events that are associated with the use of immune checkpoint inhibitors. These include mortality, endocrinopathies, and dermatitis, autoimmune, gastrointestinal and hematological disorders.

Surrogate outcomes

1. Overall response, progression-free survival, and event-free remission.
2. Common severe adverse events as described by National Cancer Institute grading system (36).

Search strategy

The search strategy will be developed using medical subheading words on MEDLINE and will be adapted to gray literature, Embase, The Cochrane Central Register of Controlled Trials databases, and ClinicalTrials.gov with the help of an experienced librarian. The search strategy will consist of the following keywords and their respective synonyms; chronic lymphocytic leukemia, anti-PD-1 drugs (nivolumab, Pembrolizumab, Pidilizumab, Atezolizumab, Avelumab), anti-PD-L1 drugs (Atezolizumab, Avelumab, Durvalumab), anti-CTLA-4 drugs (Ipilimumab, Tremelimumab) anti-LAG-3 and anti-TIM-3 drugs and adverse events.

Study selection

The study screening and selection process will be carried out by 2 independent reviewers (AN and SRN) to eliminate risk of bias and inconsistencies with regards to reviewers' inclusion and exclusion of studies. Each reviewer will screen tittle, abstract, and full texts in contrast to the inclusion criteria. The exclusion criteria for title and abstract screening phase will include duplicate of the same study, reviews, observation studies, and studies that reported nonimmune checkpoint-related CLL therapeutic drugs. In cases of disagreements, a third reviewer (TMN) will be consulted for arbitration. The level of inter-rater agreement will be determined by using the Cohen's kappa inter-rater reliability (37). A kappa value of < 0.00 will be interpreted as a poor strength of agreement, 0.00–0.20 as slight agreement, 0.21–0.40 as fair agreement, 0.41–0.60 as moderate agreement, 0.61–0.80 as substantial agreement, and 0.81–1.00 as perfect agreement.

Data management

Data collection process

The reviewers (AN and SRN) will develop a data extraction form that will be used in the collection relevant data items. To reduce data entry errors, selected studies will be independently assessed by two reviewers (AN and TMN), the third reviewer (BBN) will be consulted for arbitration in case of any disagreements.

Data items

Extracted data items will include the author's name, year of publication, sample size, duration of follow-up, outcome measures, age, gender, immune checkpoint receptors targeted by the drugs, dosage, adverse events reported, and overall survival rate.

Data simplification

Studies will be grouped according to the type of immune checkpoint inhibitor used. In addition, studies will be grouped based on the immune checkpoint receptor targeted (PD-1, PD-L1, CTLA-4, LAG-3, TIM-3). Studies that report on immune checkpoint inhibitor combined with other conventional drugs will be pooled. The adverse events will be grouped and graded into grades 1 to 4 based on their severity. Group considered 1 and 2 are mild and moderate whilst groups 3, 4, and 5 are severe (36).

Risk of bias in individual studies

To evaluate the potential risk of bias in RCTs, Cochrane collaboration tool for assessing bias (38) and Downs and Black checklist (39) will be used. Two independent reviewers (AN and SRN) will appraise all included studies and a third reviewer (PVD) will be consulted in cases of disagreements.

Data synthesis

A summary of findings table will be used to provide a synthesis of the main outcomes of included studies. Moreover, if the included studies are homogeneous in terms of the type of immune checkpoint inhibitor used and participant characteristics, data will be analyzed with Rev Manager (Version 5.3) to conduct a meta-analysis. To measure statistical heterogeneity between studies, I^2 and Chi squared statistical tests will be used (40, 41). An I^2 value of > 50% will be considered substantial heterogeneity (42). To find the sources of heterogeneity within the included studies, a subgroup analysis and meta-regression comparing the study estimates from different study-level characteristics, quality, intervention type (type of immune checkpoint inhibitor), and the reported effect measure of adverse events will be conducted.

Cumulative evidence

The Grading of Recommendations, Assessment, Development and Evaluation (GRADE) assessment tool (43) will be used to assess the overall quality of evidence. Moreover, the quality of each included study will be independently evaluated by two authors (AN, SRN). The third author (TMN) will adjudicate in cases of disagreements. The quality of evidence will be assessed based on several factors such as study limitations, indirectness of results, and publication or reporting bias. The evidence of each outcome will be rated as high, moderate, low, or very low.

Discussion

Immune checkpoint inhibitors have been shown to be effective in the treatment of CLL, its association with adverse events is controversial (13, 30) and has not been critically assessed. Therefore, this systemic review and meta-analysis aims to evaluate the risk-benefit of using immune checkpoint inhibitors as a therapeutic strategy for patients with CLL. Findings from this study will give a better understanding on the effectiveness of immune checkpoint inhibitors as well as paving way for strategic development of effective therapies and management of patients with CLL.

Authors' contributions

AN, TMN, PVD, and BBN conceptualized, designed, and drafted this manuscript. All authors including SRN wrote and approved the final manuscript. BBN is the guarantor of the review.

References

1. Hao T, Li-Talley M, Buck A, Chen W. An emerging trend of rapid increase of leukemia but not all cancers in the aging population in the United States. Scientific reports. 2019;9(1):1-13.
2. Slager SL, Rabe KG, Achenbach SJ, Vachon CM, Goldin LR, Strom SS, et al. Genome-wide association study identifies a novel susceptibility locus at 6p21. 3 among familial CLL. Blood. 2011;117(6):1911-6.
3. Miranda-Filho A, Piñeros M, Ferlay J, Soerjomataram I, Monnereau A, Bray F. Epidemiological patterns of leukaemia in 184 countries: a population-based study. The Lancet Haematology. 2018;5(1):e14-e24.
4. DeSantis CE, Miller KD, Dale W, Mohile SG, Cohen HJ, Leach CR, et al. Cancer statistics for adults aged 85 years and older, 2019. CA: a cancer journal for clinicians. 2019.
5. Combest AJ. Overview of the Recent Developments in Chronic Lymphocytic Leukemia, Part 1. J Hematol Oncol Pharm. 2016;2:54-6.
6. Sall A, Toure AO, Sall FB, Ndour M, Fall S, Sene A, et al. Characteristics of chronic lymphocytic leukemia in Senegal. BMC hematology. 2016;16:10.
7. Koffi KG, Nanho DC, Tolo A, N'Dathz E, Kouakou B, Meite N, et al. [Chronic lymphocytic leukemia in Subsaharian Africa: clinical outcome experience of Cote d'Ivoire]. Bulletin du cancer. 2009;96(9):901-6.
8. Malam-Abdou B, Brah S, Djibrilla A, Andia A, Chefou M, Sani MM, et al. Leucémie Lymphoïde Chronique au Niger: une Étude de 99 cas au Service d'Onco-Hématologie de l'Hôpital National de Niamey. HEALTH SCIENCES AND DISEASES. 2018;19(2).
9. Omoti C, Awodu O, Bazuaye G. Chronic lymphoid leukaemia: clinico-haematological correlation and outcome in a single institution in Niger Delta region of Nigeria. International journal of laboratory hematology. 2007;29(6):426-32.
10. Salawu L, Bolarinwa R, Durosinmi M. Chronic lymphocytic leukaemia: a-twenty-years experience and problems in Ile-Ife, South-Western Nigeria. African health sciences. 2010;10(2).
11. Dieye TN, Ndiaye FS, Diop S, Mathiot C, Gattiolat C-H, May E, et al. Characteristics Of Chronic Lymphocytic Leukemia (CLL) In Senegal. Clinical Features, Cytology, Immunophenotype, Cytogenetic Abnormalities and Altered Expression Of Micro-RNA. Am Soc Hematology; 2013.
12. Hallek M. Chronic lymphocytic leukemia: 2017 update on diagnosis, risk stratification, and treatment. American journal of hematology. 2017;92(9):946-65.
13. Jain N, Basu S, Thompson PA, Ohanian M, Ferrajoli A, Pemmaraju N, et al. Nivolumab Combined with Ibrutinib for CLL and Richter Transformation: A Phase II Trial. Blood cells, molecules & diseases. 2016:128.
14. Hogg SJ, Vervoort SJ, Deswal S, Ott CJ, Li J, Cluse LA, et al. BET-Bromodomain Inhibitors Engage the Host Immune System and Regulate Expression of the Immune Checkpoint Ligand PD-L1. Cell reports. 2017;18(9):2162-74.
15. Joller N, Peters A, Anderson AC, Kuchroo VK. Immune checkpoints in central nervous system autoimmunity. Immunological reviews. 2012;248(1):122-39.
16. Dyck L, Mills KH. Immune checkpoints and their inhibition in cancer and infectious diseases. European journal of immunology. 2017;47(5):765-79.
17. Palma M, Gentilcore G, Heimersson K, Mozaffari F, Nasman-Glaser B, Young E, et al. T cells in chronic lymphocytic leukemia display dysregulated expression of immune checkpoints and activation markers. Haematologica. 2017;102(3):562-72.
18. Yi JS, Cox MA, Zajac AJ. T-cell exhaustion: characteristics, causes and conversion. Immunology. 2010;129(4):474-81.
19. Awan FT, Byrd JC. Chronic lymphocytic leukemia. Abeloff's Clinical Oncology: Elsevier; 2019. p. 1850-71. e5.
20. Lanasa MC. Novel insights into the biology of CLL. ASH Education Program Book. 2010;2010(1):70-6.

21. Coiffier B, Lepretre S, Pedersen LM, Gadeberg O, Fredriksen H, van Oers MH, et al. Safety and efficacy of ofatumumab, a fully human monoclonal anti-CD20 antibody, in patients with relapsed or refractory B-cell chronic lymphocytic leukemia: a phase 1-2 study. Blood. 2008;111(3):1094-100.
22. Cartron G, de Guibert S, Dilhuydy MS, Morschhauser F, Leblond V, Dupuis J, et al. Obinutuzumab (GA101) in relapsed/refractory chronic lymphocytic leukemia: final data from the phase 1/2 GAUGUIN study. Blood. 2014;124(14):2196-202.
23. Hillmen P, Robak T, Janssens A, Babu KG, Kloczko J, Grosicki S, et al. Chlorambucil plus ofatumumab versus chlorambucil alone in previously untreated patients with chronic lymphocytic leukaemia (COMPLEMENT 1): a randomised, multicentre, open-label phase 3 trial. Lancet (London, England). 2015;385(9980):1873-83.
24. Zaja F, Vianelli N, Sperotto A, Patriarca F, Tani M, Marin L, et al. Anti-CD20 therapy for chronic lymphocytic leukemia-associated autoimmune diseases. Leukemia & lymphoma. 2003;44(11):1951-5.
25. Huhn D, von Schilling C, Wilhelm M, Ho AD, Hallek M, Kuse R, et al. Rituximab therapy of patients with B-cell chronic lymphocytic leukemia. Blood. 2001;98(5):1326-31.
26. Gassner FJ, Zaborsky N, Catakovic K, Rebhandl S, Huemer M, Egle A, et al. Chronic lymphocytic leukaemia induces an exhausted T cell phenotype in the TCL 1 transgenic mouse model. British journal of haematology. 2015;170(4):515-22.
27. Lad D, Hoeppli R, Huang Q, Garcia R, Xu L, Toze C, et al. Regulatory T-cells drive immune dysfunction in CLL. Leukemia & lymphoma. 2018;59(2):486-9.
28. De Matteis S, Molinari C, Abbati G, Rossi T, Napolitano R, Ghetti M, et al. Immunosuppressive Treg cells acquire the phenotype of effector-T cells in chronic lymphocytic leukemia patients. Journal of translational medicine. 2018;16(1):172.
29. Riches JC, Davies JK, McClanahan F, Fatah R, Iqbal S, Agrawal S, et al. T cells from CLL patients exhibit features of T-cell exhaustion but retain capacity for cytokine production. Blood. 2013;121(9):1612-21.
30. Younes A, Brody J, Carpio C, Lopez-Guillermo A, Ben-Yehuda D, Ferhanoglu B, et al. Safety and activity of ibrutinib in combination with nivolumab in patients with relapsed non-Hodgkin lymphoma or chronic lymphocytic leukaemia: a phase 1/2a study. The Lancet Haematology. 2019;6(2):e67-e78.
31. Ding W, LaPlant BR, Call TG, Parikh SA, Leis JF, He R, et al. Pembrolizumab in patients with CLL and Richter transformation or with relapsed CLL. Blood. 2017;129(26):3419-27.
32. Arenbergerova M, Fialova A, Arenberger P, Gkalpakiotis S, Jirasek T, Srp A, et al. Killing two birds with one stone: response to pembrolizumab in a patient with metastatic melanoma and B-cell chronic lymphocytic leukaemia. Journal of the European Academy of Dermatology and Venereology : JEADV. 2018;32(2):e72-e4.
33. Mato AR, Svoboda J, Prak ETL, Schuster SJ, Tsao P, Dorsey C, et al. Phase I/II study of Umbralisib (TGR-1202) in combination with Ublituximab (TG-1101) and Pembrolizumab in patients with relapsed/refractory CLL and Richter's transformation. Am Soc Hematology; 2018.
34. Archibald WJ, Meacham PJ, Williams AM, Baran AM, Victor AI, Barr PM, et al. Management of melanoma in patients with chronic lymphocytic leukemia. Leukemia research. 2018;71:43-6.
35. Moher D, Shamseer L, Clarke M, Ghersi D, Liberati A, Petticrew M, et al. Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement. Systematic reviews. 2015;4:1.
36. Health UDo, Services H. Common terminology criteria for adverse events v 4.0 (CTCAE). National Institutes of Health, National Cancer Care Institute. 2010.
37. Landis JR, Koch GG. The measurement of observer agreement for categorical data. biometrics. 1977:159-74.
38. Higgins JP, Altman DG, Gotzsche PC, Juni P, Moher D, Oxman AD, et al. The Cochrane Collaboration's tool for assessing risk of bias in randomised trials. BMJ (Clinical research ed). 2011;343:d5928.

39. Downs SH, Black N. The feasibility of creating a checklist for the assessment of the methodological quality both of randomised and non-randomised studies of health care interventions. Journal of epidemiology and community health. 1998;52(6):377-84.
40. Cochran WG. The Combination of Estimates from Different Experiments. Int Biometric Soc 1954.
41. Higgins JP, Thompson SG. Quantifying heterogeneity in a meta-analysis. Stat Med. 2002;21(11):1539-58.
42. Schroll JB, Moustgaard R, Gotzsche PC. Dealing with substantial heterogeneity in Cochrane reviews. Cross-sectional study. BMC medical research methodology. 2011;11:22.
43. Balshem H, Helfand M, Schunemann HJ, Oxman AD, Kunz R, Brozek J, et al. GRADE guidelines: 3. Rating the quality of evidence. Journal of clinical epidemiology. 2011;64(4):401-6.

2.3 Systematic review

Effectiveness of chemoimmunotherapy and the associated major severe adverse events in adult patients with chronic lymphocytic leukemia: A systematic review of randomized controlled trials

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Conceptualization, A.N., B.B.N.; Methodology, A.N., B.B.N., P.V.D.; Formal Analysis, A.N., B.B.N.; Investigation, A.N., B.B.N., P.V.D.; Data Curation, A.N., B.B.N.; Writing – Original Draft Preparation, A.N.; Writing – Review & Editing, A.N., B.B.N., P.V.D.; Visualization, A.N., B.B.N., P.V.D.; Supervision, B.B.N., P.V.D.; Project Administration, A.N.

Abstract

Background

Immunotherapy has improved progression-free survival (PFS) and overall survival (OS) of patients with chronic lymphocytic leukaemia (CLL). It is often combined with conventional chemotherapy or targeted therapy, but the most effective combination with the fewest severe adverse events remains unclear. This systematic review assessed randomized controlled trials (RCTs) on the effectiveness and severe adverse events of chemoimmunotherapy in patients with CLL.

Methods

This systematic review was reported using the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) 2020 guidelines. Database searches were conducted using Elton B. Stephens Company Host (EBSCOhost) and PubMed from inception till until February 2024. The primary outcomes of this systematic review included major severe adverse events, (PFS and OS rates associated with the use of chemoimmunotherapy in patients with CLL. The risk of bias of the included studies was evaluated using a modified Downs and Black checklist.

Results

The systematic review included 14 studies with 7,005 patients (mean age 58, range 28-92). Findings suggest that targeted therapy combined with immunotherapy is more effective than chemoimmunotherapy in treatment-naïve and high-risk CLL patients. Chemoimmunotherapy had higher incidents of severe adverse events, such as infections, neutropenia, thrombocytopenia, and leukopenia, compared to targeted therapy with immunotherapy.

Conclusions

The cumulative evidence presented suggest that chemoimmunotherapy is associated with major severe adverse events such as infections, neutropenia, thrombocytopenia and leukopenia. Moreover, targeted therapy combined with immunotherapy is more effective in treatment-naïve patients with CLL or high-risk CLL compared to chemoimmunotherapy.

Keywords: Adverse events; chronic lymphocytic leukemia, chemoimmunotherapy, immunotherapy, monoclonal antibodies, progression-free survival.

1. Background

Chronic lymphocytic leukemia (CLL) is a lymphoproliferative monoclonal disease that is characterized by an increase of functionally incompetent B lymphocytes (1). The global prevalence of CLL is approximately 3.5 cases per 100 000 people (2). Due to the limited number of studies reporting on CLL in low-and middle income countries (3, 4), the incidence of CLL remains poorly reported. In high-income countries the incidence of CLL is estimated to be 4–6/100 000 people per year and accounts for approximately 37% of all cases of leukemia (5, 6).

Chemoimmunotherapy in the form of combination of fludarabine with cyclophosphamide and rituximab (FCR) is a first-line treatment for young and physically fit patients with CLL (7, 8). FCR treatment improves the progression-free survival and overall survival of patients with unmutated immunoglobulin heavy chain variable region (IGHV) gene, del(17p) or TP53 mutation, and elevated beta-2 microglobulin levels (7, 9). However, majority of patients with CLL are not eligible for FCR as they are elderly and often have other comorbidities (10). Moreover, six-cycles of FCR treatment induce grade C myelosuppression and infections in patients with CLL (11). In a subset of patients with CLL, the combination of chlorambucil with obinutuzumab, or ofatumumab and rituximab improves the overall response (OR) and overall survival (OS) rates (12-14). Obinutuzumab and ofatumumab improves the progression-free survival (PFS) rate of CD20-positive, unmutated IGHV gene, del(17p) or TP53 mutation patients with CLL (15-18). Notably, anti-CD20 monoclonal therapy depletes mature B cells (19, 20) and has been associated with the occurrence of severe adverse events such as neutropenia and thrombocytopenia (12, 21-23). Several clinical trials have reported on the ineffectiveness of anti-CD20 based drugs when used as monotherapy in previously untreated patients with CLL (18, 24). In fact, in the Chlorambucil plus ofatumumab versus chlorambucil alone in previously untreated patients with chronic lymphocytic leukaemia (COMPLEMENT 1) trial, the effectiveness of immunotherapy drugs was observed when used in combination on previously untreated patients with CLL with del(17p) mutation (12).

To date, systematic reviews on the effectiveness and safety of immunotherapy combinations have been conducted in patients with CLL (25, 26). In fact, first line treatment with targeted therapy, Bruton's

tyrosine kinase (BTK) inhibitor (ibrutinib), given either alone or in combination with rituximab or obinutuzumab improves PFS when compared to conventional chemotherapy in high-risk, 11q deletion, unmutated IGHV patients with CLL (26). The aim of this systematic review was to provide a synthesis of evidence from randomized controlled trials (RCTs) reporting on the effectiveness of chemoimmunotherapy and the associated major severe adverse events in patients with CLL.

2. Methods

This systematic review was prepared according to the Preferred Reporting Items for Systematic Review and Meta-analysis (PRISMA) 2020 guidelines (27). The checklist is attached as Supplementary file 2.3.1. The protocol was registered in the international Prospective Register of a Systematic Review (PROSPERO), registration number: CRD42020156926, and has already been published (28).

Objectives

1. To assess major severe adverse events associated with the use of chemoimmunotherapy in patients with CLL.
2. To estimate the effectiveness of chemoimmunotherapy by determining PFS and OS of patients with CLL.

Review questions

The systematic review was conducted to answer the following questions:

1. Is chemoimmunotherapy more effective in patients with CLL?
2. What are the major severe adverse events associated with the use of chemoimmunotherapy in patients with CLL?

2.1. Search strategy

The search strategy was developed in PubMed and EBSCOhost platform without language restrictions. The search was conducted using medical subheading (MeSH) words on MEDLINE and adapted grey literature and Clinicaltrials.gov. The search terms were also adapted on the EBSCOhost platform with Cumulative Index to Nursing and Allied Health Literature (CINAHL), Academic Search Complete, Health Source: Nursing/Academic Edition databases from inception up to February 2024 and was

restricted to randomised controlled trials for study inclusion. The search strategy was conducted by two independent researchers and consisted of the following keywords and their respective synonyms: “chronic lymphocytic leukemia”, "rituximab", "obinutuzumab", "ofatumumab", "alemtuzumab", "bevacizumab", "zanubrutinib", "acalabrutinib", "ibrutinib", "navitoclax" and "venetoclax" (supplementary file 2.3.2).

2.2. Inclusion and exclusion criteria

This systematic review only included open-label, phase II or III RCTs reporting on the immunotherapy in patients with CLL. Reviews, preclinical studies, and observational studies were excluded, but screened for relevant primary studies.

Participants

Adult patients with CLL.

Intervention

chemoimmunotherapy: fludarabine plus cyclophosphamide and rituximab; bendamustine and rituximab; chlorambucil and obinutuzumab; chlorambucil and ofatumumab; fludarabine cyclophosphamide and alemtuzumab.

Comparator

1. Patients with CLL receiving chemoimmunotherapy vs. targeted therapy combined with immunotherapy (ibrutinib or zanubrutinib or acalabrutinib or navitoclax or venetoclax combined with rituximab or obinutuzumab or ofatumumab or alemtuzumab or bevacizumab).
2. Patients with CLL receiving chemoimmunotherapy vs. conventional chemotherapy.

Outcome

1. ≥2-year Progression-Free Survival (PFS) and overall survival (OS).
2. Major severe adverse events: defined according to National Cancer Institute grading system (29).

2.3. Data items and extraction

Two independent reviewers (AN and PVD) extracted relevant data items including author details, the country where the study was conducted, year of publication, average age of patients, sample size, gender, type of intervention, and main findings of each study. The Mendeley Reference Manager Version (1.19.4) software was used to identify and remove duplicates as well as to manage extracted information.

2.4. Quality assessment and risk of bias

Two independent reviewers (AN and PVD) evaluated the risk of bias using the modified Downs and Black checklist (30). The tool assesses the risk of bias within the following domains; reporting bias, external validity, internal validity, and selection bias. A third reviewer (BBN) was consulted for arbitration. Cohen's kappa inter-rater reliability was used to assess the level of inter-rater agreement (31). A kappa value of 0 or less was regarded as a poor strength of agreement, while 0.00 - 0.20 was regarded as slight agreement, 0.21 - 0.40 as fair agreement, 0.41 - 0.60 as moderate agreement, 0.61 - 0.80 as substantial agreement and 0.81 - 1.00 as perfect agreement.

2.5. Certainty of evidence

The quality of the cumulative evidence was evaluated using the grading of recommendations assessment, development, and evaluation (GRADE) tool (32). The GRADE tool evaluates the quality of evidence using the following domains; consistency, directness, precision, and publication bias. Based on these domains, the level of certainty of the evidence for each outcome was rated as low, moderate, and high (Table 2.3.4-2.3.5).

3. Results

3.1. Selected studies

A total number of 2 248 studies were retrieved through the Academic search complete, CINAHL, MEDLINE, and ClinicalTrials.gov databases. After screening, a total of 14 studies were eligible for inclusion (Figure 2.3.1). The included RCTs reported on the effectiveness of chemoimmunotherapy and the associated major adverse events in patients with CLL. Among the excluded studies, 814 records were duplicates, 1 376 studies were not the population of interest, 8 were not RCTs, 30 studies were not the intervention of interest, 2 studies were reviews, 4 were trial protocols.

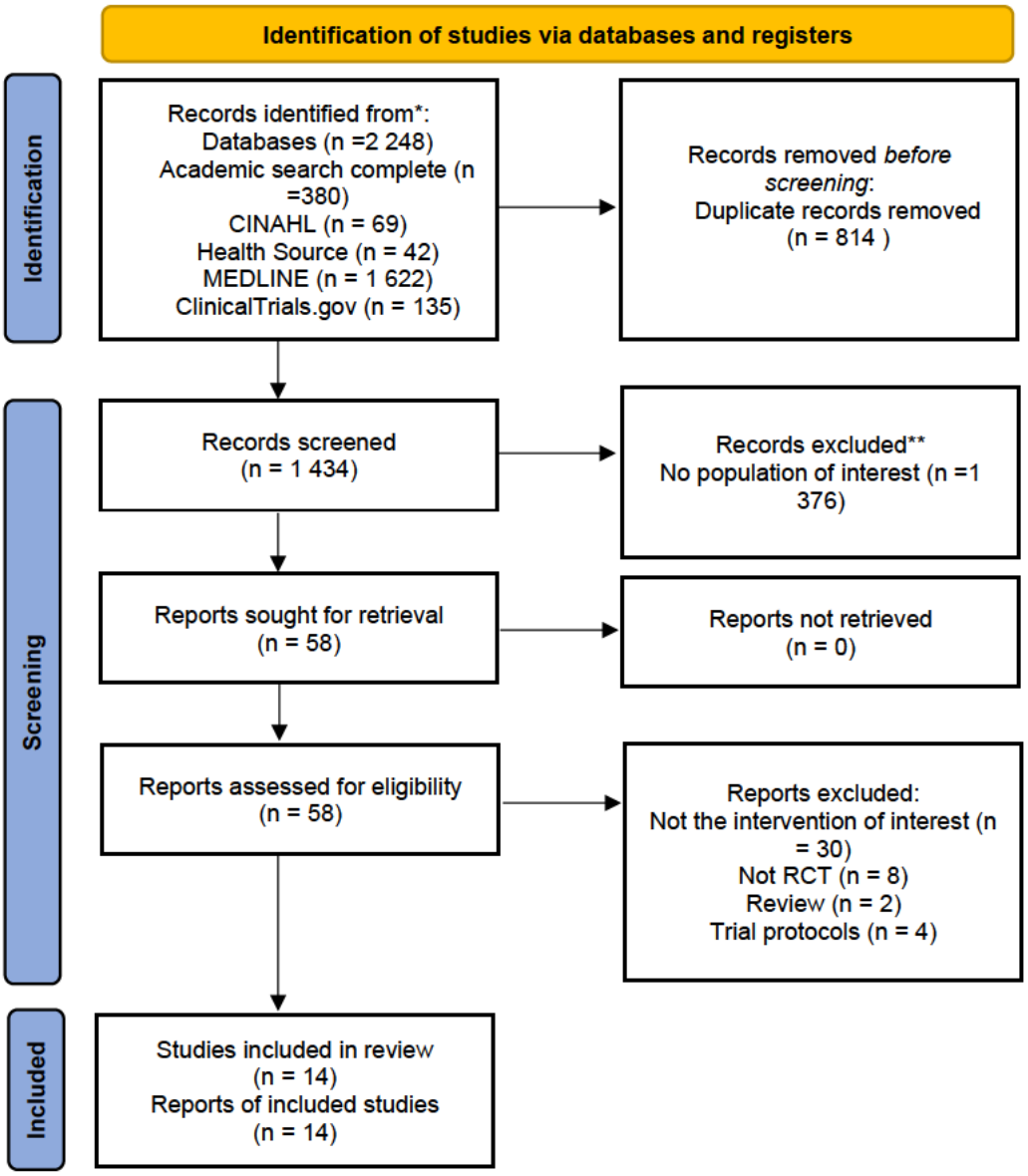

Figure 2.3.1. PRISMA diagram. Initial screening on major databases retrieved 2 248 studies, of which 14 were included on the review. Excluded studies included duplicates, studies not reporting population of interest, studies that are not randomized controlled trials, studies not reporting intervention of interest, reviews, and trial protocols. CINAHL (Cumulated Index to Nursing and Allied Health Literature), MEDLINE (Medical Literature Analysis and Retrieval System Online), RCT (randomized controlled trial).

3.2. Characteristics of the included studies

The included studies were published between 2010 and 2023. The systematic review studies comprised a total of 7 005 patients with a pooled mean age of 58±8.7 years, with 1:1.9 female to male ratio. The included studies were conducted either in the United States (21.4%, n=3), Germany (42.9%, n=6), United Kingdom (14.3%, n=2), Australia (7.1%), n=1), Spain (7.1%, n=1), and Denmark (7.1%, n=1). The included studies reported on the effectiveness of chemoimmunotherapy and the associated adverse events in patients with CLL (Table 2.3.1-2.3.2).

3.3. Risk of bias assessment

The Downs and Black checklist was used to assess the risk of bias in the included studies. Overall all the included studies (n=14) had low reporting bias with a median score of 9 (8-10) out of the possible score of 10 (overall interrater agreement was 96.7% with kappa = 0.94), fair for external validity with a median score of 1 (1-2) out of possible 3 (overall interrater agreement was 84.85%, kappa= 0.70), good for internal validity domain with a median score of 5 (3-6) out of possible 7 (overall interrater agreement was 84.85%, kappa= 0.70) and good for selection bias with a median score of 4 (3-5) out of possible 6 (overall interrater agreement was 51.52%, kappa= 0.03) (supplementary file 3). Cohen's kappa was used to assess interrater reliability per domain.

3.4. The effectiveness chemoimmunotherapy and the associated major severe adverse events compared to targeted therapy combined with immunotherapy in patients with CLL

Targeted therapy combined with immunotherapy, such as rituximab and ibrutinib have been tested for their effectiveness to improve the 5-year PFS and OS in treatment-naïve patients with CLL (33). Even though mono-immunotherapy such as obinutuzumab, has demonstrated improved overall response rate (ORR) in treatment-naïve patients with CLL (18), targeted combined with immunotherapy uses the synergistic potential of several pathways, resulting to improved PFS and OS (15, 34). Within the current reporting, a total of eight RCTs reported on the effectiveness chemoimmunotherapy and the associated major severe adverse events compared to targeted combined with immunotherapy in patients with CLL (Table 2.3.1). Notably, majority of the studies (62.5%, n=5) reported on patients with CLL with less

burden of coexisting conditions (≤6 score on the Cumulative Illness Rating Scale) (33-37). Only one study reported on patients with relapsed or refractory CLL (38). Three studies compared chemoimmunotherapy of rituximab and bendamustine with targeted combined with immunotherapy (34, 37, 38) while another 3 studies compared chemoimmunotherapy of FCR (33-35), and another three studies compared chemoimmunotherapy of obinutuzumab and chlorambucil (15, 16, 36).

Most of these studies demonstrated improved 2-year PFS and OS in treatment-naïve patients with CLL receiving targeted combined with immunotherapy compared to chemoimmunotherapy (Table 2.3.1). Subgroup analysis showed that patients with high-risk CLL, defined by unmutated immunoglobulin heavy chain (IGHV), deletion of 17p or 11q or 13q or mutated TP53 or high beta-2 microglobulin (B2M) benefited more from targeted combined with immunotherapy compared to chemoimmunotherapy. Ibrutinib and rituximab was more effective (improved PFS) than FCR in patients with high-risk CLL (33, 35). Moreover, venetoclax and rituximab was more effective (improved PFS and OS) in patients with relapsed or refractory CLL and high-risk CLL compared to chemoimmunotherapy of bendamustine and rituximab (38). Chemoimmunotherapy was associated with high incidents of major severe adverse events, particularly infections, neutropenia, thrombocytopenia and leukopenia compared to targeted and immunotherapeutic combination (Table 2.3.1 and 2.3.3).

Table 2.3.1: An overview of included studies reporting on the effectiveness chemoimmunotherapy and the associated adverse events compared to targeted therapy combined with immunotherapy in patients with chronic lymphocytic leukemia (CLL).

Author, Year	Country	Participants	Patient stratification and trial phase	Intervention	Main findings
Eichhorst et al., 2023	Germany	Treatment-naïve, medical fit patients with CLL and low burden of coexisting conditions (≤6 score on the Cumulative Illness Rating Scale) and Without del(17p) or TP53 mutation (n =926), with a mean age 61±14.3 (Male: 72%)	Binet stage A/B (37.7%) and C (35.7%). Patients without del(13q) (44.6%). Patients with unmutated IGHV gene (56.1%). Patients with elevated beta-2 microglobulin (63.8%) Phase III	Received targeted and immunotherapeutic combination of venetoclax (0.4 g) and rituximab (0.5 g/m^2) or venetoclax (0.4 g) and obinutuzumab (1 g) or venetoclax (0.4 g), obinutuzumab (1 g) and ibrutinib (0.420 g); in comparison to chemoimmunotherapy of fludarabine (0.025 g/m^2) cyclophosphamide (0.250 g/m^2) and rituximab (0.5 g/m^2) (FCR) or rituximab (0.5 g/m^2) and bendamustine (0.090 g/m^2). Patients were monitored for 5 years	Targeted and immunotherapeutic combination in the form of venetoclax–obinutuzumab–ibrutinib (90.5% 3-year PFS and 98.4% 3-year OS) or venetoclax–obinutuzumab (87.7% 3-year PFS and 94.2% 3-year OS) was more effective in patients with CLL in comparison to chemoimmunotherapy which showed lower efficacy (75.5% 3-year PFS and 87.4% 3-year OS). Patients with unmutated IGHV gene receiving venetoclax–obinutuzumab–ibrutinib (86.6% 3-year PFS) or venetoclax–obinutuzumab (82.9% 3-year PFS) or venetoclax–rituximab (76.4% 3-year PFS) had an improved PFS compared with those on chemoimmunotherapy (65.5% 3-year PFS). However, treatment groups had comparable OS. Major severe adverse events in a form of infections were more common in chemoimmunotherapy group (18.5%) compared to targeted and immunotherapeutic combination group (21.2%, 10.5% and 13.2%, respectively).
Hillmen et al., 2023	United Kingdom	Treatment-naïve, medical fit patients with CLL and no coexisting conditions (n =771), with mean age of 62.3±8.1 (Male: 73%)	Binet stage A/B (55%) and C (45%). Patients without del(13q) (35%). Patients with unmutated IGHV gene (50%). Phase III	Received targeted and immunotherapeutic combination of ibrutinib (0.420 g) and rituximab (0.5 g/m^2) in comparison to chemoimmunotherapy of fludarabine (0.024 g/m^2) cyclophosphamide (0.150 g/m^2) and rituximab (0.5 g/m^2) (FCR). Patients were monitored for 5 years	Targeted and immunotherapeutic combination was more effective in patients with CLL (85.6% 4-year PFS), while inducing 8% deaths in comparison to chemoimmunotherapy which showed lower efficacy (73.0% 4-year PFS) and 8% deaths. The overall survival was similar in both treatment groups (92.1 vs 93.5% 4-year OS). Patients with unmutated IGHV gene receiving targeted and immunotherapeutic combination had an improved PFS compared with those on chemoimmunotherapy (HR:0.41). Moreover, chemoimmunotherapy group had high incidents of major severe adverse event, leukopenia (54%) compared to targeted and immunotherapeutic combination group (14%).
Shanafelt et al., 2022	United States	Treatment-naïve patients with CLL, and no coexisting conditions (n =529), with mean age of 56.7±7.5 (Male: 67.3%)	Rai stage III or IV (43.1%). Patients with del13q (33.8%) Patients with unmutated IGHV (71.1%). Phase III	Received targeted and immunotherapeutic combination of ibrutinib (0.420 g) and rituximab (0.5 g/m^2) in comparison to chemoimmunotherapy of fludarabine (0.025 g/m^2) cyclophosphamide (0.250 g/m^2) and rituximab (0.5 g/m^2) (FCR). Patients were monitored for 6 years	Targeted and immunotherapeutic combination was more effective in patients with CLL (78% 5-year PFS and 95% 5-year OS), while also inducing 3.7% deaths in comparison to chemoimmunotherapy which showed lower efficacy (51% 5-year PFS and 89% 5-year OS) and 3.6% death rate. Patients with unmutated IGHV gene receiving targeted and immunotherapeutic combination had an improved PFS (75% 5-year PFS) compared with those receiving chemoimmunotherapy (33% 5-year PFS). Moreover, chemoimmunotherapy group had high incidents of major severe adverse events, neutropenia (45.6% vs. 28.4%) and leukopenia (40.5% vs. 6.5%).

Al-Sawaf et al., 2020	Germany	Treatment-naïve, medical unfit patients with CLL, and coexisting conditions (>6 score on the Cumulative Illness Rating Scale), (n =432), with mean age of 71.6±8.1 (Male: 66.9%)	Binet stage B and C (79%). Patients with unmutated IGHV gene (56.5%). Patients with elevated beta-2 microglobulin levels (94.7%). Phase III.	Received targeted and immunotherapeutic combination of venetoclax (0.4 g) and obinutuzumab (1 g) in comparison to chemoimmunotherapy of chlorambucil (0.0005 g/kg) and obinutuzumab (1 g). Patients were monitored for 3 years	Targeted and immunotherapeutic combination was more effective in patients with CLL and coexisting conditions (81.9% 3-year PFS), while also inducing 1% deaths in comparison to chemoimmunotherapy which showed lower efficacy (49.5% 3-year PFS) and 1% death rate. The overall survival was similar in both treatment groups. In addition, the major severe adverse event, neutropenia was comparable in both groups (53% vs. 48%).
Sharman et al., 2020	United States	Treatment-naïve with CLL and low burden of coexisting conditions (≤6 score on the Cumulative Illness Rating Scale), (n =356), with mean age of 70.6±7.3 (Male: 61.0%)	Rai stage III or IV (23%). Patients with unmutated IGHV (63%). Patients with del(17)(p13.1) (9%). Patients with del(11)(q22.3) (18%). Patients with TP53 (11%). Phase III.	Received targeted and immunotherapeutic combination of acalabrutinib (0.1 g) and obinutuzumab (1 g) in comparison to chemoimmunotherapy of chlorambucil (0.0005 g/kg) and obinutuzumab (1 g). Patients were monitored for 5 years	Targeted and immunotherapeutic combination was more effective in patients with CLL (93% 2-year PFS and 95% 2-year OS), while also inducing 5% deaths in comparison to chemoimmunotherapy which showed lower efficacy (47% 2-year PFS and 92% 2-year OS) and 9% death rate. Patients with unmutated IGHV gene receiving targeted and immunotherapeutic combination had an improved PFS (91% 2-year PFS) compared with those on chemoimmunotherapy (76% 2-year PFS). Moreover, patients with del(17)(p13.1), del(11)(q22.3) and mutated TP53 receiving targeted and immunotherapeutic combination had an improved PFS (88%, 87%, 95%, respectively 2-year PFS) compared with those on chemoimmunotherapy (22%, 24%, 19%, respectively 2-year PFS). There were high incidents of major severe neutropenia (41% vs. 30%) in chemoimmunotherapy group and low incidence of infection (8% vs. 21%).
Moreno et al., 2019	Spain	Treatment-naïve with CLL and coexisting conditions (>6 score on the Cumulative Illness Rating Scale), (n =229), with mean age of 71±7.7 (Male: 63.8%)	Rai stage III or IV (52%). Patients with del17p, TP53 mutation, del11q, or unmutated IGHV (65%). Phase III	Received targeted and immunotherapeutic combination of ibrutinib (0.420 g) and obinutuzumab (1 g) in comparison to chemoimmunotherapy of chlorambucil (0.0005 g/kg) and obinutuzumab (1 g). Patients were monitored for 4 years	Targeted and immunotherapeutic combination was more effective in patients with CLL (79% 4-year PFS and 86% 2.5-year OS), while also inducing 1% deaths in comparison to chemoimmunotherapy which showed lower efficacy (36% 4-year PFS and 85% 2.5-year OS) and 1% death rate. Patients with del17p, del11q, TP53 mutations, or unmutated IGHV receiving targeted and immunotherapeutic combination had an improved PFS compared with those receiving chemoimmunotherapy (HR:0.15). Both treatments were comparable in terms of most common severe adverse event in the form of neutropenia (36.3 vs. 46.1%) and thrombocytopenia (18.6% vs. 10.4%).

Seymour et al., 2018	Australia	Patients with relapsed or refractory CLL (389), with mean age of 67±15.8 (Male: 73.8%)	Patients without del(17p) (26.9%). Patients with unmutated IGHV gene (68.3%). Patients with mutated TP53 status (26.3%). Phase III	Received targeted and immunotherapeutic combination of venetoclax (0.4 g) and rituximab (0.5 g/m^2) in comparison to chemoimmunotherapy of bendamustine (0.090 g/m^2) and rituximab (0.5 g/m^2). Patients were monitored for 4 years	Targeted and immunotherapeutic combination was more effective in patients with CLL (84.9% 2-year PFS and 91.9% 2-year OS), while inducing 5.2% deaths in comparison to chemoimmunotherapy which showed lower efficacy (36.3% 2-year PFS and 86.6% 2-year OS) and 5.9% deaths. Patients with chromosome 17p deletion receiving targeted and immunotherapeutic combination had an improved PFS (81.5% 2-year PFS) compared with those on chemoimmunotherapy (27.8% 2-year PFS). Moreover, targeted and immunotherapeutic combination group had high incidence of major severe adverse event, neutropenia (57.7%) compared to chemoimmunotherapy group (38.8%).
Woyach et al., 2018	United States	Treatment-naïve patients with CLL, and no coexisting conditions (n =365), with mean age of 73±5.3 (Male: 66.8%)	Patients with high-risk disease according to modified Rai stage (54%). Patients with del13q14.3 (36%) Patients with unmutated IGHV (61%). Phase III	Received targeted and immunotherapeutic combination of ibrutinib (0.420 g) and rituximab (0.375 g/m^2) in comparison to chemoimmunotherapy of bendamustine (0.090 g/m^2) and rituximab (0.375 g/m^2). Patients were monitored for 4 years	Targeted and immunotherapeutic combination improved the lives of patients with CLL (88% 2-year PFS), while also inducing 7% deaths in comparison to chemoimmunotherapy which showed lower efficacy (74% 2-year PFS) and 1% death rate. The overall survival was comparable between the treatment groups (94% vs 95% 2-year OS). The major severe adverse events neutropenia (40%, 21%), thrombocytopenia (5%, 15%), anemia (6%, 12%), infection (20%, 15%) and hypertension (34%, 15%) were comparable between the groups.

3.4. The effectiveness chemoimmunotherapy and the associated major severe adverse events compared to conventional chemotherapy in patients with CLL

The first-line therapy, FCR have previously showed improved overall response rates, PFS and OS in patients with patients with unmutated IGHV gene, del(11q), del(13q) CLL (39). Chlorambucil in combination with rituximab or obinutuzumab or ofatumumab was compared to chlorambucil monotherapy in 3 studies (12, 13, 40). These studies showed an improved effectiveness (PFS) in treatment-naïve patients with CLL and coexisting conditions receiving chemoimmunotherapy compared to conventional chemotherapy. Moreover, chemoimmunotherapy was more effective in patients with unmutated IGHV gene and high B2M (12).

Chemoimmunotherapy of fludarabine and alemtuzumab was more effective (improved PFS and OS) in patients with relapsed or refractory CLL (41). Moreover, patients with advanced disease (Rai stage III or IV) benefitted more from chemoimmunotherapy compared conventional chemotherapy of fludarabine. However, this improved effect was associated with major severe adverse events such as neutropenia leukopenia, lymphopenia, thrombocytopenia and anaemia in both treatment-naïve patients with CLL and patients with relapsed or refractory CLL (Table 2.3.2 and 2.3.3).

Table 2.3.2: An overview of included studies reporting on the chemoimmunotherapy and the associated adverse events in patients with chronic lymphocytic leukemia (CLL) compared to conventional chemotherapy.

Author, Year	Country	Participants	Patient stratification and trial phase	Intervention	Main findings
Hillmen et al., 2015	United Kingdom	Treatment-naïve patients with CLL, and coexisting conditions (n =447), with mean age of 66.3±14.3 (Male: 63.1%)	Binet stage B (36%) and C (31%). Patients with unmutated IGHV gene (56%). Patients with 12q or 13q deletion or 6q deletion (54%). Patients with elevated beta-2 microglobulin levels (75%). Phase III	Received chemoimmunotherapy of chlorambucil (0.01 g/m^2) and ofatumumab (1 g) in comparison to conventional chemotherapy of chlorambucil (0.01 g/m^2). Patients were monitored for 4 years.	Chemoimmunotherapy was more effective in patients with CLL (22.4 months median PFS), while also inducing 2% deaths in comparison to conventional chemotherapy which showed lower efficacy (13.1 months median PFS) and 2% death rate. Moreover, patients with unmutated IGHV gene and elevated beta-2 microglobulin levels receiving chemoimmunotherapy had an improved PFS compared with those on conventional chemotherapy. However, chemoimmunotherapy group had high incidence of major severe adverse event, neutropenia (26%) compared to (14%).
Christian et al., 2014	Denmark	Treatment-naïve, patients with CLL with no severe coexisting conditions (n =272), with mean age not reported (Male: 74.6%)	Binet stage B (54.8%) and C (34.2%). Patients with unmutated IGHV gene (79.8%). Patients with elevated beta-2 microglobulin levels (45.6%). Phase III	Received chemoimmunotherapy of fludarabine (0.040 g/m^2), cyclophosphamide (0.250 g/m^2) and alemtuzumab (0.030 g) (FCA) in comparison to conventional chemotherapy of fludarabine (0.040 g/m^2) and cyclophosphamide (0.250 g/m^2) (FC). Patients were monitored for 5 years.	Chemoimmunotherapy was more effective in patients with high-risk CLL (53% 3-year PFS and 85% 3-year OS), while also inducing 3.8% deaths in comparison to conventional chemotherapy which showed lower efficacy (37% 3-year PFS and 76% 3-year OS) and 4.3% death rate. Patients in Binet stage C receiving chemoimmunotherapy had an improved PFS compared with those receiving chemoimmunotherapy (HR:0.53). The major severe adverse event, neutropenia was comparable among the groups (9.6 vs. 10.3%).
Lee et al., 2014	Germany	Treatment-naïve, CD20-positive patients with CLL, and coexisting conditions (n =356), with mean age of 68.8±12.3 (Male: 60.4%)	Binet stage B (42%) and C (36%). Patients with unmutated IGHV gene (61%). Patients with del(13p) (30%). Phase III	Received chemoimmunotherapy of chlorambucil (0.0005 g/kg) and obinutuzumab (1 g); in comparison to conventional chemotherapy of chlorambucil (0.0005 g/kg). Patients were monitored for 5 years	Chemoimmunotherapy was more effective in patients with CLL (median PFS 23 months), while also inducing 0% deaths in comparison to conventional chemotherapy which showed lower efficacy (median PFS 11.1 months) and 0% death rate. The median OS was not reached. However, chemoimmunotherapy group had the major severe adverse event, neutropenia accounting 34% compared to 16%.

Goede et., 2014	Germany	Treatment-naïve, CD20-positive patients with CLL, and coexisting conditions (n =781), with mean age 73±12.5 (Male: %not reported)	Binet stage B (41.4%) and C (36.2%). Patients with unmutated IGHV gene (60.4%). Phase III	Received chemoimmunotherapy of chlorambucil (0.0005 g/kg) and obinutuzumab (1 g) or chlorambucil (0.0005 g/kg) and rituximab (0.5 g/m^2); in comparison to conventional chemotherapy of chlorambucil (0.0005 g/kg). Patients were monitored for 3 years	Chemoimmunotherapy was more effective in patients with CLL (26.7 and 16.3 months median PFS), while also inducing 4% and 6% deaths in comparison to conventional chemotherapy which showed lower efficacy (11.1 months median PFS) and 9% death rate. Overall survival medians were not reached. However, chemoimmunotherapy group had the major severe adverse event, neutropenia accounting 31% vs. 16%.
Hallek et al., 2010	Germany	Treatment-naïve, physically fit, CD20-positive patients with CLL, and no coexisting conditions (n =817), with mean age of 58±12.5 (Male: 74.3%)	Binet stage B and C (95%). Patients with unmutated IGHV gene (63%). Patients with del(13p) (54%). Phase III	Received chemoimmunotherapy of fludarabine (0.025 g/m^2), cyclophosphamide (0.250 g/m^2) and rituximab (0.5 g/m^2) (FCR) in comparison to conventional chemotherapy of fludarabine (0.025 g/m^2) and cyclophosphamide (0.250 g/m^2) (FC). Patients were monitored for 5 years	Chemoimmunotherapy was more effective in patients with CLL (65% 3-year PFS and 87% 3-year OS), while also inducing 2% deaths in comparison to conventional chemotherapy which showed lower efficacy (45% 3-year PFS and 83% 3-year OS) and 3% death rate. Patients with unmutated IGHV gene, del(11q), del(13q) receiving chemoimmunotherapy had an improved PFS and OS compared with those receiving conventional chemotherapy. However, chemoimmunotherapy group had the major severe adverse events, with neutropenia accounting 34% and leukocytopenia accounting 24% compared to conventional chemotherapy (21% and 12%, respectively).
Elter et al., 2011	Germany	Patients with relapsed or refractory CLL (335), with mean age of 60.4±9.3 (Male: 65%)	Binet stage B (53%) and C (32%). Patients with 13q deletion (31%). Patients with elevated beta-2 microglobulin levels (33.5%). Phase III	Received chemoimmunotherapy of fludarabine (0.03 g/m^2) and alemtuzumab (0.03 g) in comparison to conventional chemotherapy of fludarabine (0.025 g/m^2)). Patients were monitored for 6 years.	Chemoimmunotherapy was more effective in patients with CLL (23.7 months median PFS and 70% 6-year OS), while also inducing 6% deaths in comparison to conventional chemotherapy which showed lower efficacy (16.5 months median PFS and 60% 6-year OS) and 7% death rate. Moreover, patients with advanced disease (Rai stage III or IV) receving chemoimmunotherapy (20.5 months median PFS) had an improved PFS compared with those on conventional chemotherapy (11.5 months median PFS). The major severe adverse events like leukopenia, lymphopenia, neutropenia, thrombocytopenia and anaemia were comparable between groups.

Table 2.3.3: Major severe adverse events affecting more than 5% of the patients.

Study ID	Major severe adverse events	Treatment regime	
		Chemoimmunotherapy	Targeted therapy combined immunotherapy
Eichhorst et al., 2023	Infection	18.5%	15%
Hillmen et al., 2023	Leukopenia	54%	14%
Shanafelt et al., 2022	Neutropenia	45.6%	28.4%
	Leukopenia	40.5%	6.5%
Al-Sawaf et al., 2020	Neutropenia	53%	48%
Sharman et al., 2020	Neutropenia	41%	30%
	infection	8%	21%
Moreno et al., 2019	Neutropenia	46.1%	36.3%
	thrombocytopenia	10.4%	18.6%
Seymour et al., 2018	Neutropenia	38.8%	57.7%
Woyach et al., 2018	Neutropenia	21%	40%
	Thrombocytopenia	15%	5%
	Anaemia	12%	6%
	Infection	15%	20%
	Hypertension	15%	34%
		Chemoimmunotherapy	**conventional chemotherapy**
Hillmen et al., 2015	Neutropenia	26%	14%
Christian et al., 2014	Neutropenia	9.6%	10.3%
Lee et al., 2014	Neutropenia	34%	16%
Goede et., 2014	Neutropenia	31%	16%
Hallek et al., 2010	Neutropenia	34%	21%
	Leukocytopenia	24%	12%
Elter et al., 2011	Leucopenia	74%	34%
	Lymphopenia	94%	33%
	Neutropenia	59%	68%
	Thrombocytopenia	11%	17%
	Anaemia	9%	17%

Table 2.3.4: Summary of findings: Use of chemoimmunotherapy in patients with chronic lymphocytic leukemia (CLL) compared to targeted therapy combined with immunotherapy.

Certainty assessment							Impact	Certainty
№ of studies	Study design	Risk of bias	Inconsistency	Indirectness	Imprecision	Other considerations		
8	randomized trials	not serious	not serious	not serious	serious	none	Three studies showed increased incidents of severe adverse events associated with the use of chemoimmunotherapy while only one study showed high incident of severe adverse events associated with targeted therapy and immunotherapy combination treatment. Majority of the studies (4) reported no significance difference.	⊕⊕⊕◯ Moderate
8	randomized trials	not serious	not serious	not serious	serious	none	Eight studies showed that target and immunotherapeutic combination was associated with improved PFS as compared with chemoimmunotherapy.	⊕⊕⊕◯ Moderate
8	randomized trials	not serious	not serious	not serious	serious	none	Five studies showed that targeted and immunotherapeutic combination improved overall survival of patients with CLL when compared to chemoimmunotherapy while 3 studies show no significant difference.	⊕⊕⊕◯ Moderate

Table 2.3.5: Summary of findings: Use of chemoimmunotherapy in patients with chronic lymphocytic leukemia (CLL) compared to conventional chemotherapy.

Certainty assessment							№ of patients		Effect		Certainty
№ of studies	Study design	Risk of bias	Inconsistency	Indirectness	Imprecision	Other considerations	chemoimmunotherapy	conventional chemotherapy	Relative (95% CI)	Absolute (95% CI)	
6	randomized trials	not serious	not serious	not serious	serious	none	Four studies showed increased incidences of severe adverse events associated with the use of chemoimmunotherapy when compared to conventional chemotherapy. Only two studies reported no significant difference.				⊕⊕⊕◯ Moderate
6	randomized trials	not serious	not serious	not serious	serious	none	All the included studies (6) showed that chemoimmunotherapy was associated with improved PFS when compared to conventional chemotherapy.				⊕⊕⊕◯ Moderate
6	randomized trials	not serious	not serious	not serious	serious	none	Three studies showed that chemoimmunotherapy was associated with improved OS when compared to conventional chemotherapy. The OS was not reached in two studies while one study did not report OS.				⊕⊕⊕◯ Moderate

5. Discussion

The aim of this systematic review was to provide a synthesis of evidence from randomized controlled trials (RCTs) reporting on the effectiveness of chemoimmunotherapy and the associated adverse events in patients with CLL. The results of our study showed that chemoimmunotherapy is effective when compared to conventional chemotherapy and less effective when compared to targeted therapy combined with immunotherapy (Table 2.3.1 and 2.3.2). Chemoimmunotherapy in the form of chlorambucil in combination with rituximab or obinutuzumab or ofatumumab improve the PFS of treatment-naïve patients with CLL (12, 13, 40). It is noteworthy that this chemoimmunotherapy was more effective for patients with high-risk CLL when compared to conventional chemotherapy (Table 2.3.2). Moreover, the treatment was associated with high incidence of severe adverse events such as neutropenia and leukocytopenia. The COMPLEMENT 1 trial showed that combination of ofatumumab and chlorambucil improve the PFS of patients with high-risk CLL who cannot tolerate fludarabine-based regimens (12). FCR was indeed proved to be more effective for young (≤ 65 years), medically-fit patients with high-risk CLL (39). The CLL11 and COMPLEMENT 1 studies demonstrated that elderly (≥65 years) with high-risk CLL and severe comorbidities benefit from chlorambucil in combination with rituximab or obinutuzumab or ofatumumab (12, 13, 40). The included studies had low risk of bias and the quality of the evidence for both the primary and secondary outcomes was moderate.

Our study findings showed that targeted therapy combined with immunotherapy improved the PFS and OS of patients with CLL when compared to chemoimmunotherapy (Table 2.3.1). In fact, Ibrutinib–Rituximab or Chemoimmunotherapy for Chronic Lymphocytic Leukemia (E1912) and Ibrutinib and rituximab versus fludarabine, cyclophosphamide, and rituximab for patients with previously untreated chronic lymphocytic leukaemia (FLAIR) trials demonstrated that ibrutinib and rituximab was more effective than FCR for both young and old treatment-naïve patients with high-risk CLL and a low burden of comorbidities (33, 35). Although chlorambucil in combination with obinutuzumab is effective and considered in high-risk patients with CLL comorbidities and intolerance to fludarabine-based

regimens (13), venetoclax, acalabrutinib and ibrutinib in combination with obinutuzumab was more effective in these patients (15, 16, 36).

The cumulative evidence suggests that chemoimmunotherapy and targeted therapy combined with immunotherapy are associated with increased incidence of severe adverse events. The major severe adverse events in the included RCTs were neutropenia (12, 13, 15, 16, 33, 36-42), anemia (37, 39, 41), thrombocytopenia (15, 37, 39, 41), leukopenia (33, 35, 39, 41), infections (34, 36, 37) and hypertension (37).

The existing data on the use of chemoimmunotherapy and targeted combined with immunotherapy in managing patients with CLL is primarily derived from European and American populations (Table 2.3.1-2.3.2). The lack of diverse patient populations limits the extrapolation of these findings to low-to-middle income countries.

6. Conclusion

The cumulative evidence indicates that while chemoimmunotherapy is more effective for high-risk patients with CLL compared to conventional chemotherapy, the targeted therapy combined with immunotherapy is superior for all patients with CLL. The major severe adverse events associated with the use of these treatments are mainly neutropenia, anemia, thrombocytopenia, leukopenia, infections, and hypertension.

Abréviations

CLL : Chronic lymphocytic leukaemia

MeSH : Medical subheadings

RCTs : Randomized controlled trials

PFS : Progression-free survival

OS : Overall survival

PRISMA : Preferred Reporting Items for Systematic Review and Meta-analysis

Declarations

None.

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Authors' contributions

Authors, AN, PVD and BBN conceptualised, designed, and drafted this manuscript. All authors wrote and approved the final manuscript. AN is the guarantor of the review.

Data Availability Statement

All data analyzed in this study are included in this published article.

Competing interests

None declared.

Ethics approval and consent to participate

Not applicable

References

1. Lanasa MCJH, the American Society of Hematology Education Program Book. Novel insights into the biology of CLL. 2010;2010(1):70-6.
2. Zhang N, Wu J, Wang Q, Liang Y, Li X, Chen G, et al. Global burden of hematologic malignancies and evolution patterns over the past 30 years. Blood Cancer Journal. 2023;13(1):82.
3. Malam-Abdou B, Brah S, Djibrilla A, Andia A, Chefou M, Sani MM, et al. Leucémie Lymphoïde Chronique au Niger: une étude de 99 cas au Service d'Onco-Hématologie de l'Hôpital National de Niamey. 2018;19(2).
4. Fall AS, Dieye TN, Ndiaye FS, Diop S, Mathiot C, Gattiolat C-H, et al. Characteristics Of Chronic Lymphocytic Leukemia (CLL) In Senegal. Clinical Features, Cytology, Immunophenotype, Cytogenetic Abnormalities and Altered Expression Of Micro-RNA. American Society of Hematology Washington, DC; 2013.
5. Yang S, Varghese AM, Sood N, Chiattone C, Akinola NO, Huang X, et al. Ethnic and geographic diversity of chronic lymphocytic leukaemia. Leukemia. 2021;35(2):433-9.
6. DeSantis CE, Miller KD, Dale W, Mohile SG, Cohen HJ, Leach CR, et al. Cancer statistics for adults aged 85 years and older, 2019. 2019;69(6):452-67.
7. Fischer K, Bahlo J, Fink AM, Goede V, Herling CD, Cramer P, et al. Long-term remissions after FCR chemoimmunotherapy in previously untreated patients with CLL: updated results of the CLL8 trial. 2016;127(2):208-15.
8. Munir T, Mohseninejad L, Xu S, Jevdjevic M, Bouwmeester W, Yang K. Zanubrutinib vs FCR in Fit Treatment-Naive Patients with Chronic Lymphocytic Leukemia: A Matching-Adjusted Indirect Comparison. Blood. 2023;142:6522.
9. Badoux XC, Keating MJ, Wang X, O'Brien SM, Ferrajoli A, Faderl S, et al. Fludarabine, cyclophosphamide, and rituximab chemoimmunotherapy is highly effective treatment for relapsed patients with CLL. Blood, The Journal of the American Society of Hematology. 2011;117(11):3016-24.
10. Shah N, Tam C, Seymour JF, Rule S. How applicable is fludarabine, cyclophosphamide and rituximab to the elderly? Leukemia & lymphoma. 2015;56(6):1599-610.
11. Herling CD, Cymbalista F, Groß-Ophoff-Müller C, Bahlo J, Robrecht S, Langerbeins P, et al. Early treatment with FCR versus watch and wait in patients with stage Binet A high-risk chronic lymphocytic leukemia (CLL): a randomized phase 3 trial. Leukemia. 2020;34(8):2038-50.
12. Hillmen P, Robak T, Janssens A, Babu KG, Kloczko J, Grosicki S, et al. Chlorambucil plus ofatumumab versus chlorambucil alone in previously untreated patients with chronic lymphocytic leukaemia (COMPLEMENT 1): a randomised, multicentre, open-label phase 3 trial. 2015;385(9980):1873-83.
13. Goede V, Fischer K, Busch R, Engelke A, Eichhorst B, Wendtner CM, et al. Obinutuzumab plus chlorambucil in patients with CLL and coexisting conditions. New England Journal of Medicine. 2014;370(12):1101-10.
14. Hillmen P, Gribben JG, Follows GA, Milligan D, Sayala HA, Moreton P, et al. Rituximab plus chlorambucil as first-line treatment for chronic lymphocytic leukemia: final analysis of an open-label phase II study. Journal of clinical oncology. 2014;32(12):1236.
15. Moreno C, Greil R, Demirkan F, Tedeschi A, Anz B, Larratt L, et al. Ibrutinib plus obinutuzumab versus chlorambucil plus obinutuzumab in first-line treatment of chronic lymphocytic leukaemia (iLLUMINATE): a multicentre, randomised, open-label, phase 3 trial. The Lancet Oncology. 2019;20(1):43-56.
16. Al-Sawaf O, Zhang C, Tandon M, Sinha A, Fink A-M, Robrecht S, et al. Venetoclax plus obinutuzumab versus chlorambucil plus obinutuzumab for previously untreated chronic lymphocytic leukaemia (CLL14): follow-up results from a multicentre, open-label, randomised, phase 3 trial. The Lancet Oncology. 2020;21(9):1188-200.

17. Flinn IW, Ruppert AS, Harwin W, Waterhouse D, Papish S, Jones JA, et al. A phase II study of two dose levels of ofatumumab induction followed by maintenance therapy in symptomatic, previously untreated chronic lymphocytic leukemia. 2016;91(10):1020-5.
18. Byrd JC, Flynn JM, Kipps TJ, Boxer M, Kolibaba KS, Carlile DJ, et al. Randomized phase 2 study of obinutuzumab monotherapy in symptomatic, previously untreated chronic lymphocytic leukemia. 2016;127(1):79-86.
19. Tsao L-C, Force J, Hartman ZC. Mechanisms of therapeutic antitumor monoclonal antibodies. Cancer research. 2021;81(18):4641-51.
20. Maloney DG. Mechanism of action of rituximab. Anti-cancer drugs. 2001;12:S1-4.
21. Robak P, Robak T. Immunotherapy combinations for chronic lymphocytic leukemia: advantages and disadvantages. Expert Opinion on Biological Therapy. 2023;23(1):21-35.
22. Ryan CE, Brander DM, Barr PM, Tyekucheva S, Hackett LR, Collins MC, et al. A phase 1b study of ibrutinib in combination with obinutuzumab in patients with relapsed or refractory chronic lymphocytic leukemia. Leukemia. 2023;37(4):835-42.
23. Zent CS, Victoria Wang X, Ketterling RP, Hanson CA, Libby EN, Barrientos JC, et al. A phase II randomized trial comparing standard and low dose rituximab combined with alemtuzumab as initial treatment of progressive chronic lymphocytic leukemia in older patients: a trial of the ECOG-ACRIN cancer research group (E1908). 2016;91(3):308-12.
24. Kipps TJ, Eradat H, Grosicki S, Catalano J, Cosolo W, Dyagil IS, et al. A phase 2 study of the BH3 mimetic BCL2 inhibitor navitoclax (ABT-263) with or without rituximab, in previously untreated B-cell chronic lymphocytic leukemia. Leukemia & lymphoma. 2015;56(10):2826-33.
25. Csanadi M, Agh T, Tordai A, Tapprich C, Voko Z, Stamatopoulos K. Secondary primary malignancies after treatment with chemo-immunotherapy in treatment-naïve patients with CLL: a systematic literature review. Expert Review of Hematology. 2022;15(3):273-84.
26. Molica S, Giannarelli D, Baumann T, Montserrat E. Ibrutinib as initial therapy in chronic lymphocytic leukemia: A systematic review and meta-analysis. European Journal of Haematology. 2020;104(5).
27. Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. 2021;372.
28. Ntsethe A, Dludla PV, Nyambuya TM, Ngcobo SR, Nkambule BBJM. The impact of immune checkpoint inhibitors in patients with chronic lymphocytic leukemia (CLL): A protocol for a systematic review and meta-analysis of randomized controlled trials. 2020;99(28).
29. Health UDo, Services H. Common terminology criteria for adverse events (CTCAE). (No Title). 2010.
30. Downs SH, Black N. The feasibility of creating a checklist for the assessment of the methodological quality both of randomised and non-randomised studies of health care interventions. Journal of epidemiology & community health. 1998;52(6):377-84.
31. Landis JR, Koch GGJb. The measurement of observer agreement for categorical data. 1977:159-74.
32. Balshem H, Helfand M, Schünemann HJ, Oxman AD, Kunz R, Brozek J, et al. GRADE guidelines: 3. Rating the quality of evidence. Journal of clinical epidemiology. 2011;64(4):401-6.
33. Shanafelt TD, Wang XV, Hanson CA, Paietta EM, O'Brien S, Barrientos J, et al. Long-term outcomes for ibrutinib–rituximab and chemoimmunotherapy in CLL: updated results of the E1912 trial. Blood, The Journal of the American Society of Hematology. 2022;140(2):112-20.
34. Eichhorst B, Niemann CU, Kater AP, Fürstenau M, Von Tresckow J, Zhang C, et al. First-line venetoclax combinations in chronic lymphocytic leukemia. New England Journal of Medicine. 2023;388(19):1739-54.
35. Hillmen P, Pitchford A, Bloor A, Broom A, Young M, Kennedy B, et al. Ibrutinib and rituximab versus fludarabine, cyclophosphamide, and rituximab for patients with previously untreated chronic lymphocytic leukaemia (FLAIR): interim analysis of a multicentre, open-label, randomised, phase 3 trial. The Lancet Oncology. 2023;24(5):535-52.

36. Sharman JP, Egyed M, Jurczak W, Skarbnik A, Pagel JM, Flinn IW, et al. Acalabrutinib with or without obinutuzumab versus chlorambucil and obinutuzumab for treatment-naive chronic lymphocytic leukaemia (ELEVATE-TN): a randomised, controlled, phase 3 trial. The Lancet. 2020;395(10232):1278-91.
37. Woyach JA, Ruppert AS, Heerema NA, Zhao W, Booth AM, Ding W, et al. Ibrutinib regimens versus chemoimmunotherapy in older patients with untreated CLL. New England Journal of Medicine. 2018;379(26):2517-28.
38. Seymour JF, Kipps TJ, Eichhorst B, Hillmen P, D'Rozario J, Assouline S, et al. Venetoclax–rituximab in relapsed or refractory chronic lymphocytic leukemia. New England Journal of Medicine. 2018;378(12):1107-20.
39. Hallek M, Fischer K, Fingerle-Rowson G, Fink AM, Busch R, Mayer J, et al. Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. The Lancet. 2010;376(9747):1164-74.
40. Lee H-Z, Miller BW, Kwitkowski VE, Ricci S, DelValle P, Saber H, et al. US Food and drug administration approval: obinutuzumab in combination with chlorambucil for the treatment of previously untreated chronic lymphocytic leukemia. Clinical Cancer Research. 2014;20(15):3902-7.
41. Elter T, Gercheva-Kyuchukova L, Pylylpenko H, Robak T, Jaksic B, Rekhtman G, et al. Fludarabine plus alemtuzumab versus fludarabine alone in patients with previously treated chronic lymphocytic leukaemia: a randomised phase 3 trial. The lancet oncology. 2011;12(13):1204-13.
42. Geisler CH, van t'Veer MB, Jurlander J, Walewski J, Tjønnfjord G, Itälä Remes M, et al. Frontline low-dose alemtuzumab with fludarabine and cyclophosphamide prolongs progression-free survival in high-risk CLL. Blood, The Journal of the American Society of Hematology. 2014;123(21):3255-62.

Prologue

This chapter is a published experimental paper in a peer-reviewed journal. We evaluated the B cell profiles and immune checkpoint expression on B cell subsets in patients with chronic lymphocytic leukemia (CLL). We aimed to assess the expression of these immune checkpoints and correlate them with an independent prognostic marker for patients with CLL (beta-2 microglobulin). In this study we reported elevated levels of programmed death-1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) on activated B cells and memory B cells. However, these levels were not associated with B2M levels, suggesting that the expression of these immune checkpoints in B cell subsets do not directly influence B2M levels.

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Brief Report

B Cell Subsets and Immune Checkpoint Expression in Patients with Chronic Lymphocytic Leukemia

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Abstract: Chronic lymphocytic leukemia (CLL) is characterized by dysfunctional B cells. Immune checkpoint molecules such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed death-1 (PD-1) are upregulated in patients with CLL and may correlate with prognostic markers such as beta-2 microglobulin (B2M). The aim of this study was to evaluate the levels of immune checkpoints on B cell subsets and to further correlate them with B2M levels in patients with CLL. We recruited 21 patients with CLL and 12 controls. B cell subsets and the levels of immune checkpoint expression were determined using conventional multi-color flow cytometry. Basal levels of B2M in patients with CLL were measured using an enzyme-linked immunosorbent assay. Patients with CLL had increased levels of activated B cells when compared to the control group, $p < 0.001$. The expression of PD-1 and CTLA-4 were increased on activated B cells and memory B cells, $p < 0.05$. There were no associations between B2M levels and the measured immune checkpoints on B cell subsets, after adjusting for sex and age. In our cohort, the patients with CLL expressed elevated levels of PD-1 and CTLA-4 immune checkpoints on activated and memory B cell subsets. However, there was no correlation between these immune checkpoint expressions and B2M levels.

Keywords: chronic lymphocytic leukemia; immune checkpoints; B cell subsets; beta-2 microglobulin; programmed death protein 1; cytotoxic T-lymphocyte-associated protein 4

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1. Introduction

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia among adults, with a global prevalence of about 3.5 cases per 100,000 people [1]. In high-income countries, CLL accounts for more than a third of all leukemia cases [1]. Notably, low-to-middle-income countries have a five- to ten-fold lower age-adjusted incidence rate of CLL compared to high-income countries [2]. CLL is a lymphoproliferative disorder that is characterized by functionally incompetent B cells with a distinct immunophenotype [3]. However, there are divergent findings regarding the predictive significance of B cell subsets in leukemia [4].

Regulatory B cells (Bregs) modulate T cell-driven anti-tumor immunity, promoting the expression of forkhead box protein 3 (FoxP3+) in regulatory T cells (T-regs), which dampens the innate and adaptive antitumor immune response [4,5]. These immunosuppressive mechanisms involve Bregs, which acquire inhibitory ligands and signal transducer and activator of transcription 3 (STAT3) phosphorylation, and the induction of interleukin 10 (IL-10) and transforming growth factor-β (TGF-β) [4,5]. The expression of inhibitory molecules such as programmed death-1 (PD-1) or programmed death-ligand 1 (PD-L1) inhibits the anti-tumor function of Bregs [4]. In patients with CLL, immune checkpoints such as T-cell immunoglobulin-3 (TIM-3), cytotoxic T-lymphocyte-associated protein 4

CHAPTER 3: Research article 1

B cell subsets and immune checkpoint expression in patients with chronic lymphocytic leukaemia

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The authors declare no potential conflicts of interest.

Author Contributions

Conceptualization, A.N., B.B.N., P.V.D.; Methodology, A.N., B.B.N., Z.A.M.; Formal Analysis, A.N., B.B.N.; Investigation, A.N., B.B.N., P.V.D.; Data Curation, A.N., B.B.N., Z.A.M.; Writing – Original Draft Preparation, A.N.; Writing – Review & Editing, A.N., B.B.N., P.V.D.; Visualization, A.N., B.B.N., P.V.D.; Supervision, B.B.N., P.V.D.; Project Administration, A.N.

Abstract

Chronic lymphocytic leukemia (CLL) is characterized by dysfunctional B cells. Immune checkpoint molecules such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and programmed death-1 (PD-1) are upregulated in patients with CLL and may correlate with prognostic markers such as beta-2 microglobulin (B2M). The aim of this study was to evaluate the levels of immune checkpoints on B-cell subsets and to further correlate them with B2M levels in patients with CLL. We recruited 21 patients with CLL and 12 controls. B cell subsets and the levels of immune checkpoint expression were determined using conventional multi-color flowcytometry. Basal levels of B2M in patients with CLL were measured using an Enzyme-linked immunosorbent assay. Patients with CLL had increased levels of activated B cells when compared to the control group, $p < 0.001$. The expression of PD-1 and CTLA-4 were increased on activated B cells and memory B cells, $p < 0.05$. There were no associations between B2M levels and the measured immune checkpoints on B cell subsets, after adjusting for sex and age. In our cohort, patients with CLL express elevated levels of PD-1 and CTLA-4 immune checkpoints on activated and memory B cell subsets. However, there is no correlation between these immune checkpoint expressions and B2M levels.

Keywords: Chronic lymphocytic leukemia; immune checkpoints; B cell subsets; beta-2 microglobulin, programmed death protein 1, cytotoxic T-lymphocyte associated protein 4.

1. Introduction

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia among adults, with a global prevalence of about 3.5 cases per 100,000 people (1). In high-income countries, CLL accounts for more than a third of all leukemia cases (1). Notably, low-to-middle income countries have a five to ten-fold lower age-adjusted incidence rate of CLL as compared to high-income countries (2). CLL is a lymphoproliferative disorder that is characterized by functionally incompetent B cells with a distinct immunophenotype (3). However, there are divergent findings regarding the predictive significance of B cell subsets in in leukemia (4).

Regulatory B cells (Breg) modulate T cell-driven anti-tumor immunity, promoting the expression of forkhead box protein 3 (FoxP3+) in regulatory T cells (T-regs), which dampens the innate and adaptive antitumor immune response (4, 5). These immunosuppressive mechanisms involve Bregs that acquire inhibitory ligands and signal transducer and activator of transcription 3 (STAT3) phosphorylation, and the induction of interleukin 10 (IL-10) and transforming growth factor-β (TGF-β) (4, 5). The expression of inhibitory molecules such as programmed death-1 (PD-1) or programmed death-ligand 1 (PD-L1) inhibit the anti-tumor function of Bregs (4). In patients with CLL, immune checkpoints such as T-cell immunoglobulin-3 (TIM-3), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and programmed death-1 (PD-1) are upregulated (6, 7). The use of immune checkpoint inhibitors (ICI) such as PD-1 antagonists, has fundamentally altered therapeutic approaches in patients with CLL (8). Targeting PD-1 has shown to improve the overall survival of patients with CLL while also reducing adverse effects (8). In patients with relapsed CLL, zanubrutinib, a Bruton's tyrosine kinase inhibitor is associated with fewer cardiac adverse events and improved progression-free survival (9).

There has been a significant transformation in the management of patients with CLL. Advances in prognostic markers, enhanced predictive capabilities for clinical outcomes have collectively made vital impact in the management of CLL. The important adverse prognostic markers which include immunoglobulin heavy-chain variable region gene (IGHV) mutation status, zeta-chain-associated protein kinase-70 (ZAP-70) and CD38 expression, and beta 2 microglobulin (B2M) levels (10). The levels of B2M are considered as an independent marker for poor prognosis in patients with CLL (11). The serum levels of B2M are elevated in patients with CLL who at an advanced stage of the disease, and these levels decrease post-treatment (12). The primary aim of this study was to determine the levels of immune checkpoint expression on peripheral B-cell subsets in patients with CLL and to further correlate these immune checkpoints with B2M levels in patients with CLL.

2. Methods and Materials

2.1 Patient recruitment

Patients and healthy control participants were recruited between July 2019 to May 2022 from King Edward VIII Hospital, a tertiary healthcare facility located in Durban, KwaZulu-Natal, South Africa. The study was conducted in accordance with the Declaration of Helsinki, and ethical approval was obtained from the University of KwaZulu-Natal Biomedical Research Ethics Committee (BE456/18), South Africa. Written informed consent was obtained from all study participants. In this study we excluded patients with CLL who were on treatment and only untreated patients were included along with age-matched healthy controls with no clinical signs of infection. The ethnicity of the participants was self-reported.

2.2 Sample collection

Peripheral blood was collected from consenting participants by venepuncture into 6mL ethylenediaminetetraacetic acid (EDTA) tubes (BD Bioscience, USA). Samples were transported at room temperature from the hospital to the laboratory.

2.3 Hematological analysis

Hematological parameters including the white blood cell count, hemoglobin and platelet count were measured using an automated Coulter AcT Diff hematology analyzer (Beckman Coulter Inc., California, United States) within 1-2 hours of peripheral blood collection.

2.4 Isolation of peripheral blood mononuclear cells (PBMCs)

The peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples by density-gradient centrifugation method, using the Ficoll-Paque PLUS (Amersham, Biosciences, Uppsala, Sweden), as previously described (13). Briefly, 3 mL of Ficoll-Paque PLUS was aliquoted into a 15mL centrifuge tube (Sigma-Aldrich, Germany) and 4 mL of whole blood was carefully layered on the Ficoll-Paque PLUS gradient and the samples were centrifuged at 400g for 40 minutes at 20°C. The PBMC layer was then collected and stored at -80°C.

2.5 T cell depletion and B cell isolation from peripheral blood mononuclear cells

To enrich the B cell population from the collected PBMCs, we performed T cell depletion and positively selected B cells using the BD IMag isolation system (BD Bioscience, USA). Briefly, 50 µL PBMCs were incubated with 5 µL of biotinylated human T lymphocyte enrichment cocktail (BD Biosciences, San Jose, CA, USA) for 15 minutes at room temperature. After the incubation, 50 µL of streptavidin particles were added to the T cell depleted PBMC samples and incubated for 30 minutes, at room temperature. The samples were then reconstituted into 1 mL of 3.2% sodium citrated buffer and samples

were placed on the BD Imag for 8 minutes. Isolated B cells were reconstituted into 100 µL PBS and stored -80 ºC.

2.6 Measurements of B cell subsets

To quantify B cell subsets, we made use of a six-colour flow cytometry panel consisting of CD38-FITC, CD152-PE, CD273-APC, CD19-PE/Cy7, CD27-APC/Cy7 and CD279-BV421 (BioLegend, San Diego, CA, USA). We acquired at least 5000 B cells ($CD19^+$ events) Figure 1A and defined memory B cells as $CD19^+CD27^+$ events (14, 15) (Figure 1B), activated B cells as $CD19^+CD27^-CD38^+$ events and activated memory B cells (15) as $CD19^+CD27^+CD38^+$ events (Figure 1C).

2.7 Measurements of immune checkpoint levels on B cell subsets

To determine the levels of immune checkpoint expression on B cell subsets we measured the expression of CD279 (PD-1), CD273 (PD-L2) and CD152 (CTLA-4) on B_{MEM}, and activated B cells. A total of 5 000 $CD19^+$ events were acquired at a medium flow rate. All the data were acquired using the BD FACSCanto II flow cytometer (BD Biosciences, San Diego, CA, USA) and analyzed using Kaluza version 1.2 (Beckman coulter, Inc Brea, CA, USA).

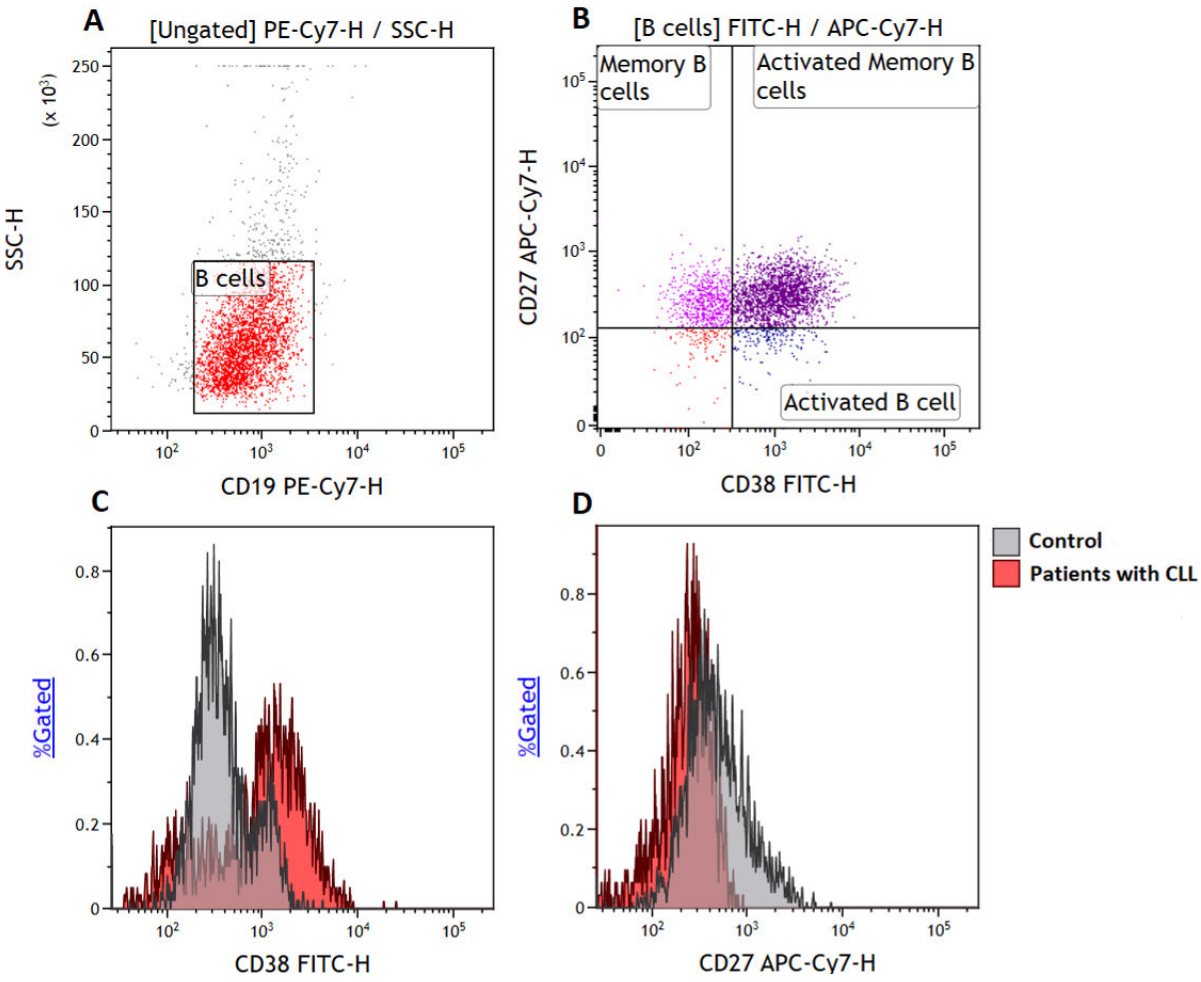

Figure 1: Gating strategy. (**A**) B cells were gated based on side scatter (SSC) and CD19 expression, (**B**) illustrates the gating of activated B cells defined as $CD19^{+}CD38^{+}CD27^{-}$, memory B cells defined as $CD19^{+}CD27^{+}CD38^{-}$ events and activated memory B cells defined as $CD19^{+}CD27^{+}CD38^{+}$ events, respectively. Histograms demonstrate the levels of activated B cells (**C**) and memory B cells (**D**) on the control group and patients with CLL. PE-Cy7 (phycoerythrin-cyanine 7), CD (cluster of differentiation), SSC (side scatter), FITC (fluorescein isothiocyanate), APC-Cy7 (allophycocyanin-cyanine 7).

2.8 Measurements of serum soluble beta-2-microglobulin (B2M) levels

To measure the plasma levels of B2M, a prognostic marker in patients with CLL (16), we made use of the beta-2-microglobulin Human enzyme-linked immunosorbent assay kit (ThermoFisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instruction.

2.9 Sample size estimation

The minimum sample size was calculated to detect a large effect size (d) of 1.14 in the expression of immune checkpoints, namely PD-1 and PD-L1 at 80% power and alpha (α) of 0.05 using GPower 3.1.94 software (Universität, Germany). To detect a large effect size between two independent means using an unpaired t-test, we required a minimum of twenty-one patients with CLL (n=21) and twelve controls (n=12).

2.10 Statistical analysis

All statistical analysis were performed using GraphPad Prism version 8 software, (GraphPad Software Inc, San Diego, CA, USA) and (17). All non-parametric data were log-transformed prior to statistical analysis and reported as mean and standard deviation. An unpaired Student's t-test was performed to compare parametric data between two groups. A repeated measures one-way ANOVA was used to compare parametric multiple data of the same group with Dunnett's as a post hoc test for multiple comparisons. To correct for multiple comparisons, a Bonferroni-corrected critical P-value of < 0.0167 was considered as statistically significant.

2.11 Data Availability

The data generated in this study are available upon request from the corresponding author.

3. Results

3.1 Patients characteristics and hematological parameters

This study comprised of 21 patients with CLL and 12 healthy controls. The mean age of the patients with CLL was 62.33 ± 13.31 years and healthy controls was 56.58± 15.67 years. The included study participants were from different ethnic groups, which comprised of African (n=30), European (n=2), and Indian (n=1). The study included 39.39% females and 60.61% males.

Based on the treatment initiation criteria, 16 patients were on a "watch and wait" approach and 5 had a treatment indication at the time of sample collection (18). In patients with CLL, the white blood cell count was significantly increased (130.4 x 10^3± 29.71) compared to controls (5.26 x 10^3 ± 1.38), p= 0.0005) (Table 1). Whereas the red blood cell count and hemoglobin were significantly reduced in patients with CLL compared to age-matched control patients ($p < 0.0001$). There were no statistically significant differences in the platelet count in patients with CLL when compared to the control group ($p = 0.1831$).

Table 1. The baseline characteristics and hematological parameters of the participants.

	Control (n=12)	Patients with CLL (21)	p-Value
Age (Years)	56.58 ± 15.67	62.33 ± 13.31	0.2714
Male, n (%)	58.33	61.9	
Female, n (%)	41.67	38.1	
White blood cell count (10^3µL)	5.26 ± 1.38	130.4 ± 29.71	0.0005
Red blood cell (10^6µL)	4.74 ± 0.94	2.10 ± 0.84	< 0.0001
Hemoglobin (g/dL)	14.13 ± 3.81	8.19 ± 2.30	< 0.0001
Platelets (10^3µL)	210.4 ± 73.14	157.5 ± 141.9	0.1831

3.2 Increased levels of activated B cells in Patients with CLL

We evaluated the levels of B cell subsets in patients with CLL (Figure 2). Notably, patients with CLL had significantly increased levels of activated B cells (57.39 ± 8.001) compared to the control group (28.47 ± 19.01; $p = 0.0002$).

Furthermore, the expression of memory B cells in patients with CLL (40.95 ± 8.353) was found to be statistically comparable to that in the control group (46.45 ± 20.90; $p = 0.3984$), as was the expression of activated memory B cells expressing (37.31 ± 7.191) in patients with CLL compared to the control group (44.25 ± 21.34; $p = 0.2956$).

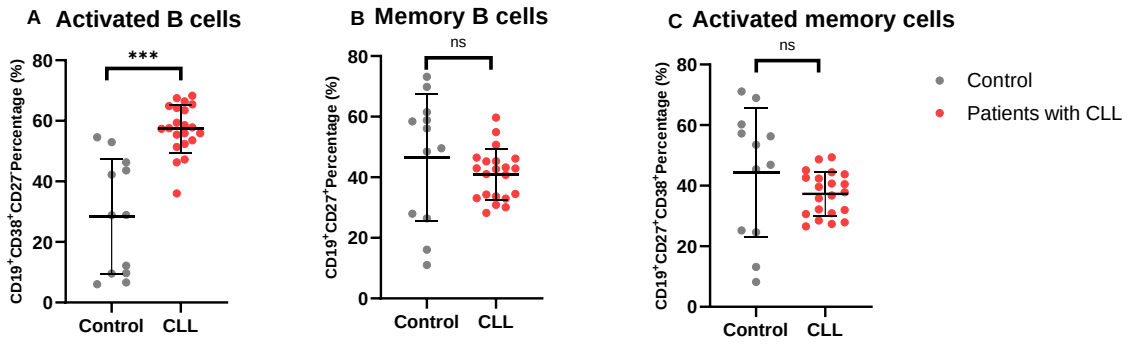

Figure 2: B Cell Subsets in patients with CLL. Figure **A-C** depict the levels of activated B cells, memory B cells and activated memory B cells in patients with CLL compared to healthy controls. The data is presented as the mean ± standard deviation (SD). CD (cluster of differentiation), CLL (chronic lymphocytic leukemia); ***shows the level of significance between groups, ns: not significant.

3.3 Elevated levels of PD-1 Expression on activated B-cells and Memory B cells in patients with CLL

Significantly elevated PD-1 expression was observed on B cells from patients with CLL (40.20 ± 6.601) compared to the control group (6.690 ± 0.8160; $p < 0.0001$) (Figure 3A). Likewise, PD-1 expression was increased on activated B cells in patients with CLL (5.325 ± 0.4397) relative to the control group (4.305 ± 0.4317; $p < 0.0001$). Moreover, a notable increase in PD-1 expression was noted on memory B cells in patients with CLL (27.44 ± 6.358) compared to the control group (14.59 ± 9.395; $p < 0.0001$).

3.4 Elevated levels of CTLA-4 on activated and memory B cells in patients with CLL

A significant increase in CTLA-4 expression was detected on B cells in patients with CLL (3.630 ± 2.897) compared to the control group (1.919 ± 1.184; $p = 0.0241$) (Figure 3B). Similarly, CTLA-4 expression was elevated on activated B cells in patients with CLL (3.631 ± 2.896) relative to the control group (1.919 ± 1.184; $p = 0.0240$). Notably, CTLA-4 expression on memory B cells in patients with CLL (3.439 ± 2.767) was higher compared to the control group (1.755 ± 1.205; $p = 0.0221$).

3.5 Decreased levels of PD-L2 Expression on activated and memory B cells in patients with CLL

Remarkably, PD-L2 expression was significantly decreased on B cells in patients with CLL (0.3967 ± 0.3343) compared to the control group (2.870 ± 2.800; $p = 0.0003$) (Figure 3C). A similar decrease in PD-L2 expression was observed on activated B cells in patients with CLL (0.3624 ± 0.2514) compared to the control group (1.137 ± 0.9655; $p= 0.0186$). Additionally, PD-L2 expression on memory B cells in patients with CLL (0.2005 ± 0.1297) was reduced compared to the control group (1.956 ± 1.706; $p= 0.0044$).

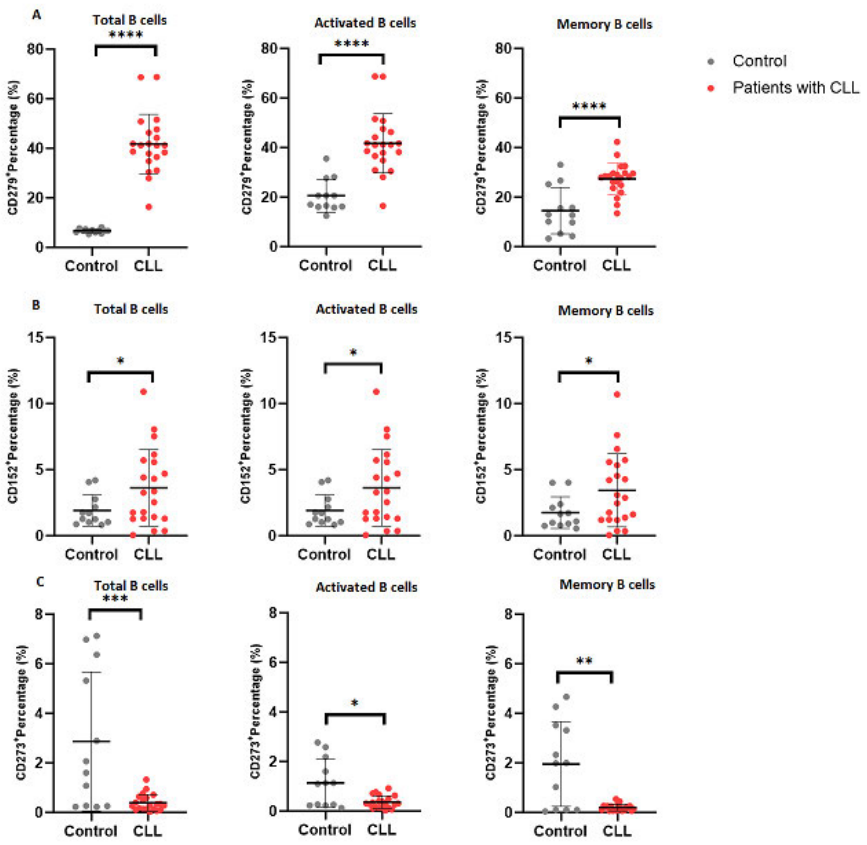

Figure 3: Immune checkpoint expression on B Cell subsets. Figure 3 depict the expression levels of (**A**) PD-1, (**B**) CTLA-4 and (**C**) on total B cells, activated B cells and memory B cells, respectively. The data is presented as the mean ± standard deviation (SD). CD (cluster of differentiation), CLL (chronic lymphocytic leukemia); *, **, *** and **** shows the level of significance between groups.

3.6 Analysis of immune checkpoint (PD-1 and CTLA-4) expression among B Cell Subsets in patients with CLL

In order to elucidate which B cell subsets were associated with heightened immune checkpoint expression, we conducted a comparative assessment of immune checkpoint expression within various B cell subsets in patients with CLL (Figure 4). PD-1 levels were significantly elevated on activated B cells when compared to memory B cells (0.5877 ± 0.04654, $p < 0.0001$). Similarly, CTLA-4 expression was found to be elevated activated when comparable to memory B cells (0.1924 ± 0.04848, $p = 0.0039$).

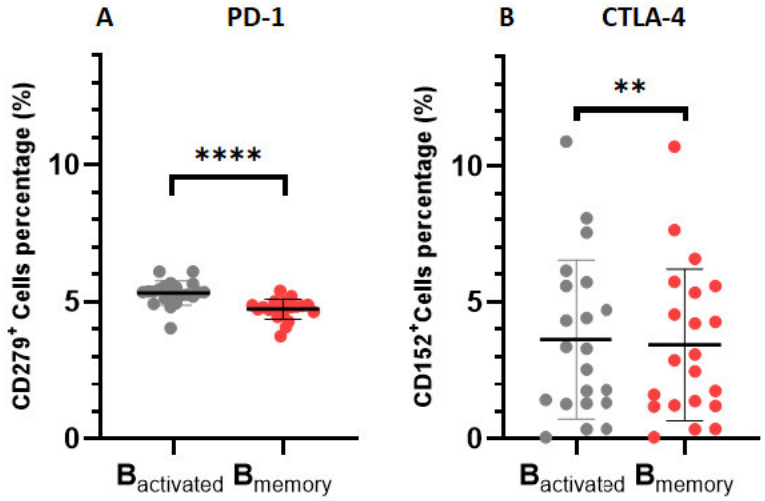

Figure 4: Immune checkpoint expression on B cell subsets. A illustrates the expression of PD-1 on activated ($CD19^+CD38^+$ cells) and memory B cells ($CD19^+CD27^+$ cells). **B** illustrates the levels of CTLA-4 expression on activated B cells and memory B cells. All data are presented as the mean ± standard deviation (SD). CD (cluster of differentiation), PD-1 (Programmed cell death protein 1), CTLA-4 (cytotoxic T-lymphocyte-associated protein 4); ** and **** shows the level of significance between groups.

3.7 β2 microglobulin levels are not associated with the expression of PD-1 or CTLA-4 on B cell subsets

The levels of B2M are independently associated with disease progression in patients with CLL (16). In our multivariable regression model, no association between B2M levels and PD-1 on B cells (β = 0.008, SE = 0.011, p= 0.524), PD-1 on activated B cells (β = 0.008, SE = 0.011, p = 0.525), PD-1 on memory B cells (β = 0.0008, SE = 0.004, p= 0.905), CTLA-4 on B cells (β = 0.004, SE = 0.002, p = 0.426), CTLA-4 on activated B cells (β = 0.004, SE = 0.002, p = 0.419), CTLA-4 on memory B cells (β = 0.004, SE = 0.002, p = 0.419) in patients with CLL (supplementary 1).

4. Discussion

The primary aim of this study was to determine the levels of immune checkpoint expression on the peripheral B-cell subsets in patients with CLL and to further correlate these immune checkpoints with B2M levels, a confirmed independent prognostic marker in patients with CLL (16). In this study, we determined the baseline expression levels of B cell subsets in patients with CLL. Patients with CLL had a higher activated B cell fraction ($CD38^+$ B cells) which has already been described in patients with CLL (19). In our study, the activated B cell had CD38 phenotype, which is one of the prognostic markers for patients with CLL (20, 21). Notably, the activated B cell profile is not restricted to peripheral circulation (22) and the expression of CD38 is associated with an increased proliferation index (23, 24).

The increased levels of $CD38^+$ activated B cells in patients with CLL may indicate patients with poor survival rate and response to therapy (19-21). Moreover, in our study, patients with CLL expressed higher CTLA-4 and PD-1 immune checkpoint proteins on activated B cell subset. The levels of PD-1 and CTLA-4 were higher on activated B cells when compared to the other B cell subsets.

The relevance of PD-1 expression on $CD4^+$ T cells has been well described in patients with CLL with an advanced stage (Rai III/IV) (25). To date the exhaustion levels of B cell subsets in patients with CLL has not been investigated. Immune system dysfunction is a hallmark of CLL, which primarily affects humoral immunity and is characterized by increased susceptibility to autoimmune disorders and secondary malignancies (26, 27).

Immune checkpoint proteins such as PD-1, PD-L1, PD-L2 and CTLA-4 are expressed on activated B cells (28, 29). The function of these immune checkpoints on B cells have not been fully evaluated. However, the binding of PD-L1 or PD-L2 to PD-1 receptors on T cells is known to induce phosphorylation of ITIM (Immunoreceptor Tyrosine-Based Inhibitory Motif) and ITSM (Immunoreceptor Tyrosine-Based Switch Motif) motifs within the PD-1 receptor (30). This phosphorylation leads to the recruitment of phosphatases like Src Homology 2 Domain-Containing Protein Tyrosine Phosphatase 2 and Src Homology 2 Domain-Containing Protein Tyrosine Phosphatase 1 (30, 31). Consequently, these phosphatases dephosphorylate key signaling molecules downstream of the T cell receptor (TCR), such as PI3K (Phosphoinositide 3-Kinase) and AKT (Protein Kinase B) (32-34). The overall effect of PD-1 binding is to inhibit the proliferation of B cells, leading to immune suppression and tumor immune evasion (35).

In our study, we further observed a significant increase in PD-1 levels on B cells and memory B cells among patients with CLL. Furthermore, PD-L2 expression was lower across the B cell subsets in patients with CLL. These findings underscore the complex and dynamic interplay between immune checkpoint molecules and distinct B cell populations within the CLL microenvironment. PD-1 function in T cells is widely studied, and is associated with T cell exhaustion in patients with CLL (36). However, its role on B cell is not known. In our study, we showed that PD-1 is expressed strongly on activated B cells. Thibult et al., (35) showed that PD-1 and its ligands (PD-L1 and PD-L2) are key regulatory proteins of B-cell activation and entry into the germinal center. Moreover, the study demonstrated that PD-1 is the inhibitor of the Toll like receptor (TLR)-ligand-mediated activation of B cells. The increased expression of PD-1 may inhibit B cell differentiation into antibody secreting cells. PD-1 has a higher affinity for PD-L1 than PD-L2 (35, 37), this may explain the significant decrease in PD-L2 expression in patients with CLL.

In the present study we observed a significant increase in CTLA-4 levels on B cells and memory B cells among patients with CLL. CTLA-4 suppresses humoral response to T cell-dependent and independent antigen (38). In addition, elevated CTLA-4 expression on B cells is associated with disease progression

in patients with CLL (39). Moreover, the elevated expression of this immune checkpoint in peripheral blood inhibit B cell activation and proliferation (40). Consistent to this study, our study found increased levels of CTLA-4 expression in B cell subsets. Therefore, the use of immune checkpoint inhibitors targeting CTLA-4 in B cells may be beneficial in patients with CLL.

In the present study, we determined the relationship between immune checkpoints on B cell subsets and B2M. There was no correlation between B2M levels and the expression of immune checkpoint molecules on B cell subsets. The elevated B2M levels are associated with an advanced CLL stage (41). In CLL, B2M levels can be elevated due to increased turnover of CLL cells or other factors. The elevated B2M is associated with advanced CLL stage (41). While immune checkpoints are dysregulated in CLL, B2M levels do not directly correlate with the expression of these checkpoints in B cell subsets. This suggests that the expression of these immune checkpoints in B cell subsets do not directly influence B2M levels. However, on CLL international prognostic index (IPI), we only investigated correlation with β2-microglobulin.

Conclusion

In our cohort, patients with CLL exhibits increased levels of PD-1 and CTLA-4 on B cell subsets in patients with CLL. However, the expression of these immune checkpoint molecules on B cell subsets do not correlate with B2M levels.

Author Contributions

Conceptualization, A.N., B.B.N., P.V.D.; Methodology, A.N., B.B.N., Z.A.M.; Formal Analysis, A.N., B.B.N.; Investigation, A.N., B.B.N., P.V.D.; Data Curation, A.N., B.B.N., Z.A.M.; Writing – Original Draft Preparation, A.N.; Writing – Review & Editing, A.N., B.B.N., P.V.D.; Visualization, A.N., B.B.N., P.V.D.; Supervision, B.B.N., P.V.D.; Project Administration, A.N.

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Disclosure

The authors declare that there are was no conflict of interest.

Ethics

The ethical approval for this study was granted by the University of KwaZulu-Natal Biomedical Research Ethics Committee (study approval no. BE456/18).

References

1. DeSantis CE, Miller KD, Dale W, Mohile SG, Cohen HJ, Leach CR, et al. Cancer statistics for adults aged 85 years and older, 2019. 2019;69(6):452-67.
2. Yang S, Varghese AM, Sood N, Chiattone C, Akinola NO, Huang X, et al. Ethnic and geographic diversity of chronic lymphocytic leukaemia. Leukemia. 2021;35(2):433-9.
3. Lanasa MCJH, the American Society of Hematology Education Program Book. Novel insights into the biology of CLL. 2010;2010(1):70-6.
4. Gupta SL, Khan N, Basu S, Soni V. B-cell-based immunotherapy: A promising new alternative. Vaccines. 2022;10(6):879.
5. Schwartz M, Zhang Y, Rosenblatt JD. B cell regulation of the anti-tumor response and role in carcinogenesis. Journal for immunotherapy of cancer. 2016;4:1-15.
6. Palma M, Gentilcore G, Heimersson K, Mozaffari F, Näsman-Glaser B, Young E, et al. T cells in chronic lymphocytic leukemia display dysregulated expression of immune checkpoints and activation markers. 2017;102(3):562-72.
7. Zhang X, Zhang H, Chen L, Feng Z, Gao L, Li QJCi. TIGIT expression is upregulated in T cells and causes T cell dysfunction independent of PD-1 and Tim-3 in adult B lineage acute lymphoblastic leukemia. 2019;344:103958.
8. Ding W, LaPlant BR, Call TG, Parikh SA, Leis JF, He R, et al. Pembrolizumab in patients with CLL and Richter transformation or with relapsed CLL. Blood, The Journal of the American Society of Hematology. 2017;129(26):3419-27.
9. Brown JR, Eichhorst B, Hillmen P, Jurczak W, Kaźmierczak M, Lamanna N, et al. Zanubrutinib or ibrutinib in relapsed or refractory chronic lymphocytic leukemia. New England Journal of Medicine. 2023;388(4):319-32.
10. Dal Bo M, Bulian P, Bomben R, Zucchetto A, Rossi F, Pozzo F, et al. CD49d prevails over the novel recurrent mutations as independent prognosticator of overall survival in chronic lymphocytic leukemia. Leukemia. 2016;30(10):2011-8.
11. Wierda W. New prognostic factors in chronic lymphocytic leukemia. Clinical Advances in Hematology & Oncology: H&O. 2009;7(1):32-3, 42.
12. Al-Rekabi AN, Alwan AF, Alobaidi NK. Assessment of beta-2 microglobulin and CD49d in patients with chronic lymphocytic leukemia pre-and posttherapy. Iraqi Journal of Hematology. 2020;9(2):155-9.
13. Jaatinen T, Laine J. Isolation of Mononuclear Cells from Human Cord Blood by Ficoll-Paque Density Gradient. Current Protocols in Stem Cell Biology. 2007;1(1):2A.1.-2A.1.4.
14. Axelsson S, Magnuson A, Lange A, Alshamari A, Hörnquist EH, Hultgren O. A combination of the activation marker CD86 and the immune checkpoint marker B and T lymphocyte attenuator (BTLA) indicates a putative permissive activation state of B cell subtypes in healthy blood donors independent of age and sex. BMC Immunology. 2020;21(1):14.
15. Joscelyn J, Ochoa-Repáraz J, Kasper L. Principles of Immunotherapy. Clinical Neuroimmunology: Springer; 2020. p. 17-42.
16. Mkhwanazi ZA, Nyambuya TM, Mfusi SA, Nkambule BB. Prognostic markers in patients with chronic lymphocytic leukaemia on targeted therapy, chemoimmunotherapy with anti-CD20 monoclonal antibody: a systematic review and meta-analysis of prognostic factors. BMC cancer. 2022;22(1):1218.
17. Team RC. A Language and Environment for Statistical Computing 2021 [Available from: https://www.r-project.org/.
18. Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Döhner H, et al. 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, The Journal of the American Society of Hematology. 2008;111(12):5446-56.

19. Damle RN, Temburni S, Calissano C, Yancopoulos S, Banapour T, Sison C, et al. CD38 expression labels an activated subset within chronic lymphocytic leukemia clones enriched in proliferating B cells. Blood, The Journal of the American Society of Hematology. 2007;110(9):3352-9.
20. Gentile M, Morabito F, Del Poeta G, Mauro FR, Reda G, Sportoletti P, et al. Survival risk score for real-life relapsed/refractory chronic lymphocytic leukemia patients receiving ibrutinib. A campus CLL study. Leukemia. 2021;35(1):235-8.
21. Amaya-Chanaga CI, Rassenti LZ. Biomarkers in chronic lymphocytic leukemia: Clinical applications and prognostic markers. Best Pract Res Clin Haematol. 2016;29(1):79-89.
22. Soma LA, Craig FE, Swerdlow SH. The proliferation center microenvironment and prognostic markers in chronic lymphocytic leukemia/small lymphocytic lymphoma. Human pathology. 2006;37(2):152-9.
23. Khoudoleeva O, Gretsov E, Barteneva N, Vorobjev I. Proliferative index and expression of CD38, Zap-70, and CD25 in different lymphoid compartments of chronic lymphocytic leukemia patients. Pathology and Laboratory Medicine International. 2011:7-16.
24. Patten PE, Buggins AG, Richards J, Wotherspoon A, Salisbury J, Mufti GJ, et al. CD38 expression in chronic lymphocytic leukemia is regulated by the tumor microenvironment. Blood, The Journal of the American Society of Hematology. 2008;111(10):5173-81.
25. Rusak M, Eljaszewicz A, Bołkun Ł, Łuksza E, Łapuć I, Piszcz J, et al. Prognostic significance of PD-1 expression on peripheral blood CD4+ T cells in patients with newly diagnosed chronic lymphocytic leukemia. Pol Arch Med Wewn. 2015;125(7-8):553-9.
26. Hallek M, Shanafelt TD, Eichhorst B. Chronic lymphocytic leukaemia. Lancet. 2018;391(10129):1524-37.
27. Forconi F, Moss P. Perturbation of the normal immune system in patients with CLL. Blood. 2015;126(5):573-81.
28. Yang Y, Li X, Ma Z, Wang C, Yang Q, Byrne-Steele M, et al. CTLA-4 expression by B-1a B cells is essential for immune tolerance. Nat Commun. 2021;12(1):525.
29. Yamazaki T, Akiba H, Iwai H, Matsuda H, Aoki M, Tanno Y, et al. Expression of programmed death 1 ligands by murine T cells and APC. J Immunol. 2002;169(10):5538-45.
30. Riley JL. PD-1 signaling in primary T cells. Immunological reviews. 2009;229(1):114-25.
31. Lorenz U. SHP-1 and SHP-2 in T cells: two phosphatases functioning at many levels. Immunological reviews. 2009;228(1):342-59.
32. Boussiotis VA, Chatterjee P, Li L. Biochemical signaling of PD-1 on T cells and its functional implications. Cancer journal (Sudbury, Mass). 2014;20(4):265.
33. Patsoukis N, Wang Q, Strauss L, Boussiotis VA. Revisiting the PD-1 pathway. Science advances. 2020;6(38):eabd2712.
34. Chemnitz JM, Parry RV, Nichols KE, June CH, Riley JL. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. The Journal of Immunology. 2004;173(2):945-54.
35. Thibult ML, Mamessier E, Gertner-Dardenne J, Pastor S, Just-Landi S, Xerri L, et al. PD-1 is a novel regulator of human B-cell activation. Int Immunol. 2013;25(2):129-37.
36. Riches JC, Davies JK, McClanahan F, Fatah R, Iqbal S, Agrawal S, et al. T cells from CLL patients exhibit features of T-cell exhaustion but retain capacity for cytokine production. Blood, The Journal of the American Society of Hematology. 2013;121(9):1612-21.
37. Ghiotto M, Gauthier L, Serriari N, Pastor S, Truneh A, Nunès JA, et al. PD-L1 and PD-L2 differ in their molecular mechanisms of interaction with PD-1. International immunology. 2010;22(8):651-60.
38. Linsley PS, Wallace PM, Johnson J, Gibson MG, Greene JL, Ledbetter JA, et al. Immunosuppression in vivo by a soluble form of the CTLA-4 T cell activation molecule. Science. 1992;257(5071):792-5.

39. Kosmaczewska A, Ciszak L, Suwalska K, Wolowiec D, Frydecka I. CTLA-4 overexpression in CD19+/CD5+ cells correlates with the level of cell cycle regulators and disease progression in B-CLL patients. Leukemia. 2005;19(2):301-4.
40. Karabon L, Partyka A, Ciszak L, Pawlak-Adamska E, Tomkiewicz A, Bojarska-Junak A, et al. Abnormal expression of BTLA and CTLA-4 immune checkpoint molecules in chronic lymphocytic leukemia patients. Journal of Immunology Research. 2020;2020.
41. Montillo M, Hamblin T, Hallek M, Montserrat E, Morra E. Chronic lymphocytic leukemia: novel prognostic factors and their relevance for risk-adapted therapeutic strategies. Haematologica. 2005;90(3):391-9.

Prologue

This chapter evaluated the effect of protein kinase C activation on B cell subsets levels and immune checkpoint expression. We further explored the effects of immune checkpoint blockage on B cell profile and immune checkpoint expression. The current study showed that protein kinase C stimulation of B cells elevate the levels of PD-1, PD-L1, PD-L2 and CTLA-4 expression on B cell subsets. We also showed that immune checkpoint blockage increases the levels of memory B cells and activated memory B cell while reducing the levels of CTLA-4, PD-1, PD-L1 and PD-L2 expression on total B cells.

CHAPTER 4: Research article 2

The B-cell function in patients with chronic lymphocytic leukemia

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Abstract

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the proliferation of dysfunctional B cells, resulting in significant immune dysregulation. Patients with CLL exhibit varied responses to B-cell receptor (BCR) targeted therapies, emphasizing the need for tailored immunotherapy approaches. This study investigated B cell function in untreated patients with CLL, and we further explored the effects of ex vivo protein kinase C activation on immune checkpoint expression and B cell profiles.

Methods

Peripheral blood samples were collected from 21 untreated patients with CLL at King Edward Hospital in South Africa, between 2019 and 2022. B cells were stimulated with phorbol myristate acetate (PMA) and ionomycin. Using flow cytometry, the study explored the levels of B cell subsets and immune checkpoint proteins programmed cell death-ligand 2 (PD-L2) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4) expression on various B cell subsets.

Results

PMA and ionomycin B cell stimulation upregulated CTLA-4, PD-1 and PD-L2 expression on B cell subsets ($p<0.05$). As expected, monoclonal antibodies targeting PD-1, PD-L1 and CTLA-4 significantly downregulated the CTLA-4 expression of B cell subsets ($p < 0.05$), and PD-L1 on total B cells ($p < 0.01$) while PD-L2 exhibited varied responses in different B cell subsets. In addition, these monoclonal antibodies increased the levels of memory B cells ($p < 0.0128$) and activated memory B cells ($p < 0.01$).

Conclusion

Protein kinase C activation on B cells stimulates immune checkpoint expression. The use of monoclonal antibodies on B cells play a critical role in the B cell function through the reduction of CD38 expressing activated B cells and upregulation of memory B cells. Moreover, the monoclonal antibody targeting PD-1, PD-L1 and CTLA-4 are effective in reducing the expression of CTLA-4 on B cell subsets.

Keywords: Chronic lymphocytic leukaemia; Immune checkpoint inhibitor; B cell subsets; Ionomycin; Phorbol myristate acetate

1. Introduction

Aberrations in adaptive immune responses, are characteristic features of chronic lymphocytic leukemia (CLL) and drive immune suppression from the early stages of the disease (1, 2). Consequently, immune dysfunction increases risk of secondary malignancies and infections, which are the main contributors to morbidity and mortality in patients with CLL (3). Although immunosuppression is a state that is already evident in early stages of CLL, disease progression or poor response to therapy is a consequence of immune dysfunction (4). It has in fact become apparent that understanding immune responses is a vital aspect to understand disease pathogenesis, which could potentially lead to the discovery of novel immunotherapies (5).

In patients with CLL, B cell function is altered (6, 7). The B-cell receptor (BCR) signaling pathway is one of the key factors contributing to the anti-apoptotic responses of malignant B cells in patients with CLL (8, 9). These signals contribute to cell survival and proliferation of malignant B cells in CLL (10). In addition, malignant B cells in patients with CLL express chemokine receptors like CXCR4, which bind to CXCL12 in the bone marrow microenvironment (11). This interaction supports malignant B cell homing to the bone marrow and provides survival signals (12).

Activated B cells secrete immune checkpoints such as programmed cell death protein 1 (PD-1) to maintain immune tolerance, to suppress pathological autoimmune and inflammatory immune responses (13). In patients with CLL, this BCR signaling pathway is disrupted (14, 15). Phorbol 12-myristate 13-acetate (PMA) activates the protein kinase C (PKC) pathway, specifically the classical PKC isoforms (PKCα, PKCβ, and PKCγ) by mimicking the action of diacylglycerol (DAG), a second messenger generated during BCR signaling (16).

Zanubrutinib, a Bruton's tyrosine kinase inhibitor, is associated with fewer incidence of adverse events and improved progression-free survival in patients with CLL (17, 18). In addition, Immune checkpoint inhibition using monoclonal antibodies (mAbs) that target the cytotoxic T lymphocyte-associated protein 4 (CTLA-4) or PD-1 pathway has reshaped the landscape of therapeutic strategies for patients with CLL. Pembrolizumab, a PD-1-targeting agent, is associated with an improved overall survival in patients with CLL (19). However, a recent study, showed that patients with CLL may have variable treatment responses to CTLA-4 inhibitors, with unfavorable treatment outcomes in patients with high CTLA-4 expression (20). CTLA-4 can regulate B-cell activation both inside and outside of the germinal center, and this regulation can occur independently of CD80 and CD86 which are important co-stimulatory molecules found on antigen-presenting cells (APCs) (21). Therefore, CTLA-4's role in inhibiting B-cell activation still need to studied. By modeling immune checkpoint blockage under different immunological activation and exhaustion settings we may stratify patients with CLL who will benefit from immunotherapy. We hypothesized that B cell stimulation would increase the levels of immune checkpoint expression on B cell subsets. The aim of this study was to investigate B cell function

in untreated patients with CLL, and to further explore the effects of ex vivo protein kinase C activation on immune checkpoint expression and B cell profiles.

2. Methods and Materials

2.1 Patient recruitment

We recruited patients at King Edward VIII Hospital, a tertiary healthcare institution in Durban, KwaZulu-Natal, South Africa from July 2019 and May 2022. Ethical approval was obtained from the University of KwaZulu-Natal Biomedical Research Ethics Committee (BE456/18), South Africa. All participants provided written informed consent.

2.2 Inclusion and exclusion criteria

We included 21 untreated patients with CLL along with 12 age-matched healthy controls with no clinical signs of infection. Treated patients with CLL were excluded. Based on the treatment initiation criteria, 16 patients were on a "watch and wait" approach and 5 had a treatment indication at the time of sample collection (22).

2.3 Sample collection

Five millilitres (5mL) of peripheral blood samples was collected from consenting participants through venipuncture, using 6 mL ethylenediaminetetraacetic acid (EDTA) tubes (BD Bioscience, USA). Subsequently, the samples were transported under room temperature conditions (20-25°C) from the hospital to the laboratory. The samples were processed within 1–2 h of sample collection.

2.4 Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples using the density-gradient centrifugation method with the use of Ficoll-Paque PLUS (Amersham, Biosciences, Uppsala, Sweden), as previously described (23). Briefly, 3 mL of Ficoll-Paque PLUS was aliquoted into a 15 mL centrifuge tube (Sigma-Aldrich, Germany), followed by the careful layering of 4 mL of whole blood onto the Ficoll-Paque PLUS gradient. This was followed by subsequent centrifugation at 400g for 40 minutes at 20°C, the collected PBMCs was stored at -80°C.

2.5 T cell depletion and B cell isolation from peripheral blood mononuclear cells

To enrich the B cell population within the obtained PBMCs, T cell depletion and positive B cell selection were conducted using the BD IMag isolation system (BD Bioscience, USA). Briefly, 50 µL of PBMCs were incubated with 5 µL of biotinylated human T lymphocyte enrichment cocktail (BD Biosciences, San Jose, CA, USA) for 15 minutes at room temperature. Following the incubation, 50 µL of streptavidin particles were introduced to the T cell-depleted PBMC samples and incubated for 30 minutes at room temperature. The samples were then reconstituted into 1 mL of 3.2% sodium citrated

buffer and placed on the BD IMag for 8 minutes. Isolated B cells were reconstituted into 100 µL phosphate-buffered saline (PBS) and stored at -80 ºC.

2.6 Stimulation and inhibition assay

To assess B-cell function in patients with CLL, B cells were treated with anti-CTLA-4, anti-PD-L1 and anti-PD-1 monoclonal antibodies at a concentration of 10µg/ml for an hour (24). Then stimulated as described previously (25, 26). Briefly, B cells were suspended in a complete medium [RPMI 1640 supplemented with 5% foetal bovine serum (FBS) and 1% liquid penicillin/streptomyosin (Biowest, USA)] and stimulated with phorbol 12-myristate 13-acetate (PMA) (12.5 ng/ml; Cayman chemical, Michigan, USA) and ionomycin (1.25 µg/ml; Cayman chemical, Michigan, USA) for 12h, at 37°C with 5% CO_2. For the stimulation and inhibition assays, the following co-culture conditions were used; (1) PMA & ionomycin stimulated B cells; (2) PMA & ionomycin stimulated B cells plus anti-PD-1; (3) PMA & ionomycin stimulated B cells plus anti-PD-L1; (4) PMA & ionomycin stimulated B cells plus anti-CTLA-4.

2.7 Measurements of B cell subsets

To quantify B cell subsets, we made use of a six-colour flow cytometry panel consisting of CD38-FITC, CTLA-4-PE, PD-L2-APC, CD19-PE/Cy7, CD27-APC/Cy7, PD-1-PB450, PD-L1-PE and Zombie Aqua-BV421 (BioLegend, San Diego, CA, USA). Viable cells were defined as Zombie Aqua dye negative (Figure 4.1A). We acquired at least 5000 B cells ($CD19^+$ events) (Figure 4.1B) and defined memory B cells (B_{MEM}) as $CD19^+CD27^+$ events (27, 28), activated B cells as $CD19^+CD27^-CD38^+$ events and activated memory B cells as $CD19^+CD27^+CD38^+$ events (28) (Figure 4.1C).

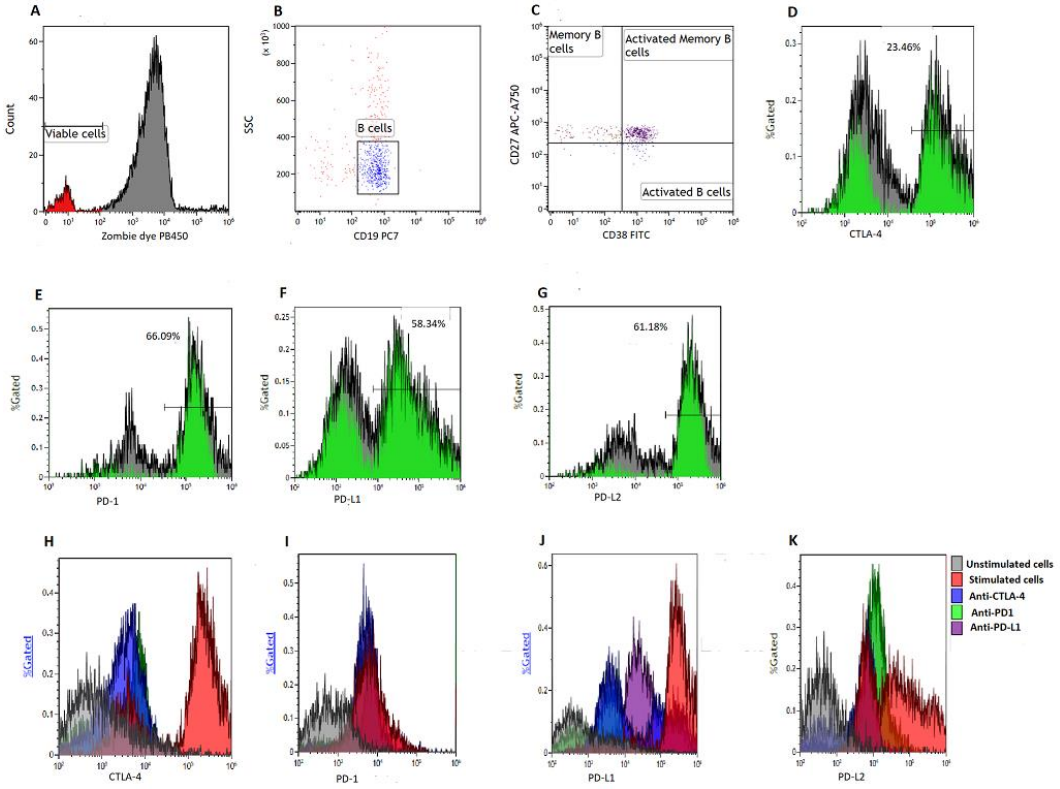

Figure 4.1: Gating strategy. B cells were isolated using magnetic bead sorting. Figure A Illustrates the gates applied to distinguish between viable and non-viable B cells based on a Zombie Aqua dye. Figure B illustrates the gating on B cells based on side scatter (SSC) and CD19 expression. Figure C illustrates the gating of activated B cells defined as $CD19^{+}CD38^{+}CD27^{-}$ events, memory B cells defined as $CD19^{+}CD27^{+}CD38^{-}$ events and activated memory B cells defined as $CD19^{+}CD27^{+}CD38^{+}$ events, respectively. Figure D-G illustrates the gating of CTLA-4, PD-1, PD-L1 and PD-L2, respectively. Histograms were used to demonstrate the levels of CTLA-4 (**H**), PD-1 **(I)**, PD-L1 **(J)**and PD-L2 expression (**K**) on various co-culture conditions. PB450 (pacific blue 450), PC7 (phycoerythrin-cyanine 7), CD (cluster of differentiation), SSC (side scatter), FITC (fluorescein isothiocyanate), APC-A750 (allophycocyanin-cyanine 750), PD-1 (programmed cell death protein 1), PD-L1 (programmed death-ligand 1), PD-L2 (programmed death-ligand 2), CTLA-4 (cytotoxic T-lymphocyte-associated protein 4).

2.8 Measurements of immune checkpoint levels on B cell subsets

To determine the levels of immune checkpoint expression on B cell subsets, we measured the expression of PD-1, PD-L1, PD-L2 and CTLA-4 on B_{MEM}, and activated B cells. A total of 5 000 $CD19^{+}$ events were acquired at a medium flow rate using the Beckman Coulter DxFLEX flow cytometer (Beckman coulter, Inc Brea, CA, USA) and analyzed using Kaluza version 1.2 (Beckman coulter, Inc Brea, CA, USA).

2.9 Sample Size Estimation

We determined the minimum sample size of patients required to detect a large effect size (d) of 1.14 in the expression of immune checkpoints, at 80% power and alpha (α) of 0.05 using GPower 3.1.94 software (Universität, Düsseldorf, Germany). To detect a large effect size between two independent means using an unpaired t-test, we needed a minimum of twenty-one patients with CLL (n = 21) and twelve controls (n = 12).

2.10 Statistical analysis

All statistical analyses were performed using GraphPad Prism version 8 software, (GraphPad Software Inc, San Diego, CA, USA). A repeated measures one-way ANOVA was used to compare parametric data with Dunnett's as a post hoc test. The Friedman test was used to compare nonparametric data of the same group with Dunn's as a post hoc test for multiple comparisons. To correct for multiple comparisons, a Bonferroni-corrected critical p-value of <0.0167 was considered as statistically significant. Non-parametric data were reported as the median IQR and parametric data was presented as mean ± SD.

3. Results

3.1 Patients characteristics

This study comprised of 21 patients with CLL, with the mean age of the 62.33 ± 13.31 years. The included study patients were multi-ethnic, and comprised of African (n=19), Indian (n=1) and European (n=1). The study included 33.33% females and 66.67% males (Table 4.1).

Table 4.1. The baseline characteristics and haematological parameters of the participants.

	Control (n=12)	Patients with CLL (21)	p-Value
Age (Years)	56.58 ± 15.67	62.33 ± 13.31	0.2714
Male,n (%)	58.33	61.9	
Female, n (%)	41.67	38.1	
White blood cell count (10^3µL)	5.26 ± 1.38	130.4 ± 29.71	0.0005
Red blood cell (10^6µL)	4.74 ± 0.94	2.10 ± 0.84	< 0.0001
Haemoglobin (g/dL)	14.13 ± 3.81	8.19 ± 2.30	< 0.0001
Platelets (10^3µL)	210.4 ± 73.14	157.5 ± 141.9	0.1831

3.2 Increased memory B cell levels and a reduction in activated B cells following immune checkpoint inhibition

In our B cell stimulation assays, there was no significant difference in the levels of activated B cells following PMA and ionomycin B cell stimulation 7.630 (8.060-7.350) when compared to the baseline levels 57.54 (64.57-52.97), p = 0.2480 (Figure 4.2A). However, there was a significant decrease in the levels of activated B cells following anti-CTLA-4 treatment 3.340 (4.085-2.410) when compared to

PMA & ionomycin stimulated B cells 7.630 (8.060-7.350), p < 0.0001; anti-PD-1 treatment 4.300 (5.240-3.300) when compared to PMA & ionomycin stimulated B cells 7.630 (8.060-7.350), p = 0.0044; and anti-PD-L1 treatment 4.630 (4.940-3.505) when compared to PMA & ionomycin stimulated B cells 7.630 (8.060-7.350), p = 0.0044.

Furthermore, there was no significant difference in the levels of memory B cells following PMA & ionomycin B cell stimulation 92.47 (92.71-91.99) when compared to the baseline levels 42.70 (45.76-33.40), p = 0.1917 (Figure 4.2B). However, there were significant increase in the levels of memory B cells following anti-CTLA-4 treatment 96.48 (97.37-95.50) when compared to PMA & ionomycin stimulated B cells 92.47 (92.71-91.99), p < 0.0001; anti-PD-1 treatment 95.65 (96.38- 94.79) when compared to PMA & ionomycin stimulated B cells 92.47 (92.71-91.99), p = 0.0064; and anti-PD-L1 treatment 95.30 (96.30-94.71) when compared to PMA & ionomycin stimulated B cells 92.47 (92.71-91.99), p = 0.0128.

Moreover, there was no significant difference in the levels of activated memory B cells following PMA & ionomycin B cell stimulation 90.81 (91.11-90.17) when compared to the baseline levels 37.82 (43.21-30.74), p = 0.2480 (Figure 4.2C). However, there were significant increase in the levels of activated memory B cells following anti-CTLA-4 treatment 95.99 (96.90-94.29) when compared to PMA & ionomycin stimulated B cells 90.81 (91.11-90.17), p = 0.0003; anti-PD-1 treatment 94.73 (95.45-93.43) when compared to PMA & ionomycin stimulated B cells 90.81 (91.11-90.17), p = 0.0044; and anti-PD-L1 treatment 94.41 (95.60- 93.66) when compared to PMA & ionomycin stimulated B cells 90.81 (91.11-90.17), p = 0.0091.

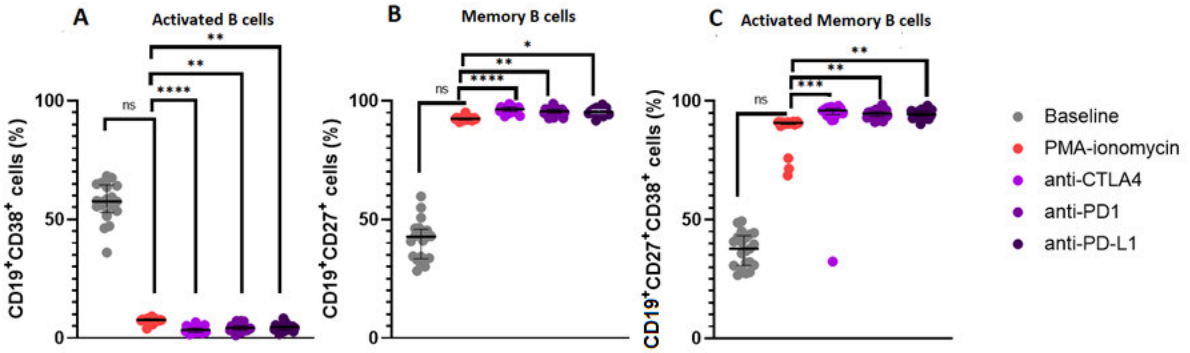

Figure 4.2: B Cell Subsets in patients with CLL upon B cell stimulation and immune checkpoint inhibition. Figure **A** illustrates the expression levels of activated B cells, **(B)** memory B cells, and **(C)** activated memory B cells. The data is presented as the median ± interquartile range (IQR). CD (cluster of differentiation), PMA (phorbol-12-myristate-13-acetate), PD-1 (programmed cell death protein 1), PD-L1 (programmed death-ligand 1), CTLA-4 (cytotoxic T-lymphocyte-associated protein 4); *, **, *** and **** shows the level of significance between groups, ns: not significant.

3.3 Increased levels of CTLA-4 expression on B cell subsets following B cell stimulation and reduction following immune checkpoint inhibition

The levels of CTLA-4 expression were significantly increased on the B cells following PMA & ionomycin B cell stimulation 81.49 (82.23-81.03) when compared to the baseline levels 3.270 (5.650-1.310), $p < 0.0001$ (Figure 4.3A). There was a significant decrease in the levels of CTLA-4 expression on B cells following anti-CTLA-4 treatment 41.09 (50.24-32.06) when compared to PMA & ionomycin stimulated B cells 81.49 (82.23-81.03), $p < 0.0001$; anti-PD-1 treatment 48.11 (64.42-41.25) when compared to PMA-ionomycin stimulated B cells 81.49 (82.23-81.03), $p = 0.0341$; and anti-PD-L1 treatment 44.92 (54.89-37.30) when compared to PMA & ionomycin stimulated B cells 81.49 (82.23-81.03), $p = 0.0004$ (Figure 4.3A).

Furthermore, levels of CTLA-4 expression were increased on activated B cells following PMA & ionomycin B cell stimulation 80.13 (80.75-79.75) when compared to the baseline levels 3.300 (5.650-1.310), $p < 0.0001$ (Figure 4.3B). However, the levels of CTLA-4 expression were decreased following anti-CTLA-4 treatment 40.36 (49.05-31.40) when compared to PMA & ionomycin stimulated B cells 80.13 (80.75-79.75), $p < 0.0001$; anti-PD-1 treatment 47.04 (63.21-40.26) when compared to PMA & ionomycin stimulated B cells 80.13 (80.75-79.75), $p = 0.0341$; and anti-PD-L1 treatment 44.13 (53.44-36.28) when compared to PMA & ionomycin stimulated B cells 80.13 (80.75-79.75), $p = 0.0003$.

Moreover, the levels of CTLA-4 expression were significantly increased on memory B cells following PMA & ionomycin B cell stimulation 77.28 (77.94-76.51) when compared to the baseline levels 2.880 (5.460-1.220), $p < 0.0001$ (Figure 4.3C). However, the levels of CTLA-4 expression were decreased following anti-CTLA-4 treatment 38.68 (47.68-30.36) when compared to PMA & ionomycin stimulated B cells 77.28 (77.94-76.51), $p < 0.0001$; anti-PD-1 treatment 45.03 (60.98-39.39) when compared to PMA & ionomycin stimulated B cells 77.28 (77.94-76.51), $p = 0.0341$; and anti-PD-L1 treatment 42.91 (51.01-35.33) when compared to PMA & ionomycin stimulated B cells 77.28 (77.94-76.51), $p = 0.0004$.

3.4 Increased levels of PD-1 expression on B cell subsets following B cell stimulation

The levels of PD-1 expression were significantly increased on the B cells following PMA & ionomycin B cell stimulation (82.61±7.960) when compared to the baseline levels (53.33±16.18), $p = 0.0132$ (Figure 4.3A). However, the PD-1 expression levels on B cells following anti-PD-1 treatment (63.50±9.042), $p = 0.0168$ and anti-PD-L1 treatment (56.18± 13.36), $p = 0.0082$ were significantly decreased when compared to that of the PMA & ionomycin stimulated B cells (82.61±7.960). The PD-1 levels on B cells following anti-CTLA-4 (57.54±21.27) were comparable to that of the PMA & ionomycin stimulated B cells (82.61±7.960), $p = 0.0781$.

Furthermore, levels of PD-1 expression were significantly increased on activated B cells following PMA & ionomycin B cell stimulation 78.41 (86.93-76.06) when compared to the baseline levels 65.68 (73.65- 60.24), p = 0.0053 (Figure 4.3B). However, the levels of PD-1 expression on activated B cells following anti-CTLA-4 treatment 73.85 (83.17-63.84), p = 0.7029; anti-PD-1 treatment 72.62 (75.78-70.26), p = 0.3838 and anti-PD-L1 treatment 73.16 (78.56 -67.25), p = 0.5327 were comparable to the PMA & ionomycin stimulated B cells 78.41 (86.93 -76.06).

In addition, the PD1 expression levels on memory B cells following PMA & ionomycin B cell stimulation 72.15 (78.82 -67.66) were increased when compared to that of the baseline levels 36.85 (47.81-33.84), p = 0.0011 (Figure 4.3C). Interestingly, the PD-1 expression levels were significantly decreased following anti-CTLA-4 treatment 50.61 (60.22-42.84), p = 0.0185. However, these levels were comparable following anti-PD-1 treatment 70.76 (77.89-64.00), p > 0.9999; and anti-PD-L1 treatment 66.13 (72.97-53.97), p > 0.9999 when compared to PMA-ionomycin stimulated B cells 72.15 (78.82-67.66).

3.5 PD-L1 expression levels on B cell subsets following B cell stimulation and reduction following immune checkpoint inhibition

The PD-L1 expression levels on B cells following PMA & ionomycin B cell stimulation 52.00 (60.05-46.62) were comparable to the baseline levels 44.75 (52.71-31.23), p = 0.0881 (Figure 4.3A). However, PD-L1 expression levels were significant decrease on B cells following anti-CTLA-4 treatment 24.45 (31.52-16.97) when compared to PMA & ionomycin stimulated B cells 52.00 (60.05-46.62), p = 0.0066; anti-PD-1 treatment 24.56 (27.35-18.94) when compared to PMA-ionomycin stimulated B cells 52.00 (60.05 -46.62), p = 0.0003; and anti-PD-L1 treatment 28.65 (31.96 -24.09) when compared to PMA & ionomycin stimulated B cells 52.00 (60.05 -46.62), p = 0.0002.

The PD-L1 expression levels on activated B cells following PMA & ionomycin B cell stimulation (51.71±13.40) were increased when compared to the baseline levels (37.49±17.29), p = 0.0231 (Figure 4.3B). However, these levels decreased following anti-PD-1 treatment (24.55±5.647) when compared to PMA & ionomycin stimulated B cells (51.71±13.40), p = 0.0005 and anti-PD-L1 treatment (31.26±8.056) when compared to PMA-ionomycin stimulated B cells (51.71±13.40), p = 0.0021. However, the levels were comparable following anti-CTLA-4 treatment (37.73±12.88) when compared to PMA & ionomycin stimulated B cells (51.71±13.40), p = 0.0963.

Furthermore, the PD-L1 expression levels on memory B cells following PMA & ionomycin B cell stimulation 59.87 (64.40-55.31) were comparable when compared to the baseline levels 58.26 (62.75-46.22), p > 0.9999 (Figure 4.3C). However, PD-L1 expression was decreased following anti-PD-1 treatment 30.35 (37.51-18.22) when compared to PMA & ionomycin stimulated B cells 59.87 (64.40-55.31), p = 0.0002; and anti-PD-L1 treatment 36.60 (49.13-26.59) when compared to PMA & ionomycin stimulated B cells 59.87 (64.40-55.31), p = 0.0298. However, PD-L1 expression was

comparable following anti-CTLA-4 treatment 42.21 (52.65-30.92) when compared to PMA & ionomycin stimulated B cells 59.87 (64.40-55.31), p = 0.6599.

3.6 Increased levels of PD-L2 expression on B cell subsets following B cell stimulation and varied expression following immune checkpoint inhibition

Levels of PD-L2 expression were significantly increased on B cells following PMA & ionomycin B cell stimulation (99.58±0.06966) when compared to the baseline levels (0.3967±0.3343), p < 0.0001 (Figure 4.3A). However, the expression of PD-L2 on B cells following anti-CTLA-4 treatment (99.33±0.3552), p = 0.0502 and anti-PD-1 treatment (99.60±0.2160), p = 0.9928 and anti-PD-L1 treatment (99.39±0.2506), p = 0.0235 was comparable to that of the PMA & ionomycin stimulated B cells (99.58±0.06966).

Furthermore, levels of PD-L2 expression were significantly increased on activated B cells following PMA & ionomycin B cell stimulation (98.26±0.1527) when compared to the baseline levels (0.3624± 0.2514), p < 0.0001 (Figure 4.3B). However, the levels of PD-L2 expression on activated B cells following anti-CTLA-4 treatment (98.55±0.6834), p = 0.2811; anti-PD-1 treatment (98.53±0.4412), p = 0.0639) and anti-PD-L1 treatment (98.41±0.3380), p = 0.2955 were comparable to the PMA & ionomycin stimulated B cells (98.26±0.1527).

In addition, the levels of PD-L2 expression on memory B cells following PMA & ionomycin B cell stimulation 82.23 (82.58-81.44) were comparable to that of the baseline levels 0.2000 (0.2600-0.08000), p = 0.4042 (Figure 4.3C). Interestingly, the PD-L2 expression was significantly increased following anti-CTLA-4 treatment 92.97 (94.62-92.24), p < 0 .0001; anti-PD-1 treatment 91.50 (92.86-89.31), p = 0.0006; and anti-PD-L1 treatment 90.66 (92.47-89.69), p = 0.0091 compared to PMA-ionomycin stimulated B cells 82.23 (82.58-81.44).

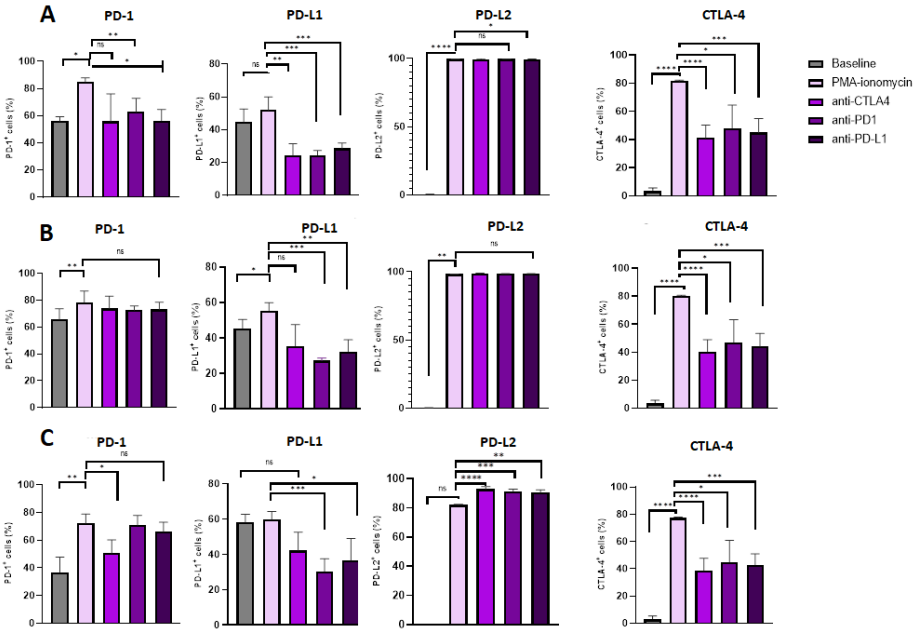

Figure 4.3: Immune checkpoint expression on B Cell subsets under different treatment conditions. The figure illustrates the levels of PD-1, PD-L1, PD-L2 and CTLA-4 expression on (A) total B cells, (B) activated B cells and (C) memory B cells, respectively. The data is presented as the median ± interquartile range (IQR). PMA (phorbol-12-myristate-13-acetate), PD-1 (programmed cell death protein 1), PD-L1 (programmed death-ligand 1), PD-L2 (programmed death-ligand 2), CTLA-4 (cytotoxic T-lymphocyte-associated protein 4); *, **, *** and **** shows the level of significance between groups, ns: not significant.

4. Discussion

The aim of this study was to investigate B cell function in untreated patients with CLL, and to further explore the effects of ex-vivo protein kinase C activation on immune checkpoint expression and B cell profiles. We previously evaluated the expression of immune checkpoints (PD-1 and CTLA-4) on B cells from patients with CLL as compared to healthy controls (29), we found that in patients with CLL, the expression of PD-1 and CTLA-4 were increased on activated B cells and memory B cells. In our study, we observed increased levels of CTLA-4, PD-1 and PD-L2 expression on total B cells and activated B cells following PMA and ionomycin B cell stimulation. The pro-survival effects of PMA on malignant B cells (30) provide a plausible explanation for increased expression of these immune checkpoints on B cell subsets. The PD-1 protein is one of the novel regulators of B cell activation (24). The PD-1 receptor interacts with PD-L1 and PD-L2 which are released upon activation of B cells (31-33). PD-L2 regulates antibody production through the inhibition of interleukin 5 (IL-5) production by T cells (34). Elevated PD-L2 expression on activated B cells in patients with CLL may lead to low levels of plasma cells. Malignant B cells expressing CTLA-4 inhibit T cell activation (35). Therefore, elevated CTLA-4 expression upon B cell activation in patients with CLL may lead to T cell exhaustion.

Immune checkpoints are important regulators of immune function and they prevent autoimmunity (36, 37). Malignant cells evade immune recognition and elimination partly due to the immunosuppression induced by upregulated immune checkpoints (38). Monoclonal antibodies that target immune checkpoint molecules have been developed and became new standard therapy for numerous malignancies, including CLL (38). Immune checkpoints such as PD-1 and CTLA-4 are dysregulated in patients with CLL (39). As a result, immune checkpoint inhibitors such as samalizumab and nivolumab targeting both B and T-cell function, are becoming more effective as a CLL therapeutic strategy (40, 41). However, there are contradictory findings on the effectiveness of immune checkpoint inhibitors in patients with CLL (19, 40, 42-44).

The present study showed that blocking CTLA-4, PD-1 and PD-L1 upregulates the levels of memory B cells and activated memory B cells. In addition, our study showed that inhibition of these immune checkpoints downregulated the CD38 expressing activated B cells. The ability of the adaptive immune system to mount quick and efficient responses to infections depends on memory B cell development (45). The accumulation of functionally incompetent matured B cells, which characterizes CLL, disrupts the normal development and function of B cells, including memory B cells. Stimulation of the memory B cell development or activation could potentially enhance the immune response against malignant B cells (46, 47). Studies have shown that higher concentrations of memory B cells in patients with CLL are associated with good prognosis (47). This may suggest that a higher percentage of memory B cells in patients with CLL could be associated with better clinical outcomes, including longer overall survival and slower disease progression. In addition, patients with CLL who have higher proportion of malignant

B cells expressing CD38 usually have a more aggressive form of the disease and a shorter overall survival (48, 49). The downregulation of activated B cells using immune checkpoint inhibitors may be beneficial for patients with CLL.

Our study demonstrated that anti-CTLA-4, anti-PD1 and anti-PD-L1 downregulates the expression of CTLA-4 and PD-L1 on the total B cell population, activated B cells and memory B cells (Figure 4.2). Whereas the expression of PD-L2 was upregulated on the memory B cells following anti-CTLA-4, anti-PD1 and anti-PD-L1. A recent study showed that CTLA-4 inhibition on CLL cell lines with an elevated expression of CTLA-4 stimulates survival of malignant cells. Whereas CTLA-4 inhibition in the low CTLA-4 expressing CLL cell lines do not affect the apoptosis (50). This suggest that anti-CTLA therapy may be unfavorable in some patients with CLL. A plausible explanation for the increased PD-L2 expression may be that PD-L2 acts as a compensatory mechanism to counteract the effect of PD-L1 blockade (51). The blocking of PD-L1 may cause malignant cells to upregulate PD-L2 and continue the suppression of the immune response. This was confirmed by the downregulation of PD-1 following immune checkpoints blockage. In addition, our study did not use monoclonal antibodies targeting PD-L2. The main limitation of this study is that monoclonal antibodies were only used as a monotherapy, we did not use combination of these therapies. Dual immunotherapy has been shown to improve immune response and overall survival rate in patients with CLL (52-54).

5. Conclusion

Protein kinase C activation on B cells stimulates immune checkpoint expression. The use of monoclonal antibodies on B cells plays a critical role on the B cell function through the reduction of CD38 expressing activated B cells and upregulation of memory B cells. Moreover, the monoclonal antibodies targeting PD-1, PD-L1 and CTLA-4 are effective in reducing the expression of CTLA-4 and PD-L1 on B cell subsets.

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Disclosure

No conflict of interest

Ethics

Ethical approval for this study was obtained from the University of KwaZulu-Natal Biomedical Research Ethics Committee (study approval no. BE456/18).

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Data Availability Statement

The data generated in this study are available upon request from the corresponding author.

References

1. Arruga F, Gyau BB, Iannello A, Vitale N, Vaisitti T, Deaglio S. Immune response dysfunction in chronic lymphocytic leukemia: dissecting molecular mechanisms and microenvironmental conditions. International journal of molecular sciences. 2020;21(5):1825.
2. Haseeb M, Anwar MA, Choi S. Molecular interactions between innate and adaptive immune cells in chronic lymphocytic leukemia and their therapeutic implications. Frontiers in immunology. 2018;9:2720.
3. Teh BW, Tam CS, Handunnetti S, Worth LJ, Slavin MAJBr. Infections in patients with chronic lymphocytic leukaemia: mitigating risk in the era of targeted therapies. 2018;32(6):499-507.
4. Morrison VAJBp, haematology rC. Infectious complications of chronic lymphocytic leukaemia: pathogenesis, spectrum of infection, preventive approaches. 2010;23(1):145-53.
5. Arruga F, Gyau BB, Iannello A, Vitale N, Vaisitti T, Deaglio SJIjoms. Immune response dysfunction in chronic lymphocytic leukemia: dissecting molecular mechanisms and microenvironmental conditions. 2020;21(5):1825.
6. Karabon L, Partyka A, Ciszak L, Pawlak-Adamska E, Tomkiewicz A, Bojarska-Junak A, et al. Abnormal expression of BTLA and CTLA-4 immune checkpoint molecules in chronic lymphocytic leukemia patients. Journal of Immunology Research. 2020;2020.
7. Solman IG, Blum LK, Hoh HY, Kipps TJ, Burger JA, Barrientos JC, et al. Ibrutinib restores immune cell numbers and function in first-line and relapsed/refractory chronic lymphocytic leukemia. Leukemia Research. 2020;97:106432.
8. Chen R, Tsai J, Thompson PA, Chen Y, Xiong P, Liu C, et al. The multi-kinase inhibitor TG02 induces apoptosis and blocks B-cell receptor signaling in chronic lymphocytic leukemia through dual mechanisms of action. Blood cancer journal. 2021;11(3):57.
9. Ondrisova L, Mraz M. Genetic and non-genetic mechanisms of resistance to BCR signaling inhibitors in B cell malignancies. Frontiers in oncology. 2020;10:591577.
10. Pascutti MF, Jak M, Tromp JM, Derks IA, Remmerswaal EB, Thijssen R, et al. IL-21 and CD40L signals from autologous T cells can induce antigen-independent proliferation of CLL cells. Blood, The Journal of the American Society of Hematology. 2013;122(17):3010-9.
11. Burger JA, editor Chemokines and chemokine receptors in chronic lymphocytic leukemia (CLL): from understanding the basics towards therapeutic targeting. Seminars in cancer biology; 2010: Elsevier.
12. Mehrpouri M. The contributory roles of the CXCL12/CXCR4/CXCR7 axis in normal and malignant hematopoiesis: A possible therapeutic target in hematologic malignancies. European Journal of Pharmacology. 2022;920:174831.
13. Han Y, Liu D, Li L. PD-1/PD-L1 pathway: current researches in cancer. American journal of cancer research. 2020;10(3):727.
14. Smith LD, Minton AR, Blunt MD, Karydis LI, Dutton DA, Rogers-Broadway K-R, et al. BCR signaling contributes to autophagy regulation in chronic lymphocytic leukemia. Leukemia. 2020;34(2):640-4.
15. Skånland SS, Karlsen L, Taskén K. B cell signalling pathways—new targets for precision medicine in chronic lymphocytic leukaemia. Scandinavian Journal of Immunology. 2020;92(5):e12931.
16. Griner EM, Kazanietz MG. Protein kinase C and other diacylglycerol effectors in cancer. Nature Reviews Cancer. 2007;7(4):281-94.

17. Molica S, Tam C, Allsup D, Polliack A. Advancements in the Treatment of CLL: The Rise of Zanubrutinib as a Preferred Therapeutic Option. Cancers (Basel). 2023;15(14).
18. Tam CS, Brown JR, Kahl BS, Ghia P, Giannopoulos K, Jurczak W, et al. Zanubrutinib versus bendamustine and rituximab in untreated chronic lymphocytic leukaemia and small lymphocytic lymphoma (SEQUOIA): a randomised, controlled, phase 3 trial. Lancet Oncol. 2022;23(8):1031-43.
19. Ding W, LaPlant BR, Call TG, Parikh SA, Leis JF, He R, et al. Pembrolizumab in patients with CLL and Richter transformation or with relapsed CLL. Blood, The Journal of the American Society of Hematology. 2017;129(26):3419-27.
20. Ciszak L, Frydecka I, Wolowiec D, Szteblich A, Kosmaczewska A. Patients with chronic lymphocytic leukaemia (CLL) differ in the pattern of CTLA-4 expression on CLL cells: the possible implications for immunotherapy with CTLA-4 blocking antibody. Tumor Biology. 2016;37(3):4143-57.
21. Sage PT, Paterson AM, Lovitch SB, Sharpe AH. The coinhibitory receptor CTLA-4 controls B cell responses by modulating T follicular helper, T follicular regulatory, and T regulatory cells. Immunity. 2014;41(6):1026-39.
22. Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Döhner H, et al. 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, The Journal of the American Society of Hematology. 2008;111(12):5446-56.
23. Jaatinen T, Laine J. Isolation of Mononuclear Cells from Human Cord Blood by Ficoll-Paque Density Gradient. Current Protocols in Stem Cell Biology. 2007;1(1):2A.1.-2A.1.4.
24. Thibult M-L, Mamessier E, Gertner-Dardenne J, Pastor S, Just-Landi S, Xerri L, et al. PD-1 is a novel regulator of human B-cell activation. 2013;25(2):129-37.
25. Bouaziz JD, Calbo S, Maho-Vaillant M, Saussine A, Bagot M, Bensussan A, et al. IL-10 produced by activated human B cells regulates CD4+ T-cell activation in vitro. European journal of immunology. 2010;40(10):2686-91.
26. Van Hoof D, Lomas W, Hanley MB, Park E. Simultaneous flow cytometric analysis of IFN-γ and CD4 mRNA and protein expression kinetics in human peripheral blood mononuclear cells during activation. Cytometry Part A. 2014;85(10):894-900.
27. Axelsson S, Magnuson A, Lange A, Alshamari A, Hörnquist EH, Hultgren O. A combination of the activation marker CD86 and the immune checkpoint marker B and T lymphocyte attenuator (BTLA) indicates a putative permissive activation state of B cell subtypes in healthy blood donors independent of age and sex. BMC Immunology. 2020;21(1):14.
28. Joscelyn J, Ochoa-Repáraz J, Kasper L. Principles of Immunotherapy. Clinical Neuroimmunology: Springer; 2020. p. 17-42.
29. Ntsethe A, Mkhwanazi ZA, Dludla PV, Nkambule BB. B Cell Subsets and Immune Checkpoint Expression in Patients with Chronic Lymphocytic Leukemia. Current Issues in Molecular Biology. 2024;46(3):1731-40.
30. Ghamlouch H, Ouled-Haddou H, Damaj G, Royer B, Gubler B, Marolleau J-P. A combination of cytokines rescues highly purified leukemic CLL B-cells from spontaneous apoptosis in vitro. PloS one. 2013;8(3):e60370.
31. Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nature immunology. 2001;2(3):261-8.
32. Kaku H, Rothstein TL. Octamer binding protein 2 (Oct2) regulates PD-L2 gene expression in B-1 cells through lineage-specific activity of a unique, intronic promoter. Genes & Immunity. 2010;11(1):55-66.
33. Yamazaki T, Akiba H, Iwai H, Matsuda H, Aoki M, Tanno Y, et al. Expression of programmed death 1 ligands by murine T cells and APC. J Immunol. 2002;169(10):5538-45.
34. McKay JT, Haro MA, Daly CA, Yammani RD, Pang B, Swords WE, et al. PD-L2 Regulates B-1 Cell Antibody Production against Phosphorylcholine through an IL-5-Dependent Mechanism. J Immunol. 2017;199(6):2020-9.

35. Do P, Beckwith KA, Cheney C, Tran M, Beaver L, Griffin BG, et al. Leukemic B Cell CTLA-4 Suppresses Costimulation of T Cells. J Immunol. 2019;202(9):2806-16.
36. Hogg SJ, Vervoort SJ, Deswal S, Ott CJ, Li J, Cluse LA, et al. BET-bromodomain inhibitors engage the host immune system and regulate expression of the immune checkpoint ligand PD-L1. 2017;18(9):2162-74.
37. Haanen J, Ernstoff M, Wang Y, Menzies A, Puzanov I, Grivas P, et al. Autoimmune diseases and immune-checkpoint inhibitors for cancer therapy: review of the literature and personalized risk-based prevention strategy. Annals of Oncology. 2020;31(6):724-44.
38. Marin-Acevedo JA, Kimbrough EO, Lou Y. Next generation of immune checkpoint inhibitors and beyond. Journal of hematology & oncology. 2021;14(1):1-29.
39. Palma M, Gentilcore G, Heimersson K, Mozaffari F, Näsman-Glaser B, Young E, et al. T cells in chronic lymphocytic leukemia display dysregulated expression of immune checkpoints and activation markers. Haematologica. 2017;102(3):562-72.
40. Younes A, Brody J, Carpio C, Lopez-Guillermo A, Ben-Yehuda D, Ferhanoglu B, et al. Safety and activity of ibrutinib in combination with nivolumab in patients with relapsed non-Hodgkin lymphoma or chronic lymphocytic leukaemia: a phase 1/2a study. The Lancet Haematology. 2019;6(2):e67-e78.
41. Mahadevan D, Lanasa MC, Farber C, Pandey M, Whelden M, Faas SJ, et al. Phase I study of samalizumab in chronic lymphocytic leukemia and multiple myeloma: blockade of the immune checkpoint CD200. Journal for immunotherapy of cancer. 2019;7(1):1-13.
42. Jain N, Basu S, Thompson PA, Ohanian M, Ferrajoli A, Pemmaraju N, et al. Nivolumab combined with ibrutinib for CLL and Richter transformation: a phase II trial. Blood. 2016;128(22):59.
43. Archibald WJ, Meacham PJ, Williams AM, Baran AM, Victor AI, Barr PM, et al. Management of melanoma in patients with chronic lymphocytic leukemia. Leukemia Research. 2018;71:43-6.
44. Mato A, Svoboda J, Luning Prak E, Schuster S, Tsao P, Dorsey C, et al. Phase I/II study of umbralisib (TGR-1202) in combination with ublituximab (TG-1101) and pembrolizumab in patients with Rel/Ref CLL and Richter's transformation. Hematological Oncology. 2019;37:119-20.
45. Akkaya M, Kwak K, Pierce SK. B cell memory: building two walls of protection against pathogens. Nature Reviews Immunology. 2020;20(4):229-38.
46. Mékinian A, Quinquenel A, Belkacem KA, Kanoun F, Dondi E, Franck E, et al. Immuno-regulatory malignant B cells contribute to Chronic Lymphocytic Leukemia progression. Cancer Gene Therapy. 2023:1-11.
47. Awan FT, Byrd JC. 99 - Chronic Lymphocytic Leukemia. In: Niederhuber JE, Armitage JO, Kastan MB, Doroshow JH, Tepper JE, editors. Abeloff's Clinical Oncology (Sixth Edition). Philadelphia: Elsevier; 2020. p. 1850-71.e5.
48. Malavasi F, Deaglio S, Damle R, Cutrona G, Ferrarini M, Chiorazzi N. CD38 and chronic lymphocytic leukemia: a decade later. Blood, The Journal of the American Society of Hematology. 2011;118(13):3470-8.
49. Manna A, Aulakh S, Jani P, Ahmed S, Akhtar S, Coignet M, et al. Targeting CD38 Enhances the Antileukemic Activity of Ibrutinib in Chronic Lymphocytic Leukemia. Clin Cancer Res. 2019;25(13):3974-85.
50. Ciszak L, Frydecka I, Wolowiec D, Szteblich A, Kosmaczewska A. Patients with chronic lymphocytic leukaemia (CLL) differ in the pattern of CTLA-4 expression on CLL cells: the possible implications for immunotherapy with CTLA-4 blocking antibody. Tumor Biology. 2016;37:4143-57.
51. Bodhankar S, Galipeau D, Vandenbark AA, Offner H. PD-1 Interaction with PD-L1 but not PD-L2 on B-cells Mediates Protective Effects of Estrogen against EAE. J Clin Cell Immunol. 2013;4(3):143.
52. Al-Sawaf O, Zhang C, Tandon M, Sinha A, Fink A-M, Robrecht S, et al. Venetoclax plus obinutuzumab versus chlorambucil plus obinutuzumab for previously untreated chronic lymphocytic leukaemia (CLL14): follow-up results from a multicentre, open-label, randomised, phase 3 trial. The Lancet Oncology. 2020;21(9):1188-200.
53. Moreno C, Greil R, Demirkan F, Tedeschi A, Anz B, Larratt L, et al. Ibrutinib plus obinutuzumab versus chlorambucil plus obinutuzumab in first-line treatment of chronic lymphocytic leukaemia

(iLLUMINATE): a multicentre, randomised, open-label, phase 3 trial. The Lancet Oncology. 2019;20(1):43-56.
54. Shanafelt TD, Wang XV, Kay NE, Hanson CA, O'Brien S, Barrientos J, et al. Ibrutinib–rituximab or chemoimmunotherapy for chronic lymphocytic leukemia. New England Journal of Medicine. 2019;381(5):432-43.

Prologue

This chapter is an experimental paper which looked at soluble immune checkpoint profiles in patients with CLL. We showed that soluble immune checkpoints such as sCD25, Tim-3, galectin-9, PD-1 and PD-L1 are increased in patients with CLL. However, in our cohort, these soluble immune checkpoints did not correlate with prognostic marker, International Prognostic Index for Chronic Lymphocytic Leukemia (CLL-IPI) score, Fluorescent in situ hybridisation (trisomy 12, 11q22, 13q14, and 17p13) status and CLL Rai staging.

CHAPTER 5: Research Article 3

Evaluating soluble immune checkpoint profiles in patient with chronic lymphocytic leukemia

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Abstract

Introduction

Chronic lymphocytic leukaemia (CLL) is a heterogenous disease, with variable patient outcomes. Not all patients with CLL require treatment and patients are kept on a watch and wait approach but a well-established criterion still need to identify patients without need of treatment (CLL-WONT). Various immunophenotypes and complement regulating protein profiles have been evaluated in the risk-stratification of patients with CLL. Multiplex assays of soluble immune checkpoints offer a less laborious more practical method of monitoring patients with CLL, but none of these panels have been validated and the prognostic value of these soluble markers remains unclear. The aim of the study was to assess the soluble immune checkpoint profiles in patients with CLL and correlate these with independent prognostic markers such as beta-microglobulin 2 (B2M) and the International Prognostic Index for Chronic Lymphocytic Leukemia (CLL-IPI).

Methods

In this prospective cross-sectional study, we recruited 21 patients with CLL and 12 controls. Six soluble immune checkpoints, soluble interleukin-2 receptor alpha (sCD25), T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3), galectin-9, programmed cell death 1 (PD-1), programmed death-ligand 1 (PD-L1) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4) using cytometric bead array-based assays. We further measured β2-microglobulin (B2M) using enzyme-linked immunosorbent assay (ELISA) kit. Lastly, we correlated the soluble immune checkpoints with prognostic and clinical measures such as B2M, CLL Rai stage, Fluorescent in situ hybridisation (trisomy 12, 11q22, 13q14, and 17p13) status and CLL-IPI score.

Results

There was a significant increase in the levels of sCD25 (IL-2Ra) in patients with CLL (6183± 893.40 pg/ml) compared to the control group (59.99±23.87 pg/ml), $p < 0.0001$. In addition, patients with CLL had higher TIM-3 levels (11.63±0.6751 pg/ml) when compared to the control group (10.33±0.2630 pg/ml), $p < 0.0001$. Furthermore, Galectin-9 levels were significantly increased in patients with CLL (725.9±221.5 pg/ml) when compared to the control group (53.42±23.55 pg/ml), $p < 0.0001$. The PD-1 levels were significantly elevated in patients with CLL (1088±129.7 pg/ml) compared to controls (417.8±57.05 pg/ml), $p < 0.0001$. Similarly, PD-L1 levels in patients with CLL were significantly increased (373.4±11.18 pg/ml) in comparison to the control group (284.0±3.689 pg/ml), $p < 0.0001$. However, CTLA-4 levels were comparable between patients with CLL and the control group, $p = 0.0542$. However, there were no associations between prognostic markers B2M levels and the measured soluble immune checkpoints.

Conclusion

Patients with CLL have increased soluble CD25, TIM-3, galectin-9, PD-1 and PD-L1 levels. However, these were not associated with clinical measures such as disease staging and the CLL-IPI.

1. Introduction

Chronic lymphocytic leukemia (CLL) is a heterogenous disease, with a varied clinical course (1) and not all patients with CLL require treatment, in fact, most patients with CLL live for many years without need of treatment (CLL-WONT) (2). Some patients may present with aggressive disease necessitating early treatment, while others have a more indolent course and may never need treatment (3). The heterogeneous clinical course of CLL makes it essential to stratify patients into appropriate risk groups to prioritize treatment (1). Currently, the most robust prognostic marker in patients with CLL is the mutational status of the immunoglobulin heavy-chain variable region (IGHV), which is valuable in predicting the clinical outcomes at diagnosis (4). Mutated IGHV is associated with a slower disease progression rate, improved overall survival rate, and a less advanced Binet stage A disease (5). However, routine testing for this biomarker is not a cost-effective option and therefore not widely implemented (6).

Chromosomal abnormalities detected via fluorescence in situ hybridization (FISH) and metaphase cytogenetic testing offer vital prognostic insights for patients with CLL, helping to predict survival outcomes and disease progression (7). Determining the FISH status at diagnosis is recommended for all patients with CLL and is valuable for monitoring the disease (8). Based on chromosomal aberrations detected by FISH, patients can be stratified into five prognostic subgroups, ranked from highest to lowest risk: del(17p), del(11q), trisomy 12, no FISH abnormalities, and del(13q) (9).

Several studies have shown that patients with del(17p) have poorer outcomes when treated with combination chemoimmunotherapy (10, 11). Notably, ibrutinib-treated patients with del(17p) show worse survival rates compared to those with del(11q) or without either abnormality (12). Interestingly, there is an apparently higher prevalence of del(17q) among Africans with CLL (13). Patients with del(11q) often present with bulky lymphadenopathy, rapid disease progression, and reduced overall survival when treated with chemoimmunotherapy (9). Therefore, patients with CLL with del(17p) or del(11q), often experience faster disease progression and poorer treatment response (11). About 20% of patients with CLL have no chromosomal abnormalities, indicating a favourable prognosis, though outcomes within this group are varied (14). Deletion of 13q14 chromosome is the most common abnormality detected by FISH, occurring in approximately 55% of cases.

The most commonly reported prognostic marker in CLL is CD38 (defined as ≥ 30% of cells expressing CD38) (15). This marker is associated with resistance to immunotherapy and reduced overall survival in patients with CLL (15). However, a previous study reporting on an Asian cohort of patients with CLL study found no association between CD38 expression and Rai staging (16). The Rai and Binet staging systems classify patients into three risk groups based on their expected overall survival (OS). Rai staging categorizes patients as low risk (stage 0), intermediate risk (stage I or II), and high risk (stage III or IV), corresponding to Binet grades A, B, and C, respectively (17). Advanced stages are

generally associated with shorter survival (17). The levels of β2-microglobulin (B2M) CD38%, and advanced Rai-stage strongly correlate with serum galectin-9 levels in patients with CLL (18). B2M is an established independent prognostic marker in CLL (19). Patients with low serum B2M levels (< 3.5 mg/L) experience significantly improved progression-free survival (PFS) and overall survival (OS) when treated with frontline fludarabine-based chemoimmunotherapy, in contrast to those with elevated B2M levels (> 3.5 mg/L) (20, 21).

Elevated levels of galectin-9 have been observed in patients with CLL, and correlate with poor prognosis (22, 23). The signalling pathway involving Galectin-9 and T cell immunoglobulin and mucin-domain containing-3 (Tim-3), plays a crucial regulatory role in CLL (23). In fact, an in-vitro study demonstrated that inhibiting the Galectin-9/Tim-3 signaling pathway in CLL partially restores the T cell subset balance (24). The levels of galectin-9 have been shown to increase with advanced Binet stage and are associated with poor prognosis (22, 23). The increased levels of expression of several inhibitory receptors such as Tim-3, programmed death-1 (PD-1), programmed death-ligand-1 (PD-L1), cytotoxic T lymphocyte associated protein-4 (CTLA-4) modulate immunosuppression in terms of cytokine production (25). The role of PD-1 in influencing the activity and progression of CLL has been hypothesised, with interactions mediated by PD-L1 playing a crucial role in the regulation of cytokine production (26, 27). Altered levels of CTLA-4 have also been reported in patients with CLL, however, these differ among patients (28). Even though increased CTLA-4 levels are a predictor of good clinical outcomes in patients with CLL, they have not been validated as independent marker of good prognosis (29).

Interleukin-2 (IL-2) binds to IL-2 receptors (CD25) and promotes the proliferation, activation, and survival of T cells (30, 31). The expression levels of CD25 in combination with IGVH mutational status are prognostic markers in patients with CLL, which are strongly associated with poor prognosis (32). Some studies have demonstrated that malignant B cell is responsible for the release of sCD25 (33, 34).

The treatment landscape for CLL has advanced significantly in recent years with the introduction of targeted therapies ushering a chemotherapy free era of CLL treatment. Conventional chemotherapy and traditional chemoimmunotherapy approaches have been largely replaced by treatments such as Bruton tyrosine kinase inhibitors (BTKis), B-cell lymphoma 2 (BCL2) inhibitors, and combinations with immunotherapy (35). The incorporation of various prognostic markers for personalised patient assessment remains a challenge. Therefore, the aim of the study was to assess soluble immune checkpoint profiles in a cohort of African patients with CLL and to further correlate them with independent prognostic markers and International Prognostic Index for Chronic Lymphocytic Leukemia (CLL-IPI).

2. Methods and materials

2.1 Patients recruitment

Participants were recruited from King Edward VIII Hospital (KEH), which is a tertiary healthcare facility situated in Durban, KwaZulu-Natal, South Africa. Ethical approval for this study was obtained from the University of KwaZulu-Natal Biomedical Research Ethics Committee (BE456/18), South Africa. All study participants provided written informed consent.

2.2 Inclusion and exclusion criteria

Treatment naïve patients with CLL were included with no clinical signs of infection. All included participants self-reported their ancestry. Patients undergoing treatments were excluded.

2.3 Sample collection

Five microliters (5mL) of blood was collected into the ethylenediamine tetra-acetic acid (EDTA) tubes and centrifuged within 1-2 hours of collection at 3000rpm for 10min at 4^{o}C (Figure 5.1). Plasma was then aliquoted and stored at -20^{o}C for analyses.

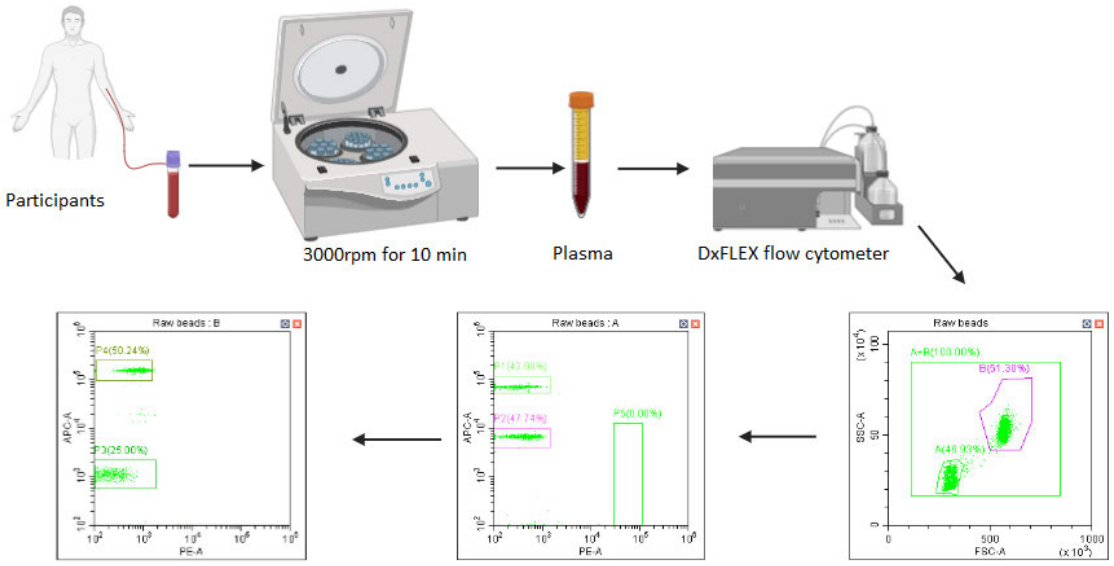

Figure 5.1: Sample collection. Figure 1 illustrates the sample collection, processing and sample analysis using flow cytometric bead array-based methods. PE (phycoerythrin), APC (allophycocyanin), FSC (forward side scatter), SSC (side scatter), rpm (revolutions per minute).

2.4 Measurement of soluble immune checkpoint profiles

The soluble immune checkpoints (PD-1, PD-L1, CTLA-4, Tim-3, galectin-9 and sCD25) were measured in plasma using BioLegend Human Immune Checkpoint Panel 1-S/P LEGENDplex™ kit (BioLegend, San Diego, CA, USA), according to the manufacturer's instructions. All samples were then

transferred to FACS tubes (BD Biosciences, San Jose, CA, USA) and data was acquired using Beckman Coulter DxFLEX flow cytometer (Beckman coulter, Inc Brea, CA, USA). The absolute concentration (pg/ml) of each analyte was determined using the BioLegend LEGENDplex™ data analysis software based on a standard curve recorded for each analyte.

2.5 Measurements of serum soluble beta-2-microglobulin (B2M) levels

To determine the plasma levels of B2M (pg/ml), an independent prognostic marker for patients with CLL (19), we made use of the beta-2-microglobulin Human enzyme-linked immunosorbent assay kit (ThermoFisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instruction.

2.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8 software, (GraphPad Software Inc, San Diego, CA, USA) and STATA 18 (36, 37). All non-parametric data were log-transformed prior to statistical analysis and reported as mean and standard deviation. An unpaired Student's t-test was performed to compare parametric data between two groups. To correct for multiple comparisons, a Bonferroni-corrected critical p-value of < 0.01 was considered statistically significant.

3. Results

3.1 Patient Characteristics and haematological Parameters

This cohort consisted of participants of African ancestry (n = 30), European (n = 2), and Indian (n = 1). The gender distribution was 39.39% females and 60.61% males (Table 5.1). This study included 21 patients with CLL who were not on treatment, and 12 healthy controls. Sixteen (n=16) of the included patients with CLL, were on a "watch and wait" approach and 5 had a treatment indication at the time of sample collection. The baseline characteristics and haematological parameters of this cohort (Table 1) has been previously described (38). Briefly, the mean age among patients with CLL was 62.33 ± 13.31 years, while it was 56.58 ± 15.67 years for the healthy controls. Furthermore, the white blood cell count was significantly elevated (130.4 × 103 ± 29.71) when compared to healthy controls (5.26 × 103 ± 1.38), $p = 0.0005$. The red blood cell count and haemoglobin levels were notably lower in CLL patients when compared to healthy controls ($p < 0.0001$). No statistically significant differences were observed in platelet counts between patients with CLL and healthy controls ($p = 0.1831$).

Table 5.1: The baseline characteristics and haematological profiles of the participants

	Control (n=12)	Patients with CLL (21)	p-Value
Gender			
Male,n (%)	7 (58.33)	13 (61.9)	
Female, n (%)	5 (41.67)	8 (38.1)	
Age (Years)	56.58±15.67	62.33±13.31	0.2714
Haematological parameters			
White blood cell count (x 10^3µL)	5.26±1.38	130.4±29.71	0.0005
Red blood cell (x 10^6µL)	4.74±0.94	2.10±0.84	< 0.0001
Haemoglobin (g/dL)	14.13±3.81	8.19±2.30	< 0.0001
Platelets (x 10^3µL)	210.4±73.14	157.5±141.9	0.1831
CD38% positive B cells	28.47±19.01	57.39±8.001	0.0002

3.2 Clinical staging and prognostic markers in patients with CLL

In patients with CLL, the diagnosis was based on standards from the International Workshop on Chronic Lymphocytic Leukaemia (39). The clinical CLL stage was determined according to the Rai classification system (17), 47.61% were on stage IV, 28.60% were on stage III and 23.81% were on stage II (Table 2). In our cohort, the most common cytogenetic abnormality was 11q22 deletion (33.33%), followed by 13q14 deletion (28.60%) and 17p13 deletion (14.30%). Only one patient had trisomy 12 (4.80%) and 19% has no abnormalities (Table 5.2). The CLL-IPI score was determined according to Hallek's calculation (56),

Table 5.2: Clinical staging and prognostic markers in patients with CLL (n=21)

Clinical parameters	
RAI Staging	
I, n (%)	0 (0)
II, n (%)	5 (23.8)
III, n (%)	6 (28.6)
IV, n (%)	10 (47.6)
FISH Status	
Trisomy 12, n (%)	1 (4.8)
Deletions	
11q22, n (%)	7 (33.3)
13q14, n (%)	6 (28.6)
17p13, n (%)	3 (14.3)
no abnormalities, n (%)	4 (19.0)
CLL-IPI	
Low risk, n (%)	14 (66.7)
Intermediate risk, n (%)	4 (19)
High risk, n (%)	3 (14.3)
Prognostic Biomarkers	
B2M mg/L	0.74± 0.30

3.3 Elevated soluble immune checkpoints levels in patients with CLL

We evaluated the levels of the soluble immune checkpoints in patients with CLL (Figure 5.2). There was a significant increase in the levels of sCD25 (IL-2Ra) in patients with CLL (6183±893.40) compared to the control group (59.99±23.87), $p < 0.0001$. In addition, patients with CLL had higher TIM-3 levels (11.63±0.6751) when compared to the control group (10.33±0.2630), $p < 0.0001$. Furthermore, Galectin-9 levels were significantly increased in patients with CLL (725.9±221.5) when compared to the control group (53.42±23.55), $p < 0.0001$.

The PD-1 levels were significantly elevated in patients with CLL (1088±129.7) compared to controls (417.8±57.05), $p < 0.0001$. Similarly, PD-L1 levels in patients with CLL were significantly increased (373.4±11.18) in comparison to the control group (284.0±3.689), $p < 0.0001$. However, CTLA-4 levels were comparable between patients with CLL (832.5±276.1) and the control group (1161±503.3), $p = 0.0542$.

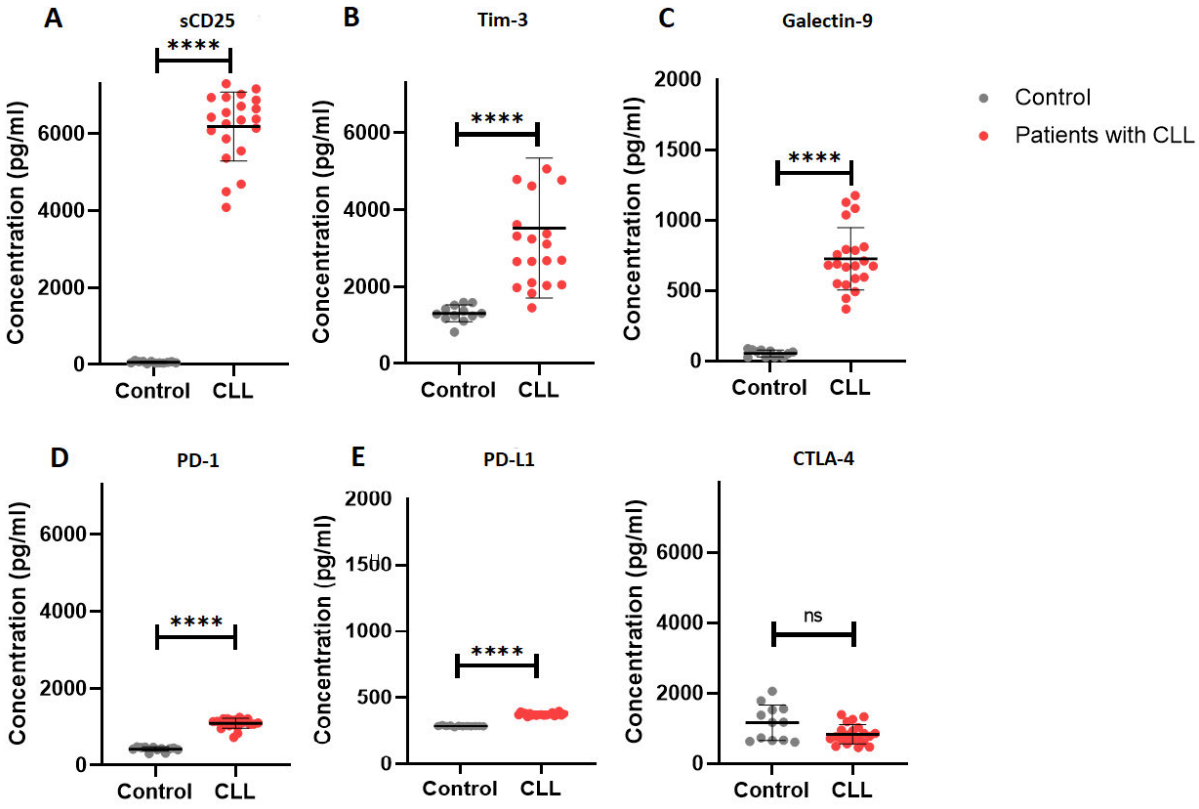

Figure 5.2: Profile of soluble immune checkpoints in patients with CLL. **A-E** illustrates the concentration (pg/ml) of sCD25, TIM-3, Galectin-9, PD-1 and PD-L1, respectively. The data is presented as the mean ± standard deviation (SD). CLL (chronic lymphocytic leukemia), sCD25 (soluble interleukin-2 receptor alpha), Tim-3 (T cell immunoglobulin and mucin domain-containing protein 3), PD-1 (programmed cell death protein 1), PD-L1 (programmed death-ligand 1), CTLA-4 (cytotoxic T-lymphocyte associated protein); **** shows the level of significance between groups, ns: not significant.

3.3 Soluble immune checkpoints are not associated with β2 microglobulin levels, FISH profiles and clinical staging and CLL-IPI score in patients with CLL

The Beta-2 microglobulin (B2M) levels show an independent correlation with disease progression in patients with CLL (19). In a multivariable regression model, no statistically significant association was observed between B2M levels and sCD25 ($\beta = -0.385$, SE = 0.8029, $p = 0.638$), TIM-3 ($\beta = 0.738$, SE = 1.603, $p = 0.651$), Galectin-9 ($\beta = 0.301$, SE = 0.168, $p = 0.091$), PD-1 ($\beta = 0.034$, SE = 0.113, p =

0.770), PD-L1(β = 0.002, SE = 0.010, p = 0.860) in patients with CLL. In addition, no statistically significant association was observed between Rai stage and sCD25 (β = -0.00003, SE = 0.0002, p = 0.891), TIM-3 (β = -0.00004, SE = 0.0001, p = 0.723), Galectin-9 (β = -0.0003, SE = 0.0012, p = 0.775), PD-1 (β = 0.0026, SE = 0.0024, p = 0.310), PD-L1 (β = 0.0085, SE = 0.0254, p = 0.742) (supplementary file 5.2).

In a logistic regression model, no statistically significant association was observed between soluble immune checkpoints and FISH profile and CLL-IPI score (Table 5.3).

Table 5.3: Odd ratios of soluble immune checkpoints with FISH profile and CLL-IPI score in patients with CLL.

Variables	Soluble immune checkpoints	Odds Ratio	95% Confidence Interval	p value
17p13 deletion	TIM-3	1.00	1.00-1.00	0.64
	Galectin-9	0.99	0.97-1.00	0.30
	PD-1	1.00	0.98-1.02	0.77
	PD-L1	0.96	0.79-1.17	0.70
	sCD25	1.00	1.00-1.00	0.88
CLL-IPI	TIM-3	0.99	0.98-1.00	0.27
	Galectin-9	0.96	0.84-1.10	0.54
	PD-1	1.00	0.99-1.00	0.43
	PD-L1	1.00	1.00-1.00	0.38
	sCD25	1.00	1.00-1.00	0.50

4. Discussion

The aim of the study was to assess the soluble immune checkpoint profiles in patients with CLL and correlate them with an independent prognostic markers and International Prognostic Index for Chronic Lymphocytic Leukemia (CLL-IPI). In our study, we found elevated levels of these soluble immune checkpoints, galectin-9, TIM-3, CD25, PD-1 and PD-L1 in patients with CLL (Figure 5.2). In malignancies elevated levels of immune checkpoints are associated with T-cell exhaustion and poor prognosis (40-43)

TIM-3 is an inhibitory protein that interacts with galectin-9 and inhibits T cell activation, and regulates immune tolerance (44). In patients with CLL, elevated levels of galectin-9 stimulate the proliferation and activation of regulatory T cells (Tregs) which supress the function of helper T cells (45). In this study we report on increased levels of both soluble TIM-3 and galectin-9 in patients with CLL. Elevated levels of galectin 9 and TIM-3 in patients with CLL may suggest a dysregulation of the immune system and possibly an unfavourable disease course. As these immune checkpoint molecules contribute to immune evasion by malignant cells, promoting tumour cell survival and growth (40, 42). The interaction between galectin 9 and Tim-3 promotes immune suppression by inhibiting the activation of helper T cells, thereby exacerbating proliferation of malignant cells (45).

Our findings are consistent with those reported by Taghiloo et al., who reported on elevated galectin-9 levels in patients with advanced-stage CLL (46). Taken together, these findings suggest that galectin-9 could serve as potential indicator of poor prognosis. Moreover, recent study found that both galectin-9 and TIM-3 were elevated in the regulatory T cells of patients with CLL in Binet C stage (45), indication that the TIM-3/Galectin-9 interaction may be associated with poor prognosis in patients with CLL. The TIM-3/Galectin-9 axis exert their function by stimulating regulatory T cell differentiation and inhibits Th17 cell differentiation (47, 48). In a recent study, the inhibition of the TIM-3/Galectin-9 pathway suppressed the function of regulatory T cells, suggesting that this pathway could be a novel target for immunotherapy in patients with CLL (45). Notably, TIM-3 aids in the immune system's defence against infections such as listeria and mycobacteria (42, 43). Hence, inhibiting TIM-3 in patients with CLL is associated with an increased risk of infection (49).

In our study, patients with CLL had increased plasma levels of sCD25, which is also known as the interleukin-2 receptor alpha chain and regulate affinity for IL-2 (50). Elevated plasma CD25 levels in patients with CLL suggests an activated state of malignant cells, as the increased CD25 expression in patients with CLL is associated with more aggressive disease characteristics, such as faster disease progression, and a poorer prognosis (32). It is worth noting that CD25 expression in patients with CLL differs among patients, and not all patients with CLL exhibit high levels of CD25 (51). Therefore, assessing CD25 levels in combination with other prognostic markers may provide a more comprehensive understanding of the disease status and risk stratification of patients.

In our study we found increased plasma levels PD-1 and PD-L1 in patients with CLL. PD-1 is a protein predominantly expressed on activated immune cells, including B cells, T cells, and natural killer cells, while PD-L1 is primarily expressed on various types of cells, including malignant cells and immune cells (52). An altered PD-1/PD-L1 axis contributes towards T-cell exhaustion in patients with CLL (53). While PD-1 serves as a marker for T cell exhaustion, the sole blockade of PD-1 has not yielded significant clinical benefits in patients with CLL (54). Hence, there is a need to identify more potent combination therapies for these patients and to stratify patients based on the likelihood of benefiting from immune checkpoint therapy. Targeting PD-1/PD-L1 axis could be beneficial for patients with CLL provided that biological markers that can predict patient responses are identified and validated. However, our findings like those previously reported (23, 55) have several limitations which include low sample size. This limits the implementation of statistical models and subgroup analysis of patients based on clinical measures such as staging and the CLL-IPI score.

Conclusion

Patients with CLL have increased sCD25, TIM-3 galectin-9, PD-1 and PD-L1 plasma levels. However, these soluble immune checkpoints may not correlate with prognostic markers such as B2M and chromosomal abnormalities. Therefore, future studies are needed to verify the significance of these soluble immune checkpoints in larger cohorts of African patients with CLL. Moreover, future prognostic biomarker validation studies including African cohorts are required to evaluate the suitability of prognostic markers and the CLL IPI in our setting.

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Disclosure

The authors declare that there are was no conflict of interest.

Ethics

The ethical approval for this study was granted by the University of KwaZulu-Natal Biomedical Research Ethics Committee (study approval no. BE456/18).

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References

1. Trivedi PJ, Patel DM, Kazi M, Varma P. Cytogenetic Heterogeneity in Chronic Lymphocytic Leukemia. Journal of the Association of Genetic Technologists. 2023;49(1):4-9.
2. Burger JA. Treatment of chronic lymphocytic leukemia. New England Journal of Medicine. 2020;383(5):460-73.
3. Braish J, Cerchione C, Ferrajoli A. An overview of prognostic markers in patients with CLL. Frontiers in Oncology. 2024;14:1371057.
4. Ghia P, Stamatopoulos K, Belessi C, Moreno C, Stilgenbauer S, Stevenson F, et al. ERIC recommendations on IGHV gene mutational status analysis in chronic lymphocytic leukemia. Leukemia. 2007;21(1):1-3.
5. Thompson PA, Bazinet A, Wierda WG, Tam CS, O'Brien SM, Saha S, et al. Sustained remissions in CLL after frontline FCR treatment with very-long-term follow-up. Blood. 2023;142(21):1784-8.
6. Vu M, Degeling K, Thompson ER, Blombery P, Westerman D, IJzerman MJ. Cost Effectiveness of Molecular Diagnostic Testing Algorithms for the Treatment Selection of Frontline Ibrutinib for Patients with Chronic Lymphocytic Leukemia in Australia. Applied health economics and health policy. 2024;22(1):107-22.
7. Nabhan C, Raca G, Wang YL. Predicting prognosis in chronic lymphocytic leukemia in the contemporary era. JAMA oncology. 2015;1(7):965-74.
8. Parikh SA, Strati P, Tsang M, West CP, Shanafelt TD. Should IGHV status and FISH testing be performed in all CLL patients at diagnosis? A systematic review and meta-analysis. Blood, The Journal of the American Society of Hematology. 2016;127(14):1752-60.
9. Döhner H, Stilgenbauer S, Benner A, Leupolt E, Kröber A, Bullinger L, et al. Genomic aberrations and survival in chronic lymphocytic leukemia. New England Journal of Medicine. 2000;343(26):1910-6.
10. Hallek M, Fischer K, Fingerle-Rowson G, Fink AM, Busch R, Mayer J, et al. Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. The Lancet. 2010;376(9747):1164-74.
11. Chavez JC, Kharfan-Dabaja MA, Kim J, Yue B, Dalia S, Pinilla-Ibarz J, et al. Genomic aberrations deletion 11q and deletion 17p independently predict for worse progression-free and overall survival after allogeneic hematopoietic cell transplantation for chronic lymphocytic leukemia. Leukemia research. 2014;38(10):1165-72.
12. Byrd JC, Furman RR, Coutre SE, Burger JA, Blum KA, Coleman M, et al. Three-year follow-up of treatment-naive and previously treated patients with CLL and SLL receiving single-agent ibrutinib. Blood, The Journal of the American Society of Hematology. 2015;125(16):2497-506.
13. Yang S, Varghese AM, Sood N, Chiattone C, Akinola NO, Huang X, et al. Ethnic and geographic diversity of chronic lymphocytic leukaemia. Leukemia. 2021;35(2):433-9.
14. Miller C, Huang Y, Hyak J, Avenarius MR, Ruppert AS, Byrd JC, et al. Normal FISH CLL represents a heterogeneous subgroup where prognosis can be refined with IGHV mutational status. Blood. 2021;138:1563.
15. Paulus A, Malavasi F, Chanan-Khan A. CD38 as a multifaceted immunotherapeutic target in CLL. Leukemia & Lymphoma. 2022;63(10):2265-75.
16. Gogia A, Sharma A, Raina V, Kumar L, Gupta R, Kumar R. Prevalence of ZAP-70 and CD 38 in Indian chronic lymphocytic leukemia patients. Indian Journal of Cancer. 2013;50(4):333-6.
17. Rai KR, Sawitsky A, Cronkite EP, Chanana AD, Levy RN, Pasternack BS. Clinical staging of chronic lymphocytic leukemia. 1975.
18. Ahmed HA, Nafady A, Ahmed EH, Hassan EEN, Soliman WGM, Elbadry MI, et al. CXC chemokine ligand 13 and galectin-9 plasma levels collaboratively provide prediction of disease activity and progression-free survival in chronic lymphocytic leukemia. Annals of Hematology. 2024;103(3):781-92.
19. Mkhwanazi ZA, Nyambuya TM, Mfusi SA, Nkambule BB. Prognostic markers in patients with chronic lymphocytic leukaemia on targeted therapy, chemoimmunotherapy with anti-CD20

monoclonal antibody: a systematic review and meta-analysis of prognostic factors. BMC cancer. 2022;22(1):1218.
20. Parikh SA, Shanafelt TD, editors. Prognostic factors and risk stratification in chronic lymphocytic leukemia. Seminars in oncology; 2016: Elsevier.
21. Thompson PA, O'Brien SM, Xiao L, Wang X, Burger JA, Jain N, et al. β2-microglobulin normalization within 6 months of ibrutinib-based treatment is associated with superior progression-free survival in patients with chronic lymphocytic leukemia. Cancer. 2016;122(4):565-73.
22. Wdowiak K, Gallego-Colon E, Francuz T, Czajka-Francuz P, Ruiz-Agamez N, Kubeczko M, et al. Increased serum levels of Galectin-9 in patients with chronic lymphocytic leukemia. Oncology letters. 2019;17(1):1019-29.
23. Alimu X, Zhang J, Pang N, Zhang R, Chen R, Zeng X, et al. Galectin-9 and myeloid-derived suppressor cell as prognostic indicators for chronic lymphocytic leukemia. Immunity, Inflammation and Disease. 2023;11(5):e853.
24. Pang N, Alimu X, Chen R, Muhashi M, Ma J, Chen G, et al. Activated Galectin-9/Tim3 promotes Treg and suppresses Th1 effector function in chronic lymphocytic leukemia. The FASEB Journal. 2021;35(7):e21556.
25. Yi JS, Cox MA, Zajac AJ. T-cell exhaustion: characteristics, causes and conversion. Immunology. 2010;129(4):474-81.
26. Grzywnowicz M, Karczmarczyk A, Skorka K, Zajac M, Zaleska J, Chocholska S, et al. Expression of programmed death 1 ligand in different compartments of chronic lymphocytic leukemia. Acta Haematologica. 2015;134(4):255-62.
27. Gamaleldin M, Ghallab O, Nadwan E, Abo Elwafa R. PD-1 and PD-L1 gene expressions and their association with Epstein-Barr virus infection in chronic lymphocytic leukemia. Clinical and Translational Oncology. 2021;23:2309-22.
28. Ciszak L, Frydecka I, Wolowiec D, Szteblich A, Kosmaczewska A. CTLA-4 affects expression of key cell cycle regulators of G0/G1 phase in neoplastic lymphocytes from patients with chronic lymphocytic leukaemia. Clinical and Experimental Medicine. 2016;16:317-32.
29. Karabon L, Partyka A, Ciszak L, Pawlak-Adamska E, Tomkiewicz A, Bojarska-Junak A, et al. Abnormal expression of BTLA and CTLA-4 immune checkpoint molecules in chronic lymphocytic leukemia patients. Journal of Immunology Research. 2020;2020.
30. Jaleil NAR, Abed WM. Plasma level of programmed death receptor ligand-1 and CD25 in chronic myeloid leukemia patients and their correlations with response to first-line therapy. Medical Journal of Babylon. 2021;18(4):398.
31. Gowda A, Ramanunni A, Cheney C, Rozewski D, Kindsvogel W, Lehman A, et al. Differential effects of IL-2 and IL-21 on expansion of the CD4+ CD25+ Foxp3+ T regulatory cells with redundant roles in natural killer cell mediated antibody dependent cellular cytotoxicity in chronic lymphocytic leukemia. MAbs. 2010;2(1):35-41.
32. Sulda ML, Kuss BJ, Hall RK, Bailey S, Macardle PJ. Clinical utility of molecular and flow cytometric markers in chronic lymphocytic leukaemia. Intern Med J. 2012;42(2):137-46.
33. Caruso C, Candore G, Cigna D, Colucci AT, Modica MA. Biological significance of soluble IL-2 receptor. Mediators of inflammation. 1993;2(1):3-21.
34. Kay NE, Burton J, Wagner D, Nelson DL. The malignant B cells from B-chronic lymphocytic leukemia patients release TAC-soluble interleukin-2 receptors. 1988.
35. Fidan K. Chronic lymphocytic leukemia. Journal of Current Hematology & Oncology Research. 2023;1(3):59-67.
36. Team RC. A Language and Environment for Statistical Computing 2021 [Available from: https://www.r-project.org/.
37. STATA18. [Available from: http://www.stata.com/.
38. Ntsethe A, Mkhwanazi ZA, Dludla PV, Nkambule BB. B Cell Subsets and Immune Checkpoint Expression in Patients with Chronic Lymphocytic Leukemia. Current Issues in Molecular Biology. 2024;46(3):1731-40.

39. Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Döhner H, et al. iwCLL guidelines for diagnosis, indications for treatment, response assessment, and supportive management of CLL. Blood, The Journal of the American Society of Hematology. 2018;131(25):2745-60.
40. Xierenguli A, Zeng X, Pang N, Zhagn R, Ma J, Zhao Y, et al. [TIM-3/galectin-9 is involved in negative regulation of T cells in patients with chronic lymphocytic leukemia]. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi. 2020;36(11):1021-5.
41. Shatrova AN, Mityushova EV, Vassilieva IO, Aksenov ND, Zenin VV, Nikolsky NN, et al. Time-dependent regulation of IL-2R α-chain (CD25) expression by TCR signal strength and IL-2-induced STAT5 signaling in activated human blood T lymphocytes. PLoS One. 2016;11(12):e0167215.
42. Du W, Yang M, Turner A, Xu C, Ferris RL, Huang J, et al. TIM-3 as a Target for Cancer Immunotherapy and Mechanisms of Action. International journal of molecular sciences. 2017;18(3):645.
43. Das M, Zhu C, Kuchroo VK. Tim-3 and its role in regulating anti-tumor immunity. Immunological reviews. 2017;276(1):97-111.
44. Sheng CC, Han FY. Immunoregulation effects of TIM-3 on tumors. Neoplasma. 2019;66(2):167-75.
45. Pang N, Alimu X, Chen R, Muhashi M, Ma J, Chen G, et al. Activated Galectin-9/Tim3 promotes Treg and suppresses Th1 effector function in chronic lymphocytic leukemia. Faseb j. 2021;35(7):e21556.
46. Taghiloo S, Allahmoradi E, Ebadi R, Tehrani M, Hosseini-Khah Z, Janbabaei G, et al. Upregulation of Galectin-9 and PD-L1 immune checkpoints molecules in patients with chronic lymphocytic leukemia. Asian Pacific journal of cancer prevention: APJCP. 2017;18(8):2269.
47. Wang J, Li C, Fu J, Wang X, Feng X, Pan X. Tim-3 regulates inflammatory cytokine expression and Th17 cell response induced by monocytes from patients with chronic hepatitis B. Scand J Immunol. 2019;89(5):e12755.
48. Oomizu S, Arikawa T, Niki T, Kadowaki T, Ueno M, Nishi N, et al. Galectin-9 suppresses Th17 cell development in an IL-2-dependent but Tim-3-independent manner. Clin Immunol. 2012;143(1):51-8.
49. Rezaei M, Tan J, Zeng C, Li Y, Ganjalikhani-Hakemi M. TIM-3 in leukemia; immune response and beyond. Frontiers in oncology. 2021;11:753677.
50. Nelson BH, Willerford DM. Biology of the interleukin-2 receptor. Advances in immunology. 1998;70:1-81.
51. Shvidel L, Braester A, Bairey O, Rahimi-Levene N, Herishanu Y, Tadmor T, et al. Cell surface expression of CD25 antigen (surface IL-2 receptor α-chain) is not a prognostic marker in chronic lymphocytic leukemia: results of a retrospective study of 281 patients. Ann Hematol. 2012;91(10):1597-602.
52. Marin-Acevedo JA, Kimbrough EO, Lou Y. Next generation of immune checkpoint inhibitors and beyond. Journal of hematology & oncology. 2021;14(1):1-29.
53. Brusa D, Serra S, Coscia M, Rossi D, D'Arena G, Laurenti L, et al. The PD-1/PD-L1 axis contributes to T-cell dysfunction in chronic lymphocytic leukemia. haematologica. 2013;98(6):953.
54. Ding W, LaPlant BR, Call TG, Parikh SA, Leis JF, He R, et al. Pembrolizumab in patients with CLL and Richter transformation or with relapsed CLL. Blood, The Journal of the American Society of Hematology. 2017;129(26):3419-27.
55. Knauf W, Langenmayer I, Ehlers B, Mohr B, Adorf D, Nerl C, et al. Serum levels of soluble CD23, but not soluble CD25, predict disease progression in early stage B-cell chronic lymphocytic leukemia. Leukemia & lymphoma. 1997;27(5-6):523-32.

56. Hallek M. International Prognostic Index for Chronic Lymphocytic Leukemia (CLL-IPI): mdcalc; 2005 [Available from: https://www.mdcalc.com/calc/4054/international-prognostic-index-chronic-lymphocytic-leukemia-cll-ipi#creator-insights.

CHAPTER 6: Synthesis

Patients with CLL express dysregulated levels of serum and cell membrane immune checkpoints, which are associated with impaired immune response (1-4). However, the levels of immune checkpoint expression on B cell subsets and their role disease progression in patients with CLL has not been elucidated. Alarmingly, to date there is limited data on B cell immunophenotyping in African cohorts of patients with CLL. Therefore, we investigated the levels of immune checkpoint expression on B cell subsets and B cell function in a cohort of South African patients with CLL. In our approach, we firstly performed a comprehensive review of available literature on patients with CLL. In our initial abstract screening and study selection we assessed the risk of bias of the included studies and noted the poor external validity of these studies, all the included studies were not conducted in Asia or Africa. Interestingly, there is a greater frequency of del(17p) among Africans which associated with rapid disease progression and poorer response to treatment (5). This was further confirmed by the majority of patients with CLL with 11q22deletion (33.3%) included in our study, this chromosomal abnormality is also associated with rapid disease progression and poorer response to treatment (6). With focus on providing cumulative evidence on major adverse events reported in patients with CLL on chemoimmunotherapy or targeted therapy combined with immunotherapy we screened available RCTs. We identified knowledge gaps on B cell function and diversity of immune checkpoint profiles in patients with CLL. Due to the lack of studies reporting on immune checkpoints in patients with CLL and the importance of immune checkpoints in immunotherapy-based patient management, our approach was to firstly registered a systematic review protocol on the impact of immune checkpoint inhibitors in patients with CLL (PROSPERO Registration: CRD42020156926) to avoid unnecessary duplication on the topic. Thereafter, we performed a systematic review on the effectiveness of chemoimmunotherapy and the associated major severe adverse events in adult patients with CLL.

In our electronic database and bibliometric searches, we observed no published RCTs reporting on African or Asian cohorts. This further highlights the disparities in the prioritization of CLL research in most low-middle income countries. Our systematic review further highlighted the importance of prognostic markers, Fluorescent in situ hybridisation (FISH) testing, and personalized treatment in the management of patients with CLL. Cumulative evidence from our systematic reviewed showed that targeted therapy combined with immunotherapy, rather than chemoimmunotherapy is associated with prolonged survival rate in patients with CLL or high-risk CLL. However, these combination therapies are associated with the development of severe hematological adverse events. We thereafter conducted an experimental study aimed at reporting the levels of immune checkpoint expression on various B cell subsets in a cohort of African patients with CLL. We further explored the effects of ex-vivo protein kinase C activation on immune checkpoint expression and B cell profiles. Moreover, we correlated the levels of immune checkpoints with independent prognostic marker, beta-2 microglobulin (B2M) (7) to

understand prognostic potential of immune checkpoints on B cell subsets. Lastly, we investigated the soluble immune checkpoint profiles in patients with CLL and correlated them with prognostic markers, Rai staging and International Prognostic Index for Chronic Lymphocytic Leukemia (CLL-IPI).

6.1 Increased immune checkpoint expression on B cell subsets

To identify the possible main source of soluble immune checkpoint levels in patients with CLL, we measured the expression of PD-1 and CTLA-4 on B cell subsets. The expression of these immune checkpoints (PD-1 and CTLA-4) was increased in activated B cells and memory B cells. These immune checkpoint regulate B cell function, in fact, PD-1 receptor binds to PD-L1 or PD-L2 to induce phosphorylation of immunoreceptor tyrosine-based inhibitory motif (8). This leads to the inhibition of B cell proliferation and immune suppression (9). Increased levels of CTLA-4 expression on B cells are associated with disease progression in patients with CLL through the suppression of humoral response to T cell-dependent and independent antigen (10, 11). Therefore, elevated levels of these immune checkpoints may play a role in the progression of CLL.

6.2 The B cell function in patients with CLL

Investigating B cell mediated immune responses is important in patients with CLL. In our ex-vivo experiments we showed that immune checkpoint expression on B cells is elevated upon protein kinase C stimulation (Fig. 6.1). We found that even though B cell subsets are altered in patients with CLL when compared to controls, B cells retained functional capacity. Our findings from the PD-1, PD-L1 and CTLA-4 blockage studies suggest that our African cohort may benefit from immunotherapy as these simulations reduced the CTLA-4 and PD-L1 expression.

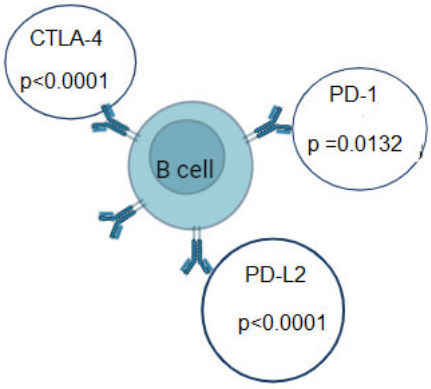

Figure 6.1: Immune checkpoint expression on stimulated B cells. PD-1 (programmed cell death protein 1), PD-L2 (programmed death-ligand 2), CTLA-4 (cytotoxic T-lymphocyte-associated protein 4).

6.3. Prognostic significance of soluble immune checkpoints profiles

In clinical settings, most of the prognostic markers are measured in serum or plasma. We therefore looked at soluble immune checkpoints that are of prognostic significance in patients with CLL. We

further correlated these soluble immune checkpoints with independent prognostic markers, Rai staging, CLL-IPI. These included soluble immune checkpoint profiles such as sCD25, Tim-3, galectin-9, PD-1 and PD-L1. Our study found that these soluble immune checkpoints are indeed elevated in patients with CLL, consistent with the literature (4, 12, 13). However, in our study we found no correlation between soluble immune checkpoints and independent prognostic markers, Rai staging, CLL-IPI. This may be due to low sample size used as some of these molecules (galectin-9) correlated with prognostic markers and Rai staging in larger cohorts (4, 12, 14), highlighting its potential as a prognostic marker for patients with CLL. However, several studies have used similar sample size in their investigation of both cell membrane and soluble immune checkpoints levels (15, 16). In addition, we calculated the minimum sample size of patients needed to detect a large effect size (d) of 1.17 in the levels of soluble immune checkpoints, at 85% power and alpha (α) of 0.05 using GPower 3.1.94 software (Universität, Düsseldorf, Germany). To detect this large effect size between two independent means using an unpaired t-test, we required a minimum of nineteen patients with CLL (n = 19) and nine controls (n = 9).

6.4 Conclusion and future direction

There are limited studies investigating immune checkpoint blockage of CTLA-4, PD-1 and PD-L2 in patients with CLL. Our findings show a promising therapeutic potential of these immune checkpoint inhibitors. The treatment and management of CLL patients has significantly advanced in recent years due to the development of targeted therapies and immunotherapies or combination. The standard of care has transitioned from conventional chemotherapy or traditional chemoimmunotherapy to targeted therapies, including Bruton tyrosine kinase (BTK) and B-cell lymphoma 2 (Bcl-2) inhibitors, which are used either alone or in combination with immunotherapy. Our study suggested that African cohorts may benefit from immune checkpoint blockade treatment strategies. However, CLL is a heterogeneous disease with a highly variable clinical course. Some patients experience aggressive disease requiring early treatment, while others have an indolent course and may never need intervention. This variability complicates disease prognosis and deeper genetic phenotyping of B cells is required to fully understand the determinants of disease progression. Therefore, incorporating several prognostic markers is crucial for individualized assessment, personalized treatment, and patient stratification. The prognostic significance of immune checkpoints should be further investigated in larger patient cohorts, particularly in another Sub-Saharan African country to confirm these findings.

The "watch and wait" period for patients with CLL in South Africa is not fixed or predetermined. In our study, based on Rai staging, 76.2% of the patients were in an advanced stage, and 23.8% had hemoglobin levels below 10 g/dL, meeting the criteria for treatment initiation (17). We recommend refining the "watch and wait" period and shifting away from conventional chemotherapy or chemoimmunotherapy towards targeted therapy combined with immunotherapy, especially for African

patients. The higher frequency of del(17p) observed in African patients likely indicates that individuals with more advanced CLL (5). Moreover, del(17p) is associated with poor clinical outcomes and reduced overall survival when treated with chemoimmunotherapy (6, 18).

References

1. Rezazadeh H, Astaneh M, Tehrani M, Hossein-Nataj H, Zaboli E, Shekarriz R, et al. Blockade of PD-1 and TIM-3 immune checkpoints fails to restore the function of exhausted CD8+ T cells in early clinical stages of chronic lymphocytic leukemia. Immunologic Research. 2020;68:269-79.
2. Taghiloo S, Allahmoradi E, Ebadi R, Tehrani M, Hosseini-Khah Z, Janbabaei G, et al. Upregulation of Galectin-9 and PD-L1 immune checkpoints molecules in patients with chronic lymphocytic leukemia. Asian Pacific journal of cancer prevention: APJCP. 2017;18(8):2269.
3. Khoudoleeva O, Gretsov E, Barteneva N, Vorobjev I. Proliferative index and expression of CD38, Zap-70, and CD25 in different lymphoid compartments of chronic lymphocytic leukemia patients. Pathology and Laboratory Medicine International. 2011:7-16.
4. Ahmed HA, Nafady A, Ahmed EH, Hassan EEN, Soliman WGM, Elbadry MI, et al. CXC chemokine ligand 13 and galectin-9 plasma levels collaboratively provide prediction of disease activity and progression-free survival in chronic lymphocytic leukemia. Annals of Hematology. 2024;103(3):781-92.
5. Yang S, Varghese AM, Sood N, Chiattone C, Akinola NO, Huang X, et al. Ethnic and geographic diversity of chronic lymphocytic leukaemia. Leukemia. 2021;35(2):433-9.
6. Chavez JC, Kharfan-Dabaja MA, Kim J, Yue B, Dalia S, Pinilla-Ibarz J, et al. Genomic aberrations deletion 11q and deletion 17p independently predict for worse progression-free and overall survival after allogeneic hematopoietic cell transplantation for chronic lymphocytic leukemia. Leukemia research. 2014;38(10):1165-72.
7. Dal Bo M, Bulian P, Bomben R, Zucchetto A, Rossi F, Pozzo F, et al. CD49d prevails over the novel recurrent mutations as independent prognosticator of overall survival in chronic lymphocytic leukemia. Leukemia. 2016;30(10):2011-8.
8. Riley JL. PD-1 signaling in primary T cells. Immunological reviews. 2009;229(1):114-25.
9. Thibult M-L, Mamessier E, Gertner-Dardenne J, Pastor S, Just-Landi S, Xerri L, et al. PD-1 is a novel regulator of human B-cell activation. International immunology. 2013;25(2):129-37.
10. Kosmaczewska A, Ciszak L, Suwalska K, Wolowiec D, Frydecka I. CTLA-4 overexpression in CD19+/CD5+ cells correlates with the level of cell cycle regulators and disease progression in B-CLL patients. Leukemia. 2005;19(2):301-4.
11. Karabon L, Partyka A, Ciszak L, Pawlak-Adamska E, Tomkiewicz A, Bojarska-Junak A, et al. Abnormal expression of BTLA and CTLA-4 immune checkpoint molecules in chronic lymphocytic leukemia patients. Journal of Immunology Research. 2020;2020.
12. Alimu X, Zhang J, Pang N, Zhang R, Chen R, Zeng X, et al. Galectin-9 and myeloid-derived suppressor cell as prognostic indicators for chronic lymphocytic leukemia. Immunity, Inflammation and Disease. 2023;11(5):e853.
13. Knauf W, Langenmayer I, Ehlers B, Mohr B, Adorf D, Nerl C, et al. Serum levels of soluble CD23, but not soluble CD25, predict disease progression in early stage B-cell chronic lymphocytic leukemia. Leukemia & lymphoma. 1997;27(5-6):523-32.
14. Wdowiak K, Gallego-Colon E, Francuz T, Czajka-Francuz P, Ruiz-Agamez N, Kubeczko M, et al. Increased serum levels of Galectin-9 in patients with chronic lymphocytic leukemia. Oncology letters. 2019;17(1):1019-29.
15. Lindqvist CA, Christiansson LH, Simonsson B, Enblad G, Olsson-Strömberg U, Loskog AS. T regulatory cells control T-cell proliferation partly by the release of soluble CD25 in patients with B-cell malignancies. Immunology. 2010;131(3):371-6.

16. Karabon L, Partyka A, Ciszak L, Pawlak-Adamska E, Tomkiewicz A, Bojarska-Junak A, et al. Abnormal Expression of BTLA and CTLA-4 Immune Checkpoint Molecules in Chronic Lymphocytic Leukemia Patients. Journal of Immunology Research. 2020;2020(1):6545921.
17. Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Döhner H, et al. iwCLL guidelines for diagnosis, indications for treatment, response assessment, and supportive management of CLL. Blood, The Journal of the American Society of Hematology. 2018;131(25):2745-60.
18. Hallek M, Fischer K, Fingerle-Rowson G, Fink AM, Busch R, Mayer J, et al. Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. The Lancet. 2010;376(9747):1164-74.

APPENDICES

Ethics approval

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

14 October 2021

Mrs V Nundkissor (201267498)
School of Laboratory Medicine and Medical Sciences
College of Health Sciences
Venishree@mut.ac.za

Dear Mrs Nundkissor

Protocol: The effect of immune check point inhibitors on Chronic Lymphocytic Leukaemia patients in KwaZulu-Natal.
Degree: MMedSc
BREC Ref No: BE456/18
New Title*: *The effect of immune check point inhibitors on lymphocytic leukaemias

We wish to advise you that your application for amendments listed below received 21 September 2021 for the above study has been **noted and approved** by a sub-committee of the Biomedical Research Ethics Committee.

Amendments noted and approved:

1. Change of title to the new title above.
2. Additional co-investigators:
   Mr. Aviwe Ntsethe (213512600)
   Miss. Zekhethelo Mkhwanazi (216015946)

The committee will be notified of the above approval at its next meeting to be held on 09 November 2021.

Yours sincerely

[redacted]

.....
Ms A Marimuthu
(for) Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
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Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx
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INSPIRING GREATNESS

Published systematic review and meta-analysis protocol

Study Protocol Systematic Review

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The impact of immune checkpoint inhibitors in patients with chronic lymphocytic leukemia (CLL)

A protocol for a systematic review and meta-analysis of randomized controlled trials

Aviwe Ntsethe, MSc[a], Phiwayinkosi Vusi Dludla, PhD[c,d], Tawanda Maurice Nyambuya, MSc[a,b], Siphamandla Raphael Ngcobo, BSc[a], Bongani Brian Nkambule, PhD[a,*]

Abstract

Introduction: The global burden of chronic lymphocytic leukemia (CLL) has constantly increased over the years, with a current incidence of 3.5 cases per 100,000 people. Although the conventional drugs used to treat CLL patients have been effective treatment failure rate in some of the patients is alarming. Therefore, as a result, novel treatment strategies with improved outcomes such as the blockade of immune checkpoints have emerged. However, consensus on the risk-benefit effects of the using these drugs in patients with CLL is controversial and has not been comprehensively evaluated. This systemic review and meta-analysis provide a comprehensive synthesis of available data assessing adverse events associated with the use of immune checkpoint inhibitors in patients with CLL as well as their influence on the overall survival rate.

Methods: This protocol for a systematic review and meta-analysis has been prepared in accordance with Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols 2015 guidelines. A search strategy will be developed using medical subject headings words in PubMed search engine with MEDLINE database. The search terms will also be adapted for gray literature, Embase, and Cochrane Central Register of Controlled Trials electronic databases. Two reviewers (AN and SRN) will independently screen studies, with a third reviewer consulted in cases of disagreements using a defined inclusion and exclusion criteria. Data items will be extracted using a predefined data extraction sheet. Moreover, the risk of bias and quality of the included studies will be appraised using the Downs and Black checklist and the quality and strengths of evidence across selected studies will be assessed using the Grading of Recommendations Assessment Development and Evaluation approach. The Cochran's Q statistic and the I^2 statistics will be used to analyze statistical heterogeneity across studies. If the included studies show substantial level of statistical heterogeneity ($I^2 > 50\%$), a random-effects meta-analysis will be performed using R statistical software.

Ethics and dissemination: The review and meta-analysis will not require ethical approval and the findings will be published in peer-reviewed journals and presented at local and international conferences. This review may help provide clarity on the risk-benefit effects of using immune checkpoint inhibitors in patients with CLL.

Systematic review registration: International prospective Register of Systematic Reviews (PROSERO) number: CRD42020156926.

Abbreviations: CLL = chronic lymphocytic leukemia, CTLA-4 = cytotoxic T-lymphocyte-associated protein 4, LAG-3 = lymphocyte-activation gene 3, PD-1 = programmed death-1, PD-L1 = programmed death-ligand 1, PRISMA-P = Preferred

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The authors have no conflicts of interest to disclose.

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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1

supplementary file 2.3.1: systematic review checklist

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	29
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	30-31
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	32
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	33
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	37
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	39
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	33
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	37
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	37
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	37
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	37
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	37-38
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	N/A
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	43-45 and 47-48
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	N/A
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	N/A
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the	43-45 and

Section and Topic	Item #	Checklist item	Location where item is reported
		model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	47-48
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	N/A
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	N/A
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	N/A
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	38
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	39
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	39
Study characteristics	17	Cite each included study and present its characteristics.	43-45 and 16-17
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	41
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	51-52
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	41
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	N/A
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	N/A
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	N/A
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	41
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	51-52
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	53
	23b	Discuss any limitations of the evidence included in the review.	54
	23c	Discuss any limitations of the review processes used.	53-54
	23d	Discuss implications of the results for practice, policy, and future research.	54
OTHER INFORMATION			

Section and Topic	Item #	Checklist item	Location where item is reported
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	33
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	33
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	33
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	54
Competing interests	26	Declare any competing interests of review authors.	54
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	33,38 and 41

From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi: 10.1136/bmj.n71

Supplementary file 2.3.2: search strategy

Table S2: Search strategy used on EBSCOHOST and PubMed (Search ran on the 24/02/2024)

Databases searched (#Hits)	**Academic Search Complete (n=380), CINAHL with Full Text (n=69), Health Source: Nursing/Academic Edition (42), MEDLINE (n=1 622), ClinicalTrials.gov (135)**
Search Strategy used	((((((((("Leukemia, Lymphocytic, Chronic, B-Cell"[Mesh]) AND "Rituximab"[Mesh]) OR "obinutuzumab" [Supplementary Concept]) OR "ofatumumab" [Supplementary Concept]) OR "Alemtuzumab"[Mesh]) OR "Bevacizumab"[Mesh]) OR "zanubrutinib" [Supplementary Concept]) OR "acalabrutinib" [Supplementary Concept]) OR "ibrutinib" [Supplementary Concept]) OR "navitoclax" [Supplementary Concept]) OR "venetoclax" [Supplementary Concept]

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Brief Report

B Cell Subsets and Immune Checkpoint Expression in Patients with Chronic Lymphocytic Leukemia

Aviwe Ntsethe [1], Zekhethelo Alondwe Mkhwanazi [1], Phiwayinkosi Vusi Dludla [2,3] and Bongani Brian Nkambule [1,*]

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Abstract: Chronic lymphocytic leukemia (CLL) is characterized by dysfunctional B cells. Immune checkpoint molecules such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed death-1 (PD-1) are upregulated in patients with CLL and may correlate with prognostic markers such as beta-2 microglobulin (B2M). The aim of this study was to evaluate the levels of immune checkpoints on B cell subsets and to further correlate them with B2M levels in patients with CLL. We recruited 21 patients with CLL and 12 controls. B cell subsets and the levels of immune checkpoint expression were determined using conventional multi-color flow cytometry. Basal levels of B2M in patients with CLL were measured using an enzyme-linked immunosorbent assay. Patients with CLL had increased levels of activated B cells when compared to the control group, $p < 0.001$. The expression of PD-1 and CTLA-4 were increased on activated B cells and memory B cells, $p < 0.05$. There were no associations between B2M levels and the measured immune checkpoints on B cell subsets, after adjusting for sex and age. In our cohort, the patients with CLL expressed elevated levels of PD-1 and CTLA-4 immune checkpoints on activated and memory B cell subsets. However, there was no correlation between these immune checkpoint expressions and B2M levels.

Keywords: chronic lymphocytic leukemia; immune checkpoints; B cell subsets; beta-2 microglobulin; programmed death protein 1; cytotoxic T-lymphocyte-associated protein 4

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1. Introduction

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia among adults, with a global prevalence of about 3.5 cases per 100,000 people [1]. In high-income countries, CLL accounts for more than a third of all leukemia cases [1]. Notably, low-to-middle-income countries have a five- to ten-fold lower age-adjusted incidence rate of CLL compared to high-income countries [2]. CLL is a lymphoproliferative disorder that is characterized by functionally incompetent B cells with a distinct immunophenotype [3]. However, there are divergent findings regarding the predictive significance of B cell subsets in leukemia [4].

Regulatory B cells (Bregs) modulate T cell-driven anti-tumor immunity, promoting the expression of forkhead box protein 3 (FoxP3+) in regulatory T cells (T-regs), which dampens the innate and adaptive antitumor immune response [4,5]. These immunosuppressive mechanisms involve Bregs, which acquire inhibitory ligands and signal transducer and activator of transcription 3 (STAT3) phosphorylation, and the induction of interleukin 10 (IL-10) and transforming growth factor-β (TGF-β) [4,5]. The expression of inhibitory molecules such as programmed death-1 (PD-1) or programmed death-ligand 1 (PD-L1) inhibits the anti-tumor function of Bregs [4]. In patients with CLL, immune checkpoints such as T-cell immunoglobulin-3 (TIM-3), cytotoxic T-lymphocyte-associated protein 4

Supplementary 1

Table S1. Age, sex adjusted multivariable regression analysis of immune checkpoints on B cell subsets with B2M.

	B2M		Gender		Age (years)	
	Coefficient Estimate (SE)	***p*-Value**	**Coefficient Estimate (SE)**	***p*-Value**	**Coefficient Estimate (SE)**	***p*-Value**
PD-1 on B cells	0.008 (0.011)	0.454	−2.811 (7.206)	0.701	−0.179 (0.276)	0.524
PD-1 on activated B cells	0.008 (0.011)	0.458	−2.825 (7.192)	0.699	−0.179 (0.275)	0.525
PD-1 on memory B cells	0.0008 (0.006)	0.897	−0.929 (3.900)	0.815	−0.018 (0.149)	0.905
CTLA-4 on B cells	0.004 (0.002)	0.098	−0.171 (1.631)	0.918	−0.051 (0.062)	0.426
CTLA-4 on activated B cells	0.004 (0.002)	0.086	−0.056 (1.548)	0.972	−0.049 (0.059)	0.419
CTLA-4 on memory B cells	0.004 (0.002)	0.086	−0.056 (1.548)	0.972	−0.049 (0.059)	0.419

Supplementary file 5.2

Table S1. multivariable regression analysis of soluble immune checkpoints with B2M and Rai stage.

	B2M		Rai stage	
	Coefficient Estimate (SE)	***p*-Value**	**Coefficient Estimate (SE)**	***p*-Value**
sCD25	-0.385 (0.8029)	0.638	-0.00003 (0.0002)	0.891
TIM-3	0.738 (1.603)	0.651	-0.00004 (0.0001)	0.723
Galectin-9	0.301 (0.168)	0.091	-0.0003 (0.0012)	0.775
PD-1	0.034 (0.113)	0.770	0.0026 (0.0024)	0.310
PD-L1	0.002 (0.010)	0.860	0.0085 (0.0254)	0.742

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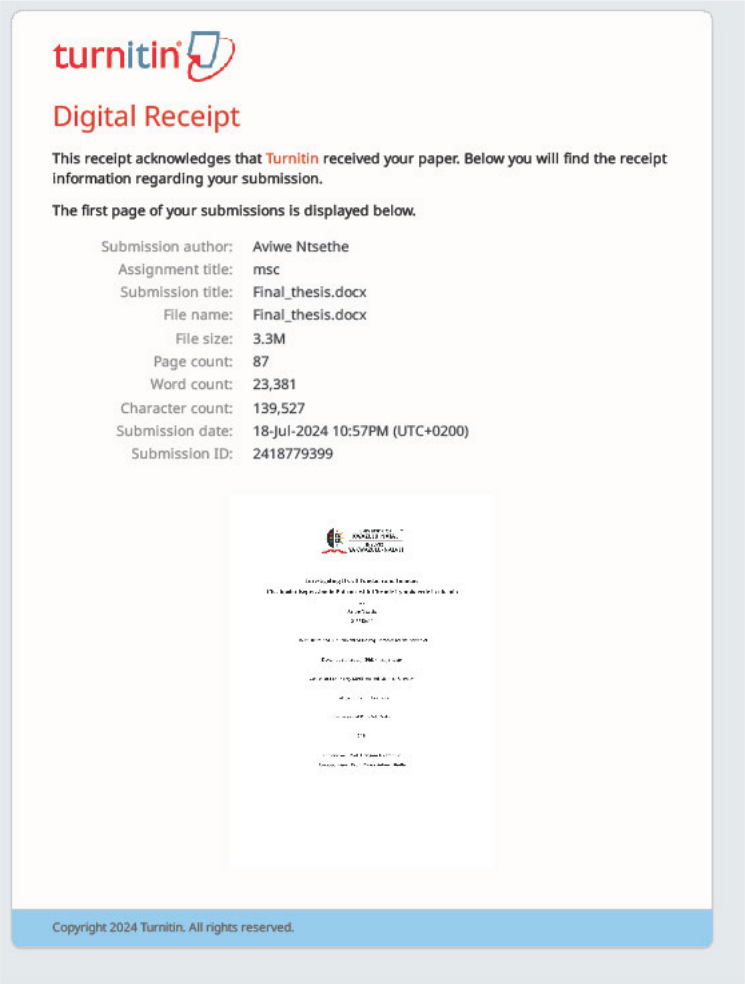

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