DOI: 10.37990/medr.1352143 Med Records 2023;5(Suppl 1):150-4

MEDICAL RECORDS-International Medical Journal

Research Article

High Salt-Induced Hyperosmolality Reduces in Vitro Survival and Proliferation of Pre-B Cells

Mehmet Yabas

Malatya Turgut Özal University, Faculty of Medicine, Department of Immunology, Malatya, Türkiye

Abstract

Aim: B cells of the adaptive immunity are critical for protection against the vast majority of pathogens through the production of specific antibodies. A number of signaling pathways and transcription factors control B cell development. Environmental factors, including diet, are also important in determining how B cell develop and function. Here, the effects of hyperosmolality induced by elevated salt on the survival, IL-7-induced proliferation and differentiation of pre-B cells were tested in vitro.

Material and Methods: The wk3 pre-B cell line generated from SLP65$^{-/-}$ mice was used. Hyperosmolality in the cell culture medium was created by increasing the salt concentration with the addition of 40 mM NaCl. Wk3 pre-B cells were cultured in standard (normal NaCl) and high salt (+40 mM NaCl) medium, followed by flow cytometric analysis.

Results: It was found that hyperosmolality caused by high salt reduced survival and induced apoptosis in wk3 pre-B cells. In addition, hyperosmolality inhibited IL-7-induced proliferation of pre-B cells. Conversely, pre-B cells treated with high salt were able to differentiate normally into IgM^{+} immature B cells when IL-7 was removed.

Conclusion: These findings suggest that the hyperosmolar microenvironment induced by high salt may play a key role in B cell development in the bone marrow.

Keywords: Hyperosmolality, pre-B cells, salt, B cells

INTRODUCTION

The development of B cells in the mammalian bone marrow is a tightly regulated process controlled by multiple factors, including transcription factors and signaling through the cytokine receptors (1). At the pro-B cell stage, a functional Igμ heavy chain results from successful immunoglobulin (Ig) heavy chain rearrangements (2). The assembly of Igμ with VpreB, lambda5, Igα and Igβ leads to the formation of a pre-BCR complex (3-6). Expression of the pre-BCR is a hallmark of pre-B cell differentiation and serves as a checkpoint (3-6). Functional pre-BCR expression initiates interleukin (IL)-7-dependent proliferation to increase pre-B cell numbers (7). The cessation of proliferation leads to the differentiation of large proliferating pre-B cells into small pre-B cells, which initiates the rearrangement of the Ig light chain gene (3-6). After successfully producing a light chain, these cells express IgM and become immature B cells (8).

In addition to transcription factors and signaling pathways, environmental factors such as diet are thought to play critical roles in the immune system (9). The consumption of table salt (sodium chloride, NaCl) has been increasing in western societies. Salt consists approximately of 40% sodium and 60% chloride, and is an important source for these minerals. Sodium, in particular, is the most abundant cation found in the human body and plays key roles in human physiology. According to the World Health Organization, a healthy individual should consume 5 grams of salt per day. However, people consume much more salt than recommended, which can lead to significant health problems such as hypertension, stroke, cardiovascular and kidney diseases (10). Due to the important biological properties of sodium, low salt intake may also have negative biological effects. Therefore, understanding the positive or negative effects of low/excessive salt intake on different biological systems is critical for public health. This study investigated the effects of hyperosmolality caused by elevated salt on pre-B cells in vitro.

CITATION

Yabas M. High Salt-Induced Hyperosmolality Reduces in Vitro Survival and Proliferation of Pre-B Cells. Med Records. 2023;5(Suppl 1):150-4. DOI:1037990/medr.1352143

Received: 29.08.2023 **Accepted:** 28.09.2023 **Published:** 11.10.2023

Corresponding Author: Mehmet Yabas, Malatya Turgut Özal University, Faculty of Medicine, Department of Immunology, Malatya, Türkiye

E-mail: mehmet.yabas@ozal.edu.tr

MATERIAL AND METHOD

Cell Culture

The cell culture and experimental procedures used in this study were performed as previously described (11). The wk3 cell line was originally generated from SLP65$^{-/-}$ mice (12) and cultured in IMDM (Sigma) supplemented with 7.5% premium FBS (Cegrogen), 1% L-Glutamine (Sigma), 1% Penicillin-Streptomycin (Sigma) and 50 µM 2-Mercaptoethanol. Supernatant from IL-7-expressing J558L cells was also added to the medium as an excess source of IL-7 (12,13). For hyperosmolality, the medium was supplemented with an extra 40 mM NaCl (Sigma) to mimic the high salt environment (14-21).

Survival Assay

10^5 wk3 cells were cultured in standard salt (normal) and high salt medium supplemented with excess IL-7 for 24, 48 and 72 h. The cells were then stained with an Apoptosis Detection Kit (Cat: 640914, Biolegend) based on the manufacturer's instructions, followed by flow cytometric analysis.

Carboxyfluorescein Succinimidyl Ester (CFSE) Labelling

For the detection of IL-7-dependent proliferation, 10^5 wk3 cells were labelled with 10 µM CellTrace™ CFSE kit (Invitrogen) in IL-7-free medium for 10 min at RT, followed by three washes with the same medium (22). The cells were then cultivated in standard salt (normal) and high salt in medium with excess IL-7 for 48 and 72 h. A dead cell marker (Cat: S34859, ThermoFisher) was then used for the exclusion of dead cells from the analysis and the remainder cells were tested for CFSE dilution by flow cytometry.

Differentiation Assay

5x10^5 wk3 cells were cultivated in standard salt (normal) and high salt medium without IL-7 for 48 and 72 h. The cells were then labelled with a FITC-conjugated anti-mouse Igκ (Clone: RMK-45, Biolegend) and a PE-conjugated anti-mouse IgM (Clone: eB121-15F9, ThermoFisher), and stained at 4ºC for 30 min. After the incubation period, the cells were resuspended in PBS supplemented with 2% FBS before their analysis on a flow cytometry.

Analysis on Flow Cytometry

An Attune Acoustic Focusing Cytometer (Applied Biosystems) was used for the flow cytometric analysis and FlowJo software (Tree Star) was used for data analysis.

Statistical Analysis

For statistical analysis of the two groups (normal and high salt), Student's t-test was used with the GraphPad Prism 5. When $p<0.05$, differences were considered significant.

RESULTS

Hyperosmolality induced by high salt diminishes pre-B cell survival

In order to test whether hyperosmolality has any effect on pre-B cells, wk3 cells were used. These cells were originated from SLP65$^{-/-}$ mice and are able to proliferate indefinitely when IL-7 is present (12). However, in the absence of IL-7, wk3 cells differentiate into immature B cells (12). In this study, hyperosmolality was created in the cell culture medium by increasing the NaCl concentration with an additional 40 mM NaCl (high salt), as previously reported (19-21,23). Wk3 cells were cultured in standard and high salt medium with excess IL-7 for 24, 48 and 72 h. The cells were labelled with Annexin-V and PI, and analyzed for cell viability and the detection of apoptotic cells by flow cytometry (Figure 1A). The analysis revealed a reduction in the proportion of live (Annexin-V$^-$PI$^-$) cells (Figure 1B) and a rise in the proportion of dead (Annexin-V$^+$PI$^+$) cells (Figure 1D) when wk3 cells were treated with an additional 40 mM salt for 24 h. Apoptosis and cell death were further induced by high salt at 48 and 72 h, as evidenced by an increase in the proportion of early apoptotic (Annexin-V$^+$PI$^-$) cells and dead (Annexin-V$^+$PI$^+$) cells (Figure 1C, D). Concomitantly, there was a more pronounced reduction in the percentage of live (Annexin-V$^-$PI$^-$) cells when cells were cultured in high NaCl medium for 48 and 72 h (Figure 1B). These results suggest that hyperosmolality caused by high salt reduces survival and increases apoptosis in wk3 pre-B cells in vitro.

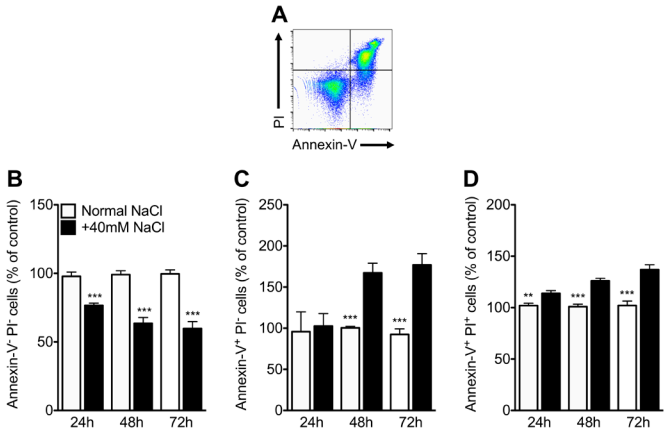

Figure 1. High salt-induced hyperosmolality diminishes survival of wk3 cells. 10^5 wk3 cells were cultivated for 24, 48 and 72 h in IL-7 containing medium supplemented with or without an extra 40 mM NaCl. Cells were labelled with Annexin-V and PI for flow cytometric analysis. **(A)** Representative flow cytometric plot showing the gating strategy for the analysis of Annexin-V and PI in cultured cells, pre-gated on singlets. The graphs show % of **(B)** live cells (Annexin-V$^-$PI$^-$), **(C)** early apoptotic cells (Annexin-V$^+$PI$^-$) and **(D)** late apoptotic/necrotic cells (Annexin-V$^+$PI$^+$) presented as percent of control. Data are representative of at least three independent experiments per group in triplicate. **p<0.01, ***p<0.001

Hyperosmolality Induced by High Salt Reduces IL-7-Induced Pre-B Cell Proliferation

Given that treatment of wk3 cells with high salt resulted in reduced survival, and that wk3 cells are capable of IL-7-induced proliferation (12,13), the impact of hyperosmolality on IL-7-induced pre-B cell proliferation was next tested. To this end, wk3 cells were labelled with CFSE and cultured in normal and high salt medium for 48 and 72 h, followed by analysis (Figure 2A). The

results demonstrated that wk3 cells treated with high salt accumulated in cell division phases 2 and 3 after 48 h of incubation (Figure 2A, B). Conversely, the proportion of proliferating cells in cell division phase 4 was reduced when wk3 cells were cultured in high salt medium for 48 h (Figure 2A, B). Similarly, wk3 cells treated with high salt had a rise in the percentage of proliferating cells in cell division phases 1, 2 and 3 and a reduction in phases 4 and 5 after 72 h of incubation (Figure 2A, C). Collectively, these findings indicate that hyperosmolality induced by high salt in wk3 cells results in a compromised or delayed in vitro response to IL-7.

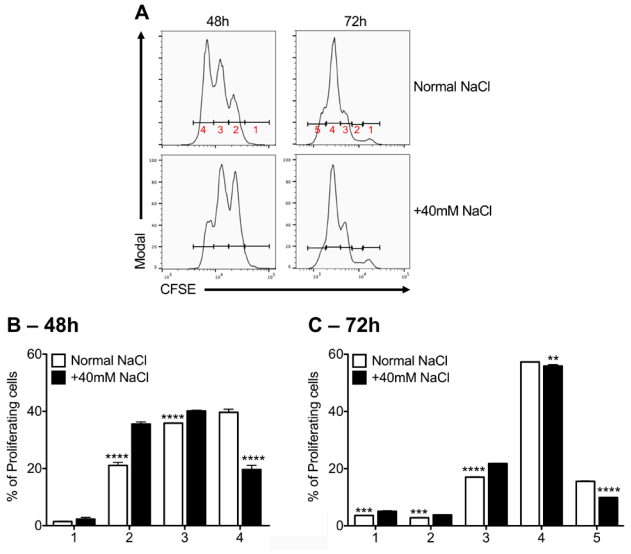

Figure 2. High salt-induced hyperosmolality reduces IL-7-induced wk3 cell proliferation. 10^5 wk3 cells were labelled with 10μM CFSE and cultivated for 48 and 72 h in IL-7 containing medium supplemented with or without an extra 40 mM NaCl and analyzed for CFSE dilution. Doublets and dead cells were excluded from the analysis using the dead cell marker. **(A)** Representative histograms show CFSE dilution on live cells. The graphs show the % of cells that have undergone cell division after **(B)** 48 h and **(C)** 72 h of incubation. Cells were gated as in **(A)**. Data are representative of at least three independent experiments per group in triplicate. **p<0.01, ***p<0.001, ****p<0.0001

Hyperosmolality Induced by High Salt has no Effect on the Differentiation of Pre-B Cells

Loss of IL-7R signaling suppresses pre-B cell proliferation and provokes the differentiation of pre-B cell into IgM⁺ B cells (12,13). In order to test whether hyperosmolality due to elevated salt has any effect on pre-B cell differentiation, wk3 cells were cultured in standard and high salt medium for 48 and 72 h in the absence of IL-7, followed by their Igκ and IgM expression. The results demonstrated that the percentage of Igκ⁺IgM⁺ cells in cells cultured in high salt medium was comparable to those cultured in standard medium after 48 h of incubation (Figure 3). In addition, wk3 cells cultured in high salt medium induced slightly more Igκ⁺IgM⁺ immature B cells after 72 h than cells cultured in medium with standard salt (Figure 3). These data indicate that high salt-induced hyperosmolality has no effect on wk3 cell differentiation into immature B cells when IL-7R signaling was terminated in vitro.

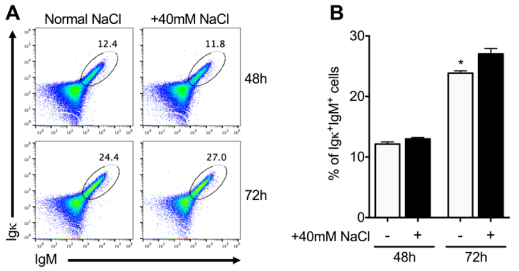

Figure 3. High salt-induced hyperosmolality has no effect on the differentiation of wk3 cells after IL-7 deprivation. $5x10^5$ wk3 cells were cultured for 48 and 72 h in medium containing normal and high salt without IL-7. Cells were labelled with Igκ, IgM and Sytox Red, followed by the flow cytometric analysis. An exclusion of doublets and dead cells from the analysis using the dead cell marker was done. **(A)** Representative flow cytometric plots of Igκ and IgM analysis in cultured cells. **(B)** The graph demonstrates % of Igκ⁺IgM⁺ immature B cells after 48 and 72 h of incubation. Data are representative of at least three independent experiments per group in triplicate. *p<0.05

DISCUSSION

It has long been recognized that there is an important link between the immune system and environmental factors such as diet (9). As salt is one of the most commonly consumed nutrients, this study investigated the impacts of high salt-induced hyperosmolality on pre-B cells in vitro. The results demonstrated a reduction in wk3 cell survival and proliferation caused by the hyperosmolar environment.

Salt is an important source of the sodium and chloride that our bodies need. Because sodium is particularly important for human physiology, through its ability to control osmotic balance and regulate the amount of extracellular fluid, the optimal amount of salt should be consumed, which is 5 grams per day for a healthy person. However, people consume much more than the recommended amount (24), which can cause significant health problems such as hypertension, stroke, cardiovascular diseases and kidney diseases (10). Recent studies have revealed that there may be an important link between salt intake and the immune system (25-27). In 2015, a study showed that macrophages activated in high salt concentrations were more effective against cutaneous Leishmania major than macrophages activated in normal salt (28). Moreover, high salt treatment increased type 1 interferon signaling in macrophages and resistance to vesicle stomatitis virus, and provided protection against vesicle stomatitis virus in mice (29). The tumor growth was also inhibited by high salt (23,30), and high salt prevents autoimmune demyelination through regulation of the permeability of the blood-brain barrier (31).

Despite these beneficial effects of high salt intake, studies have reported that high sodium chloride intake triggers autoimmune development by inducing pathogenic T17 helper cells (TH17) (15,32). In addition, high salt exacerbated central nervous system autoimmunity by activating pro-inflammatory macrophages (33). Moreover, dietary intake of high salt has also been shown to reduce the antibacterial responses of neutrophils against pyelonephritis (18), and to inhibit the suppressive capacity

of regulatory T cells (17).

In B cells, hyperosmolality induced by high level of salt inhibits the survival and LPS-dependent proliferation of peripheral mature B cells isolated from mice (20). Interestingly, hyperosmolality led to a rise in B activation and the generation of more antibody-producing plasma cells (20). Similarly, in this study high salt-induced hyperosmolality also diminished the survival of wk3 cells and induced apoptosis. In addition, wk3 cells showed an impaired or delayed IL-7-induced proliferation. Taken together, these data suggest that the hyperosmolar environment created by elevated salt has a negative effect on the survival and proliferation of pre-B cells as well as LPS-stimulated mouse splenic mature B cells.

Salt has been suggested to control the development and function of immune cells by modulating various signaling pathways (25, 34). Hyperosmolality induced by increased salt has been shown to increase the p38-dependent NFAT5 response in peripheral B cells, T cells and macrophages (15, 20, 28, 35). It is therefore possible that increased salt may have similar mechanistic effects in pre-B cells. Furthermore, since IL-7R signaling is important for pre-B cell survival and proliferation (36, 37), and hyperosmolality caused a block in IL-7-dependent proliferation of wk3 cells, an alternative mechanism responsible for the phenotype reported in this study would be the effects of hyperosmolality induced by increased salt on signaling through the IL-7R. It is plausible that salt could affect IL-7R (IL-7Rα and γc) expression on the cell surface, the phosphorylation of its downstream molecules (such as JAK1, JAK3 and STAT5) and/or the transcription factors regulated by IL-7R signaling. Future studies will help to determine how high salt induced hyperosmolality mechanistically regulates pre-B cell survival and proliferation.

CONCLUSION

In conclusion, this study shows that hyperosmolality induced by increased salt negatively affects pre-B cell survival and proliferation in vitro. Elucidating the impacts of a high salt diet on B cell biology may help to understand the pathophysiology of B cell-related disorders, including autoimmune diseases. As a limitation, it should be noted that this in vitro study used a pre-B cell line derived from SLP65-deficient mice.

Financial disclosures: *The authors declared that this study has received no financial support.*

Conflict of Interest: *The authors declare that they have no competing interest.*

Ethical approval: *Not applicable.*

Acknowledgments: *The author thanks Dr. Sebastian Herzog, the Medical University of Innsbruck, Austria for providing wk3 cells, the Department of Genetics and Bioengineering at Trakya University, Türkiye for the opportunity to conduct the experiments in their research facilities, and the Technology Research Development Application and Research Center of Trakya University, Türkiye for the use of the flow cytometry.*

REFERENCES

1. Nutt SL, Kee BL. The transcriptional regulation of B cell lineage commitment. Immunity. 2007;26:715-25. Erratum in: Immunity. 2007;27:361.
2. Hardy RR, Hayakawa K. B cell development pathways. Annu Rev Immunol. 2001;19:595-621.
3. Herzog S, Reth M, Jumaa H. Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling. Nat Rev Immunol. 2009;9:195-205.
4. Clark MR, Mandal M, Ochiai K, Singh H. Orchestrating B cell lymphopoiesis through interplay of IL-7 receptor and pre-B cell receptor signalling. Nat Rev Immunol. 2014;14:69-80.
5. Hendriks RW, Middendorp S. The pre-BCR checkpoint as a cell-autonomous proliferation switch. Trends Immunol. 2004;25:249-56.
6. Reth M, Nielsen P. Signaling circuits in early B-cell development. Adv Immunol. 2014;122:129-75.
7. Rolink AG, Winkler T, Melchers F, Andersson J. Precursor B cell receptor-dependent B cell proliferation and differentiation does not require the bone marrow or fetal liver environment. J Exp Med. 2000;191:23-32.
8. Samitas K, Lotvall J, Bossios A. B cells: from early development to regulating allergic diseases. Arch Immunol Ther Exp (Warsz). 2010;58:209-25.
9. Calder PC. Nutrition and immunity: lessons for COVID-19. Nutr Diabetes. 2021;11:19.
10. Kotchen TA, Cowley AW Jr, Frohlich ED. Salt in health and disease--a delicate balance. N Engl J Med. 2013;368:1229-37.
11. Yabas M, Bostanci A, Aral S. ATP11C promotes the differentiation of pre-B cells into immature B cells but does not affect their IL-7-dependent proliferation. Immunol Res. 2023;71:609-16.
12. Flemming A, Brummer T, Reth M, Jumaa H. The adaptor protein SLP-65 acts as a tumor suppressor that limits pre-B cell expansion. Nat Immunol. 2003;4:38-43.
13. Lindner SE, Lohmuller M, Kotkamp B, et al. The miR-15 family reinforces the transition from proliferation to differentiation in pre-B cells. EMBO Rep. 2017;18:1604-17.
14. Binger KJ, Gebhardt M, Heinig M, et al. High salt reduces the activation of IL-4- and IL-13-stimulated macrophages. J Clin Invest. 2015;125:4223-38.
15. Kleinewietfeld M, Manzel A, Titze J, et al. Sodium chloride drives autoimmune disease by the induction of pathogenic TH17 cells. Nature. 2013;496:518-22.
16. Wilck N, Matus MG, Kearney SM, et al. Salt-responsive gut commensal modulates TH17 axis and disease. Nature. 2017;551:585-9.
17. Hernandez AL, Kitz A, Wu C, et al. Sodium chloride inhibits the suppressive function of FOXP3+ regulatory T cells. J Clin Invest. 2015;125:4212-22.
18. Jobin K, Stumpf NE, Schwab S, et al. A high-salt diet compromises antibacterial neutrophil responses through hormonal perturbation. Sci Transl Med. 2020;12:eaay3850.
19. Machnik A, Neuhofer W, Jantsch J, et al. Macrophages regulate salt-dependent volume and blood pressure by a vascular endothelial growth factor-C-dependent buffering mechanism. Nat Med. 2009;15:545-52.

20. Cvetkovic L, Perisic S, Titze J, et al. The impact of hyperosmolality on activation and differentiation of B lymphoid cells. Front Immunol. 2019;10:828.

21. Wiig H, Schroder A, Neuhofer W, et al. Immune cells control skin lymphatic electrolyte homeostasis and blood pressure. J Clin Invest. 2013;123:2803-15.

22. Quah BJ, Parish CR. New and improved methods for measuring lymphocyte proliferation in vitro and in vivo using CFSE-like fluorescent dyes. J Immunol Methods. 2012;379:1-14.

23. He W, Xu J, Mu R, et al. High-salt diet inhibits tumour growth in mice via regulating myeloid-derived suppressor cell differentiation. Nat Commun. 2020;11:1732.

24. Tan M, He FJ, Wang C, MacGregor GA. Twenty-four-hour urinary sodium and potassium excretion in China: a systematic review and meta-analysis. J Am Heart Assoc. 2019;8:e012923.

25. Jobin K, Muller DN, Jantsch J, Kurts C. Sodium and its manifold impact on our immune system. Trends Immunol. 2021;42:469-79.

26. Toussirot E, Bereau M, Vauchy C, Saas P. Could sodium chloride be an environmental trigger for immune-mediated diseases? An overview of the experimental and clinical evidence. Front Physiol. 2018;9:440.

27. Wilck N, Balogh A, Marko L, et al. The role of sodium in modulating immune cell function. Nat Rev Nephrol. 2019;15:546-58.

28. Jantsch J, Schatz V, Friedrich D, et al. Cutaneous Na+ storage strengthens the antimicrobial barrier function of the skin and boosts macrophage-driven host defense. Cell Metab. 2015;21:493-501.

29. Zhang WC, Du LJ, Zheng XJ, et al. Elevated sodium chloride drives type I interferon signaling in macrophages and increases antiviral resistance. J Biol Chem. 2018;293:1030-9.

30. Willebrand R, Hamad I, Van Zeebroeck L, et al. High salt inhibits tumor growth by enhancing anti-tumor immunity. Front Immunol. 2019;10:1141.

31. Na SY, Janakiraman M, Leliavski A, Krishnamoorthy G. High-salt diet suppresses autoimmune demyelination by regulating the blood-brain barrier permeability. Proc Natl Acad Sci U S A. 2021;118:e2025944118.

32. Wu C, Yosef N, Thalhamer T, et al. Induction of pathogenic TH17 cells by inducible salt-sensing kinase SGK1. Nature. 2013;496:513-7.

33. Hucke S, Eschborn M, Liebmann M, et al. Sodium chloride promotes pro-inflammatory macrophage polarization thereby aggravating CNS autoimmunity. J Autoimmun. 2016;67:90-101.

34. Li X, Alu A, Wei Y, et al. The modulatory effect of high salt on immune cells and related diseases. Cell Prolif. 2022;55:e13250.

35. Kino T, Takatori H, Manoli I, et al. Brx mediates the response of lymphocytes to osmotic stress through the activation of NFAT5. Sci Signal. 2009;2:ra5.

36. Milne CD, Paige CJ. IL-7: a key regulator of B lymphopoiesis. Semin Immunol. 2006;18:20-30.

37. Corfe SA, Paige CJ. The many roles of IL-7 in B cell development; mediator of survival, proliferation and differentiation. Semin Immunol. 2012;24:198-208.