Investigation of B cell lines and primary human B cells for in Vitro Immunological Enhancement

Sujith Sri Surya R [1], Leela Kakithakara Vajravelu [1], Balamurali Venkatesan [1*], Jayaprakash Thulukanam[1]

[1] Department of Microbiology, SRM Medical College Hospital and Research Centre, SRM Institute of Science and Technology, Kattankulathur, Chengalpattu district, Tamilnadu, India.

*Corresponding Author

Balamurali Venkatesan, Department of Microbiology, SRM Medical College Hospital and Research Centre, SRM Institute of Science and Technology, Kattankulathur, Chengalpattu district, Tamilnadu, India.

Email: balajai96@gmail.com

Abstract

Introduction: Even while immunotherapeutic drugs lengthen patients' lives, they have considerable adverse effects. B cells meet a similar function during immunological responses, and they are now only the target of a very small number of drugs. B cells respond differently based on strength and type of the receiving stimulatory signals.

Aim of the study: To analyse cell lines of human B cells and different B cell stimuli in order to develop an in vitro model that is particularly suitable for investigating the immunological activation of B cells and that is simple to modify for drug discovery and screening.

Materials and Method: Realistic stimulatory conditions may be simulated in vitro. The ligation of CD40-CD40L that are promoting the differentiation and clonal proliferation of B cell has been mimicked by anti-CD40 antibodies that are hostile. Initially, weak responses to stimuli by B cells is used here as it has detrimental effects on their capacity to multiply and generate Ig and cytokines. LPS had no impact with the exception of a slight increase in IL6 and IL8. The majority of cell surface indicators significantly upregulated after ODN2006 stimulation.

Results: CD86 which is a costimulatory marker is a great sensitive that has emerged. By using flow cytometry readout to activate B cells, ODN2006 as well as costimulatory cell surface markers, front-line screen for discovering new B cell active components is made possible.

Conclusion: When the adoptively transplanted tumour-primed B lymphocytes are then activated in vitro, they can aid in the regression of preexisting tumours. This involves a group of distinct effector cells as it may increase effectiveness of B cell therapy adoptively.

Keywords: CD40-CD40L ligation, ODN2006-stimulated B cells, Clonal proliferation, B cells.

1. INTRODUCTION

Even while immunotherapeutic drugs lengthen patients' lives, they have considerable adverse effects [1,2]. Additionally, clinical development of numerous innovative small molecule

immunotherapeutic medicines are diminishing despite growing pharmaceutical industry investment levels [3]. Additionally, the majority of commercially available immunotherapeutic drugs (e.g., tacrolimus – the inhibitors of calcineurin, tacrolimus and everolimus (mTOR inhibitors), CD3 antagonists like daclizumab and basiliximab, and muromonab as well as cyclosporine A) work by inhibiting T cell activation [4]. Despite this, B cells carry out the same function in immunological responses and are only now being targeted by a very small number of drugs. [5]. B cells can act in a variety of ways as an effector. Igs are created to guarantee that invading germs and dead cells are removed [6]. B cells which attach with major histocompatibility complex (MHC) molecules through epitopes and capture antigens are called effective antigen-presenting cells [7]. By cytokine generating and changing degree of expression among several markers of cellular membrane. The activated B cells could effectively associate with various effector cells for generating immunological responses which are regulated as well as focused. [8,9].

B cells have a crucial, albeit underappreciated, role as pathogenic agents among cancer, autoimmune diseases, graft-versus-host diseases, and rejection of transplants. [10]. Only just a handful of immunomodulatory medications that are specific to B cell (like bortezomib, rituximab and belimumab,) and primarily reducing drugs are now available in clinical settings. [11]. As a result, a gap in the market for innovative drugs in this field has been noted. The study on regulatory models on B cells may resulted in detection of newer targets or compounds by therapeutic ability for B cells. Therefore, the main target of the present research work is for analysing cells lines of human B cells as well as different stimulus of B cells in order to develop model in vitro which are particularly adequate for investigating the immunological activation of B cells and that is simple to modify the screening and discovery of drugs.

2. MATERIALS AND METHODS:

Cell Culture Media:

Gentamicin sulphate (5 g/mL) and RPMI 1640 + foetal calf serum (10%) are both present in RPMI 1640 medium. DMEM (Dulbecco's Modified Eagle's Medium) culture media with DMEM, gentamicin sulphate (5 g/mL), and inactivated foetal calf serum (10%).

Cell Lines and Cells:

Blood samples have been collected from the healthy participants at the Chengalpattu Tertiary Care Centre with the proper consent. Human peripheral blood mononuclear cells (PBMCs) has been acquired through centrifuging venous blood that had been heparinized in a density gradient centrifuge. Based on the instruction by manufacturer, freshly separated human PBMCs are used to isolate extremely pure peripheral human B cells using CD19 magnetic beads and magnetic columns (EasySepTM Direct Human Naive B Cell Isolation Kit, India). To analyse and show that isolated naive B cells were 95% pure, flow cytometry was used. Suspend the necessary number of cells in full culture medium. Human MRC-5 B cell lines are kept in culture flasks with complete RPMI 1640 media with 5% CO2 and at 37°C.

Stimulatory Conditions - In Vitro:

Human primary B cells are stimulated in several typical in vitro stimulatory environments. As stimulatory reagents, haptens of 2,4,6-trinitrophenol connected to bovine serum albumin (TNP-BSA), 2,4,6-trinitrophenol haptens connected to recombinant human IL4 and IL21, TNP-Ficoll, anti-CD40, recombinant human IL2 and IL10, anti-IgM, pansorbin, and TNP-

BSA were all employed. At different concentrations, the chemicals were used to stimulate primary B cells; effective concentrations of the reagents were also utilised for the assays that followed.

Production of Ig:

In order to assess Ig production, human CD19+ cells have been freshly extracted and transferred into a microtitre plate containing 384-well with 25 000 cells per well within 55 L complete DMEM. After 7 days of stimulation, the supernatant was extracted in concordance with the instructions given by manufacturer. Also, AlphaLISA human IgG and IgM kits were used for assessing IgM and IgG. A stimulus was considered to have a moderate effect if IgG production increased by less than five times, modestly by five to twenty times, and strongly by twenty times or more. A weak reaction to the stimulus was defined as an increase in IgM production less than fivefold, between five and tenfold for a slight impact, and greater than fifteenfold.

Cytokine production:

Freshly isolated B cells (human CD19+) have been transferred to 96-well plates having the density of nearly 50,000 cells per well in 220 L DMEM entirely and stimulated under a range of stimulatory settings. Then, AlphaLISA human IL6 and IL8 kits was employed in accordance with the manufacturer's instructions, and IL6 and IL8 were estimated for 2 days. A stimulus was regarded to have a minor effect if increased IL6 production by less than fivefold, modest effect if it increased by between five and twentyfold, and a major effect if it increased by more than twentyfold. If IL8 production increased by below five times the stimuli was produced with milder effects. If it rose five to 10 times, the effect would be moderate; if it grew more than ten times, the effect would be considerable.

Proliferation of B Cell:

In addition to being isolated freshly and transferred to 96-well plates with 50 000 cells per well and packed inside 220 L complete DMEM, human CD19+ B cells are also stimulated under various activation conditions. Ten Ci of 3H thymidine has been administered among wells during its course of the 18 hours with incubation for 3 days. On glass filter paper, the cells were gathered into a 96-well configuration. Radioactivity was measured using a scintillation counter following drying. If the proliferation increase was less than five times, between five and twenty times, or more than twenty times, the effect of the stimulus was considered to be mild, moderate, or high, respectively.

Flow Cytometry:

Human CD19+ B cells are freshly separated after stimulation for 24 hours, as previously mentioned. A 3-color Becton Dickinson FACS Calibur analysis equipment was used to analyse all cell surface markers after plating human B cell lines to 96-well plates 50000 cells as density per well in 220 L of DMEM entirely. Before a 30-minute incubation at 4°C, antibodies that have been conjugated to fluorescein are diluted in cold PBS (phosphate-buffered saline). Suspension of cells were done for analysis into PBS containing 2% paraformaldehyde following incubation as well as two rounds of cold PBS washing. To gauge the potency of an effect, mean fluorescence intensity (MFI) was utilised. When there was an increase of less than 1.5 times of MFI, it was deemed to have a mild influence; when it grew by 1.5 to 2 times, it was deemed to have a moderate effect; and when it climbed by more than 2 times, it was deemed to have a significant impact.

Reaction of Human Mixed Lymphocytes:

The human mixed lymphocyte response (MLR) assay employed responder cells as stimulator cells and freshly obtained human PBMCs as growth-inhibited RPMI 1788 cells. 96 nM mitomycin C was used to stop the proliferative phase of RPMI 1788 cells at temperature of 37 °C for nearly for 20 minutes. Following three washes with RPMI 1640 containing antibiotics, stimulator cells were diluted to the necessary concentration in the entire RPMI 1640 solution. PBMCs and stimulator cells were co-cultivated for six days at 37°C and 5% CO2 in an 8:3 ratio into the whole of RPMI 1640 medium. During the last 18 hours of culture, 10 10 Ci 3H thymidine is added to the responder cells to measure their DNA synthesis. On glass filter paper, the cells were assembled into a 96-well format. After drying, radioactivity was determined using a scintillation counter.

WST-1 Viability Assay:

A WST-1 viability test was used on a B cell-cell line to assess the cytotoxic and cytostatic chemicals. Utilising a 96-well plate format and the cell proliferation reagent WST-1, viability across cell populations and cell proliferation were measured spectrophotometrically. Formazan production was measured using a scanning multiwall spectrophotometer. Different amounts of the chemicals were given to the cell lines. In the control wells, 10 L of WST-1 and Triton X-100 reagent were added, and followed by 48 hours of incubation at 37 °C with CO2 of 5%. The formazan dye absorbance at 540 nM was determined by the EnVision 2103 Multilabel Reader.

3. RESULTS:

Immune Stimulation - In Vitro.

B cells respond differently based on strength and type of the receiving stimulatory signals. Realistic stimulatory conditions may be simulated in vitro. To determine which stimulatory phrase has the most prominent and all-pervasive effects of immunostimulants on pure primary human B cells, many stimulative terms were evaluated in vitro.

Results of phenotypic analysis on primary human B cells under various stimulatory conditions in vitro:

The haptens were altered by T-independent antigen TNPFicoll's altered has no effect on B cell or Ig proliferation, cytokine generation, or the expression of cell surface markers. Ig and IL6 production were moderately stimulated by TNP-BSA, a T cell/CD40L dependent stimulus, as well as B cell proliferation. There was a stronger stimulation of IL8 production. MHC class I and CD69 expression were somewhat elevated after TNP BSA stimulation.

Upon ligation of the antigen, BCR has been induced by anti-IgM antibodies to assemble in vivo. Anti-IgM antibodies made only in vitro are inefficient B cell activators. When IL4 was present IL6 and IgG rose production, and CD69 and CD83 stimulation markers are drastically upregulated whereas CD40 expression only slightly increased. Anti-IgM IL4 likewise had negligible to no effect on the others.

CD40-CD40L ligation that are promoting differentiation and proliferation of clonal B cell has been mimicked by anti-CD40 antibodies that are hostile. Initially, weak responses to stimuli by B cells is used here as it has detrimental effects on their capacity to multiply and generate Ig and cytokines. Combining IL4 and IL21, however, produced moderate to significant effects on Ig production, cellular expression, and proliferation. A significant increase in IL6

was observed when anti CD40 antibodies were combined with agonistic IL4. Human terminal B cells developed significantly more quickly in response to IL21, which induces IgG and IgM production somewhat weaker. Cytokine production and appearance of other surface markers remain unaffected [12].

Then it was looked at if three TLR agonistic ligands might excite B cells. The exterior membrane of gram-negative organisms is mostly composed of LPS, a ligand of TLR4. LPS had little effect, other than for a slight increase in IL6 and IL8. It was expected that human B cells would not respond due to their lower expression of TLR4 than murine B cells. Non-B cells are very rarely contaminated, so little impact is evident on IL6 and IL8. [13].

Resiquimod, an artificial agonist for TLR8 and TLR7 and is a member of the imidazoquinolinamine family. As well as stimulating cell surface expression of a diverse array of costimulatory and stimulatory indicators, this potent B cell activator influenced IgM, IL6, and IL8 synthesis. Cellular proliferation and IgG production were only very weakly impacted.

Synthetic oligonucleotide ODN2006 has been identified by TLR9s in humans as a class B CpG ODN with unmethylated CpG dinucleotides. Resiquimod performed better in terms of IgG production and cellular proliferation than ODN2006. But compared to resiquimod, IL8 production was less noticeable. The majority of cell surface indicators significantly upregulated after ODN2006 stimulation.

Cells of Staphylococcus Pansorbin were heat-killed, cells were formalin-fixed having a protein A covering which might help B lymphocytes by cross-linking Igs on its surface. When coupled with IL2 and IL10, pansorbin markedly boosted the production of cytokines, Ig, and B cells. CD40, CD80, MHC class I and class II markers, as well as expression of CD70, CD69, CD86 and CD83, were unaffected. [14].

However, there was no stimulation of IgG production, and there was hardly any change in the surface markers expression. Among every condition on stimulation which were investigated, ODN2006 has been selected as the stimulus which is ideal since they may increase B cell proliferation, the generation of IgG and IgM, cytokines release, and activation markers elevation.

Analysis of the cell surface marker expression in Various Human B Cell Lines Following ODN2006 Stimulation:

Primary B cells are not suited for repeated testing due to the diversity across subpopulations following B cell separation and purification from peripheral blood. To address specific issues, a homogeneous B cell line that is immortalized may be utilized. Because ODN2006 showed that they had the most activating impact on polyclonal B cells, human B cell lines RPMI 8866 and RPMI 1788 are examined for their susceptibility to ODN2006 stimulation in vitro.

Prior to and during a 24-hour period of TLR9 agonist ODN2006 activation, The expressive MHC class I and class II molecules CD69, CD40, CD80, CD86, CD83, and CD70, are examined by flow cytometry on human B cell lines. Nearly all of the analyzed indicators were unaffected by ODN2006's activation of RPMI 8866 and RPMI 1788.

4. DISCUSSION:

The enormous requirement in targeting B cells using specific immunosuppressant therapies is equivalent to the necessity of in vitro state immunoassay which might allow for identifying

the significant drugs. [15,16]. Even though they have significant limitations, in vitro experiments are essential for comprehending complex biological processes that result in new predictions and discoveries. In that examination, an upgrade that prompts phenotypically significant changes in a steady B-cell model should be applied. [17]. To do this, the current study involved the investigation of capacity of multiple stimuli to activate primary human B cells through analysing a variety of outcomes, such as the production of IgG and IgM, cell proliferation, release of cytokine, as well as the activation-related markers that are overexpressed. This is the first study to our knowledge about comparison of those stimulus across many different conclusions. Human polyclonal B cells were activated in vitro with the "broadest" phenotypical modification form, ODN2006, among the several stimuli investigated.

With the exception of mitochondrial DNA, CpG motifs are recognised in oligodeoxynucleotides by the pattern recognition receptor TLR9 as a molecular pattern associated with pathogens. The immune system responds to bacterial and viral infections by activation of molecules, proinflammatory secretions (type I interferon, TNF, and IL6), immunological regulatory (IL10) cytokines, and TLR9 signalling by activating immune cellular interactions. These interactions lead to adaptive, innate, cellular, and humoral immunity. Based on the pharmacological tests we conducted, TLR9 activation of NF-B, tyrosine, serine/threonine kinases, and PI3K pathways was confirmed. [18-20].

The greatest option for reproducible tests is not primary B cells because of the unpredictability in the various blood donations made by people, the variations that result from separating and periphery blood purification, poor yield purification, as well as short lifetime. Thus, by getting beyond the aforementioned limitations, monoclonal splitting cells may result in the primary B cell substitution, stabilising as well as making experiment easier to conduct. [21,22]. CD40, CD70, CD80, and CD86 were found to activate CD4+ T cells more efficiently than costimulatory molecules in vitro and in vivo. In actuality, IgG production is regulated by its signaling role. Activation of CD80/CD86 is essential to regulate the synthesis of IgG1 through B cells which are activated previously. [23-25].

When naïve B cells with people having typical or varied immunodeficiencies are employed to promote the synthesis of CD86 and CD70 costimulatory molecules, their expression was substantially inhibited as well as stays at lesser level even by homologous helper CD4+ T cells. LPS had little effect, with the exception of a slight increase in IL6 and IL8. The majority of cell surface indicators significantly upregulated after ODN2006 stimulation. Similar to the X-linked immunodeficiency known as hyper-IgM syndrome, inadequate CD40-CD40L contacts lead to problems of Ig class switching, a critical mechanism for the development of antigen-dependent B cells, memory B cells, and plasma cells. A variety of pharmacological chemicals were examined in order to tackle the test in the expectation of medications that would target B cells. [26].

In terms of sensitivity, CD86 seems to be the most sensitive marker. In our study, The pathways for Bruton's tyrosine kinases, PI3K/AKT, and NF-B were verified. Human primary B cells generate IgG following activation with ODN2006, supporting the relevance of these tests for discovering IgG production inhibitors.

5. CONCLUSION:

In repeated and thorough tests, the B cell line was employed for circumventing its limitations for employing primary human B cells. It is possible to find significant novel active B cell compounds using in vivo immunoassay at state by ODN2006-activated B cells, flow cytometry outcomes activation, and costimulatory surface markers activation, or by fine-tuning the mechanism of action of known immunomodulators.

ACKNOWLEDGEMENT:

None

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

None

References

[1] Sujith Ravi , Leela Kakithakara Vajravelu , Balamurali Venkatesan , Jayaprakash Thulukanam. (2022). Cancer Adoptive Immunotherapy – An In Vitro Mediation Of Activated B Cells By Regressing Tumour Cells. Journal of Pharmaceutical Negative Results, 1705–1709.

[2] Karkhah A, Javanian M, Ebrahimpour S. The role of regulatory T cells in immunopathogenesis and immunotherapy of viral infections. Infect Genet Evol. 2018 Apr;59:32-37.

[3] Zhang Z, Zhou L, Xie N, Nice EC, Zhang T, Cui Y, Huang C. Overcoming cancer therapeutic bottleneck by drug repurposing. Signal Transduct Target Ther. 2020 Jul 2;5(1):113.

[4] Parlakpinar H, Gunata M. Transplantation and immunosuppression: a review of novel transplant-related immunosuppressant drugs. Immunopharmacol Immunotoxicol. 2021 Dec;43(6):651-665.

[5] Cyster JG, Allen CDC. B Cell Responses: Cell Interaction Dynamics and Decisions. Cell. 2019 Apr 18;177(3):524-540.

[6] Namm JP, Li Q, Lao X, Lubman DM, He J, Liu Y, Zhu J, Wei S, Chang AE. B lymphocytes as effector cells in the immunotherapy of cancer. J Surg Oncol. 2012 Mar 15;105(4):431-5.

[7] Sujith Ravi, Leela Kakithakara Vajravelu, Balamurali Venkatesan, Jayaprakash Thulukanam, & Pavithra Selvan. (2022). Comparison Of Primary Human B Cells And B Cell Lines After Invitro Immune Stimulation. Journal of Pharmaceutical Negative Results, 13, 1900–1904.

[8] Yap HY, Tee SZ, Wong MM, Chow SK, Peh SC, Teow SY. Pathogenic Role of Immune Cells in Rheumatoid Arthritis: Implications in Clinical Treatment and Biomarker Development. Cells. 2018 Oct 9;7(10):161.

[9] Bugatti S., Vitolo B., Caporali R., Montecucco C., Manzo A. B cells in rheumatoid arthritis: From pathogenic players to disease biomarkers. BioMed Res. Int. 2014;2014:681678.

[10] Sarantopoulos S, Stevenson KE, Kim HT, Bhuiya NS, Cutler CS, Soiffer RJ, Antin JH, Ritz J. High levels of B-cell activating factor in patients with active chronic graft-

versus-host disease. Clin Cancer Res. 2007 Oct 15;13(20):6107-14.
[11] Barnas JL, Looney RJ, Anolik JH. B cell targeted therapies in autoimmune disease. Curr Opin Immunol. 2019 Dec;61:92-99.
[12] Sic H, Speletas M, Cornacchione V, Seidl M, Beibel M, *etal.* An Activating Janus Kinase-3 Mutation Is Associated with Cytotoxic T Lymphocyte Antigen-4-Dependent Immune Dysregulation Syndrome. Front Immunol. 2017 Dec 15;8:1824.
[13] Vaure C, Liu Y. A comparative review of toll-like receptor 4 expression and functionality in different animal species. Front Immunol. 2014 Jul 10;5:316.
[14] Uebele J, Habenicht K, Ticha O, Bekeredjian-Ding I. Staphylococcus aureus Protein A Induces Human Regulatory T Cells Through Interaction With Antigen-Presenting Cells. Front Immunol. 2020 Sep 30;11:581713.
[15] Ludwig RJ, Vanhoorelbeke K, Leypoldt F, *etal.* Mechanisms of Autoantibody-Induced Pathology. Front Immunol. 2017 May 31;8:603.
[16] Liu C, Chu D, Kalantar-Zadeh K, George J, Young HA, Liu G. Cytokines: From Clinical Significance to Quantification. Adv Sci (Weinh). 2021 Aug;8(15):e2004433.
[17] Van Belle K, Herman J, Boon L, Waer M, Sprangers B, Louat T. Comparative In Vitro Immune Stimulation Analysis of Primary Human B Cells and B Cell Lines. J Immunol Res. 2016;2016:5281823.
[18] Cobb D, Guo S, Smeltz RB. CpG and interleukin-15 synergize to enhance IFN-γ production by activated CD8+ T cells. Biomed Res Int. 2013;2013:924023. doi: 10.1155/2013/924023
[19] Orlacchio A, Mazzone P. The Role of Toll-like Receptors (TLRs) Mediated Inflammation in Pancreatic Cancer Pathophysiology. Int J Mol Sci. 2021 Nov 25;22(23):12743.
[20] Iskra S, Kalla M, Delecluse HJ, Hammerschmidt W, Moosmann A. Toll-like receptor agonists synergistically increase proliferation and activation of B cells by epstein-barr virus. J Virol. 2010 Apr;84(7):3612-23.
[21] Taddio MF, Castro Jaramillo CA, Runge P, Blanc A, *etal.* In Vivo Imaging of Local Inflammation: Monitoring LPS-Induced CD80/CD86 Upregulation by PET. Mol Imaging Biol. 2021 Apr;23(2):196-207
[22] Gujer C, Sandgren KJ, Douagi I, Adams WC, *etal.*. IFN-α produced by human plasmacytoid dendritic cells enhances T cell-dependent naïve B cell differentiation. J Leukoc Biol. 2011 Jun;89(6):811-21.
[23] Tefferi A, Hanson CA, Inwards DJ. How to interpret and pursue an abnormal complete blood cell count in adults. Mayo Clin Proc. 2005 Jul;80(7):923-36. doi: 10.4065/80.7.923.
[24] Arandi N, Mirshafiey A, Jeddi-Tehrani M, *etal.* Evaluation of CD4+CD25+FOXP3+ regulatory T cells function in patients with common variable immunodeficiency. Cell Immunol. 2013 Feb;281(2):129-33.
[25] Zhang Y, Wang Y, Zhang M, Liu L, Mbawuike IN. Restoration of Retarded Influenza Virus-specific Immunoglobulin Class Switch in Aged Mice. J Clin Cell Immunol. 2016 Apr;7(2):403.
[26] Dondapati SK, Pietruschka G, Thoring L, Wüstenhagen DA, Kubick S. Cell-free synthesis of human toll-like receptor 9 (TLR9): Optimization of synthesis conditions

and functional analysis. PLoS One. 2019 Apr 25;14(4):e0215897.