Original Article

Comparison Of Primary Human B Cells And B Cell Lines After Invitro Immune Stimulation

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Doi: 10.47750/pnr.2022.13.S05.299

Abstract

Immunotherapeutic medications have significant negative effects as they increase the life expectancy of patients. B cells perform the equivalent task with immunological reactions as well as they are now only the target of a relatively limited number of medications. Depending on the type and intensity of receiving the stimulatory signals, B cells behave by a certain way. In vitro simulations of true stimulatory environments are possible. Anti-CD40 antibodies that are antagonistic imitate the CD40-CD40L ligation, which promotes B cell clonal proliferation and differentiation. The initial B cells only responded weakly to the stimuli we utilized in the experiments, which had negative consequences on their ability to proliferate and produce Ig and cytokines. The ability of three TLR agonistic ligands to stimulate B cells was then investigated. Lipopolysaccharide (LPS) is a ligand of TLR4 and makes up the Gram-negative organism's external membrane in major way. With an exception to minor rise in IL6 and IL8, LPS had no impact. Upon stimulation with ODN2006, the majority of cell surface markers showed significant upregulation. On nearly all of the indicators tested, RPMI 8866 and RPMI 1788 were stimulated with ODN2006 showed no significant impact. The most sensitive marker appears to be the costimulatory marker CD86. B cells are stimulated by ODN2006 and costimulatory cell surface markers by flow cytometry readout to be able to use as an effective and reliable front-line screen in order to find novel active components of B cell.

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

INTRODUCTION:

Immunotherapeutic medications have significant negative effects as they increase the life expectancy of patients [1,2]. Additionally, despite rising levels of investment in the pharmaceutical sector, clinical development and several novel immunotherapeutic drugs of small molecules are declining [3]. Furthermore, the majority of commercially available immunotherapeutic medications target the inhibition of T cell activation (e.g., mTOR inhibitors such as everolimus and sirolimus, cyclosporine A and calcineurin inhibitors like tacrolimus and costimulation-blocking antibodies example abatacept and belatacept, CD3 antagonists such as CD25/IL2-R antagonists like daclizumab and basiliximab) and muromonab [4]. Despite this, B cells perform the equivalent task with immunological reactions as well as they are now only the target of a relatively limited number of medications [5]. The roles that B lymphocytes play as an effector are diverse. Igs are produced to ensure the removal of invasive pathogens and dead cells [6]. Effective antigen-presenting cells are B cells that bind to the molecules of major histocompatibility complex (MHC) with epitopes and capturing of antigens together with antigen-specific B cell receptor (BCR) [7]. It transmits epitope towards the relevant T cells, by producing cytokines and altering the level of expression of certain cellular membrane indicators, B cells that are activated can efficiently interact with other effector cells to produce an immune response that is more controlled and targeted [8,9]. The nosogenic factors in a variety of medical conditions such as autoimmune illnesses, cancer, graft-versus-host diseases, and transplant rejection, B cells have a significant role however poorly realized [10]. There is currently only a very limited group of immunomodulatory drugs which are B cell-specific (such as belimumab, rituximab and bortezomib) that are

clinically accessible as well as mostly decreasing drugs [11]. Therefore, a gap has been observed in the market for novel medications in this area. The investigation of B cell regulatory models may result in the discovery of novel targets or molecules having promise as B cell therapeutics. Hence, objective from the current work is to examine human B cell lines and various stimuli of B cells for the purpose of finding a model in vitro specifically appropriate for studying the immune activation of B cell which is easily adaptable for drug discovery and screening.

MATERIALS AND METHODS:

Media for Cell Culture:

RPMI 1640 medium contains RPMI 1640 + fetal calf serum (10%) and antibiotics at a concentration of gentamicin sulphate (5 g/mL). DMEM culture media (Dulbecco's Modified Eagle's Medium) included Foetal Calf Serum (10%) that are inactivated with heat, DMEM along with gentamicin sulphate of 5 g/mL.

Cell Lines and Cells:

At the Chengalpattu tertiary care center, blood samples from healthy volunteers were taken with appropriate consent. Heparinized venous blood was centrifuged by using a density gradient centrifuge to collect human peripheral blood mononuclear cells (PBMCs). In line with the manufacturer's directions, highly pure peripheral human B cells are isolated with new human PBMCs with the help of magnetic columns and CD19 magnetic beads (EasySepTM Direct Human Naive B Cell Isolation Kit, India). According to flow cytometry analysis, the naïve B cells that were extracted around 95% pure. In complete culture media, suspend cells at the required concentration. There were employed human MRC-5 B cell lines from India. The cell lines are retained in full medium of RPMI 1640 in culture flasks at the temperature of 37°C and 5% of CO2.

Stimulatory Conditions - In Vitro:

Multiple common stimulatory in vitro settings are used for triggering primary B cells from humans. Anti-CD40, anti-IgM, pansorbin, 2,4,6-trinitrophenol hapten linked with bovine serum albumin (TNP-BSA) and 2,4,6-trinitrophenol hapten linked with Ficoll (TNP-Ficoll), as well as recombinant human IL4 and IL21, recombinant human IL2 and IL10 were utilized as stimulatory reagents. The chemicals were employed in stimulating primary B cells at various concentrations, also effective concentrations of the reagents were used for subsequent tests.

Production of Ig:

Human CD19+ B cells were newly isolated and put into 384-well plate having 25 000 cells into every well in a 55 μL full DMEM medium to measure Ig production. Following 7 days of stimulation, the manufacturer's recommendations were followed to remove the supernatant for IgM and IgG analysis using the AlphaLISA human IgG and IgM kits. Less than a 5-fold rise in IgG production was deemed a mild impact of the stimulus, between a 5 and a 20-fold raise was deemed to have a modest impact, also beyond a 20-fold raise was deemed a strong effect. Less than a 5-fold rise in IgM production was regarded as a mild response to the stimulus, between 5 and 10 folds for modest impact, and exceeding beyond 10 folds which is a significant impact.

Production of Cytokine:

Human CD19+ B cells are isolated newly and plated into 96-well plate having 50 000 cells into every well as density in 220 μL whole DMEM media as well as stimulated under various stimulatory conditions. Using the AlphaLISA human IL6 and IL8 kits in accordance with manufacturer's directions, an investigation of IL6 and IL8 was carried out subsequently for two days. Less than a 5-fold rise in IL6 production was deemed a mild impact of the stimulus, between a 5 and 20-fold rise was deemed a modest impact, and exceeding beyond a 20-fold rise was deemed a significant effect. Less than a fivefold rise in IL8 production was deemed a mild effect of the stimulus, a five to tenfold increase deemed a modest impact, and in excess of a tenfold increase deemed significant impact.

Proliferation of B Cell:

Human CD19+ B cells are newly isolated and plated into 96-well plate having 50 000 cells into every well as density in 220 μL whole DMEM media also it is stimulated under various activatory conditions. During the final 18 hours of the final three days of incubation, Ten μCi of 3H thymidine was added to the wells. The cells were collected in a 96-well configuration on glass filter paper. After drying, a scintillation counter was used to measure radioactivity. A stimulus was deemed to have a mild impact if the increase in proliferation was less than five times, a moderate effect if it was between five and twenty times, and a strong effect if it was more than twenty times.

Flow Cytometry:

As previously explained, following the stimulation for 24 hours, human CD19+ B cells are newly isolated. 96-well plate were plated with cells of human B cell lines at 50000 cells into every well with 220 μL whole DMEM medium and all the markers of cell surface are analyzed by apparatus of a 3-color Becton Dickinson FACSCalibur. Phycoerythrin-cyanine 5 (Pe-Cy5), Phycoerythrin (PE), or fluorescein isothiocyanate (FITC)-labeled antibodies to MHC class I, MHC class II, CD40, CD69, CD70, CD80, CD83, and CD86. Fluorescein-conjugated antibodies are diluted in cold phosphate-buffered saline (PBS) and followed by 30 minutes of incubation at 4°C. Cells were suspended in PBS containing 2%

paraformaldehyde for analysis following the incubation and 2 rounds of cold PBS washing. A stimulus was deemed to have a mild effect if the mean fluorescence intensity (MFI) increased by less than 1.5 times, a moderate effect if it increased between 1.5 to 2 times, and a strong effect if it increased by more than 2 times.

Human Mixed Lymphocyte Reaction:

From the assay of human mixed lymphocyte reaction (MLR), newly obtained human PBMCs served as RPMI 1788 cells which are growth-inhibited and the responder cells as stimulator cells. 96 nM mitomycin C was administered to RPMI 1788 cells to stop their proliferative process for 20 minutes at 37°C. The dilution of stimulator cells was done according to required concentration in the whole RPMI 1640 media following 3 washes with RPMI 1640 having antibiotics. responder human PBMCs and stimulator cells were cocultivated at an 8:3 ratio into the whole RPMI 1640 media for 6 days at the temperature of 37°C and 5% of CO2. By adding 10 10 μCi 3H thymidine during the final 18 hours of culture, synthesis of DNA in the responder cells are measured. The cells were collected in a 96-well configuration on glass filter paper. After drying, a scintillation counter was used to measure radioactivity.

WST-1 Viability Assay:

The cytostatic and cytotoxic substances were examined with an assay called WST-1 viability, on B cell-cell line. Viability among cell populations and the proliferation of cells were assessed spectrophotometrically utilizing the format of 96-well plate and were carried out using WST-1, a soluble tetrazolium salt which is a cell proliferation reagent. A scanning multiwall spectrophotometer was used to quantify the formazan that was produced. The cell lines received various dosages of substances. Triton X-100 and WST-1 reagent of 10 L were put in to the control wells and every well were incubated at the temperature of 37°C after 48 hours and 5% of CO2. EnVision 2103 Multilabel Reader was used to measure the formazan dye's absorbance at 540 nM.

RESULTS:

Immune Stimulation - In Vitro.

Depending on the type and intensity of receiving the stimulatory signals, B cells behave by a certain way. In vitro simulations of true stimulatory environments are possible. On pure primary human B cells, various in vitro stimulative terms were examined in an effort to identify the one that produces the most pronounced and comprehensive impacts of immunostimulants.

Phenotypic results among several Stimulatory Conditions In Vitro on Primary Human B Cells:

The T-independent antigen TNPFicoll, which had its hapten changed, did not stimulate the proliferation of B cell, Ig or production of cytokine, or cell surface marker expression. TNP-BSA, which is T cell/CD40L dependent stimulation, moderately induced B cell proliferation as well as the generation of Ig and IL6. More strongly stimulated IL8 production was seen. Following TNP BSA stimulation, MHC class I and CD69 expression were moderately increased.

Anti-IgM antibodies cause the BCR to aggregate in vivo upon ligation of antigen. The ineffective activators of B cells are Anti-IgM antibodies that are in vitro on their own. The synthesis of IL6 and IgG increased when IL4 was present, and the expression CD69 and CD83 stimulation markers were dramatically upregulating while expression of CD40 increased only marginally. In addition, anti-IgM with IL4 had minimal to no effect on the other cell surface indicators. When human B cells were stimulated with anti-IgM antibodies, IgM production could not be quantified.

Anti-CD40 antibodies that are antagonistic imitate the CD40-CD40L ligation, which promotes B cell clonal proliferation and differentiation. The initial B cells only responded weakly to the stimuli we utilised in the experiments, which had negative consequences on their ability to proliferate and produce Ig and cytokines. However, moderate to substantial impacts on Ig production, expression of a various markers of cell surface and proliferation, were seen when coupled with IL4 or IL21. Combining agonistical IL4 with anti CD40 antibodies significantly boosted the production of IL6, but not IL8. The expression of CD69 was greatly raised by IL21, a highly effective inductor of differentiation of terminus B cell differentiation in humans, and a modest inducer for the production of IgG and IgM. Both the cytokine generation and the manifestation of other cell surface indicators are unaffected [12].

The ability of three TLR agonistic ligands to stimulate B cells was then investigated. LPS is a ligand of TLR4 and makes up the gram negative organism external membrane in major way. Apart from a minor rise in IL6 and IL8, LPS had no impact. Given that human B cells do not express much TLR4 compared to murine B cells, this lack of reactivity was not surprising. The extremely modest fraction of contaminating non-B cells is likely responsible for the minimal impact on IL6 and IL8 [13].

The synthetic agonist of TLR7 and TLR8 is Resiquimod and it belongs to the family of imidazoquinolinamines. IgM, IL6, and IL8 production as well as the expression of a number of costimulatory and stimulatory markers of cell surface were all strongly influenced by this powerful B cell activator. The synthesis of IgG and cellular proliferation were very mildly affected.

ODN2006 is a class B CpG ODN, a kind of synthetic oligonucleotide that is recognised by the human TLR9 and contains CpG dinucleotides that are unmethylated in specific series contexts (CpG motifs). Among the synthesis of IgG and cellular proliferation, ODN2006 shown more efficacy than resiquimod. However, IL8 generation was less obvious than it was with resiquimod. Upon stimulation with ODN2006, the majority of cell surface markers showed significant upregulation. Staphylococcus aureus pansorbin cells are formalin-fixed, heat-killed cells with a protein A coat that may enable B lymphocytes by surface Igs that are cross-linking. Pansorbin significantly increased the production of cytokines, Ig, and

B cells when combined with IL2 and IL10. MHC class I and class II, CD40, and CD80 markers were unaffected, but CD69, CD70, CD83, and CD86 expression has been markedly increased [14].
However, there was no induction of IgG synthesis, and the expression of the cell surface indicators barely altered. ODN2006 was chosen as the preferred stimulus out of all the stimulation conditions examined since it may stimulate the proliferation of B cell, IgM and IgG production, the release of cytokine, and elevation of activation markers.
Analysis on Various Human B cell Lines in the expression of Cell Surface Marker following the stimulation of ODN2006:
Due to the differences in human blood donors and the variety of B cell subpopulations following B cell separation and purification from peripheral blood, primary B cells aren't suitable with repeated tests. Certain issues might be resolved by using a homogenous, immortalized B cell line. Human B cell lines, RPMI 8866, and RPMI 1788 are examined on behalf of its sensitivity to in vitro stimulation of ODN2006 because ODN2006 proved that they have the most extensive activatory impact on polyclonal B cells. The emphasis was on the cell surface markers expression since malignant B cell lines commonly exhibit deviations in the production of cytokine and Ig depending on their stage of development.
Before and after activation of TLR9 agonist ODN2006 for 24 hours, human B cell lines are examined for the expressive MHC class I and class II, CD40, CD69, CD70, CD80, CD83, and CD86 using flow cytometry. On nearly all of the indicators tested, activation of RPMI 8866 and RPMI 1788 using ODN2006 showed no significant impact.

DISCUSSION:

The requirement of wide immunoassay in vitro state which may make the detection of such prospective medications easier is analogous to the great unmet demand to target B cells by particular immunosuppressive treatments [15,16]. In vitro tests are crucial for gaining an understanding of complicated biological processes that lead to novel predictions and inventions, despite the fact as they have some limitations. A stimulus that causes phenotypically significant alterations in a B-cell model which is stable must be used in that experiment [17]. To achieve this, we examined the ability of distinct stimuli to stimulate primary B cells of humans by examining numerous results of activation, including the formation of IgM and IgG, the proliferation of cells, the release of cytokine, and the overexpression of several markers for activation. This is, as far as we are aware, the first publication to compare these stimuli on a variety of activation outcomes. "broadest" phenotypical alteration form was seen in polyclonal B cells of humans after activating them in vitro with TLR9 agonist ODN2006 among numerous stimuli examined.
The CpG motifs in oligodeoxynucleotides are rare in vertebrate genomes, with the exception of the mitochondrial DNA, and the pattern recognition receptor TLR9 detects them as pathogen-associated molecular patterns. By encouraging antibody-secreting cells differentiation and cellular proliferation, immune cellular interactions by upregulating molecules, TLR9 signaling, immune regulatory (IL10) cytokines, and increased proinflammatory secretions (type I interferons, TNF, and IL6) cause adaptive and innate, cellular and humoral immunity activation upon bacterial and viral infections. The pharmacological drugs we evaluated verified activation of the molecular pathways by TLR9 with tyrosine, NF-κB, serine/threonine kinases, and PI3K [18-20].
Primary B cells ain't the best choice in case of repeatable experiments due to variability among the many blood donations by humans, the diversities following the separation and purifying of the blood from the periphery, purification at low yield, and the low lifespan. Therefore, by overcoming the aforementioned restrictions, monoclonal splitting cells might replace main B cell will stabilize and simplify the experiment [21,22]. Cell surface markers CD40, CD70, CD80, and CD86 were merely beyond costimulatory molecules in activating CD4+ T lymphocytes, according to in vitro and in vivo research. In fact, they are also important signalling molecules for controlling Ig synthesis, especially IgG. In order to control the production of IgG1 by formerly activated B cells, CD80/CD86 activation is crucial [23-25].
When BCR cross-linking is used to induce the production of CD86 and CD70 costimulatory molecules in naive B cells from individuals with usual varying immunodeficiency, the expression is severely hindered and remains low still with homologs helper CD4+ T cells. With an exception to minor rise in IL6 and IL8, LPS had no impact. Upon stimulation with ODN2006, the majority of cell surface markers showed significant upregulation. Similar to the hyper-IgM syndrome which is X-linked immunodeficiency, insufficient CD40-CD40L interactions result in abnormalities of Ig class switching, which is crucial mechanism for B cell maturation that are antigen-dependent and memory B cells and plasma cell production. In order to confront the test in the anticipation of targeting drugs of B cell, a number of pharmacological compounds were evaluated [26].
The most sensitive marker appears to be the costimulatory marker CD86. We verified that the PI3K/AKT, NF-B, and Bruton's tyrosine kinase pathways were present in our experiment. The ability of inhibitors of these pathways to prevent human primary B cells from producing IgG following ODN2006 activation supports the applicability of the assays for identifying IgG production inhibitors.

CONCLUSION:

The B cell line has been used to get around the drawbacks of using primary B cells of humans in repeated as well as extensive assays. Immunoassay at in vivo state uses ODN2006-activated B cells and activation of outputs of flow cytometry as well as the markers of cell surface that are costimulatory could be used in effective and reliable front-line screening to find significant novel active compounds of B cells or fine-tuning the action mechanism of recognized immunomodulators.

ACKNOWLEDGEMENT:

None

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