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DEVELOPMENT OF TWO METHODS FOR DETECTION OF IL-23 STIMULATION AND INHIBITION

Faculty of Medicine and Health Technology
Bachelor's Thesis
February 2024

TIIVISTELMÄ

Veera Räkköläinen: Kahden menetelmän kehittäminen IL-23:n stimulaation ja inhiboinnin havaitsemiseen
Kandidaatintutkielma
Tampereen yliopisto
Bioteknologian ja Biolääketieteen tekniikan tutkinto-ohjelma
Helmikuu 2024

Tutkimuksen tarkoituksena oli kehittää kaksi erilaista menetelmää interleukiini 23:n (IL-23) stimulaation ja inhiboinnin havaitsemiseen. IL-23:n stimulaatio ja inhibointi voidaan havaita monesta eri signalointireitin kohdasta. Tutkimuksessa käytetyt menetelmät havaitsevat stimulaatiota ja inhibointia kahdesta signalointireitin kohdasta mittaamalla eri muuttujia.

IL-23 on proinflammatorinen sytokiini, joka signaloi Janus Kinaasi/signaalin välittäjä ja transkriptioaktivaattori (JAK/STAT) signalointireitin välityksellä. Janus kinaasi proteiiniperheeseen kuuluvat JAK1, JAK2, JAK3 ja tyrosiini kinaasi 2(TYK2). IL-23 signalointireitti alkaa sytokiinin sitoutumisella reseptoriinsa. Sitoutuminen reseptoriin saa aikaan reseptorin konformaation muutoksen ja reseptoriin kiinnittyneiden TYK2 ja JAK2 proteiinien lähentymisen, jolloin niiden on mahdollista fosforyloida ja aktivoida toisensa. Fosforyloituneet TYK2 ja JAK2 fosforyloivat reseptorin hännät luoden sitoutumispaikan kahdelle STAT3 proteiinille. TYK2 ja JAK2 fosforyloivat sitoutuneet STAT3 proteiinit, jotka irrottautuvat reseptorista ja dimerisoituvat matkalla tumaan, jossa ne säätelevät sytokiinivälitteisten geenein ilmentymistä.

Menetelmät, joilla IL-23 signalointia tutkitaan, ovat virtaussytometria ja entsyymivälitteinen immunosorbenttimääritys (enzyme linked immunosorbent assay, ELISA). Virtaussytometria menetelmä hyödyntää mitattavien näytteiden fosforylaatio signaaleja. Kyseisessä kokeessa mitataan solujen sisäisten fosforyloitujen STAT3 proteiinien antamaa signaalia IL-23 stimuloidusta veri näytteestä. ELISA:lla mitataan solujen kasvatusliuoksen interleukiini 22 (IL-22) pitoisuus. IL-22 on IL-23 stimulaation aikaan saaman signalointireitin tuote T-lymfosyyteissä, joita näyte veren mononukleaarisista valkosoluista sisältää. Sytokiinin inhibitiota tutkitaan brepocitinibillä. Brepocitinib on adenosiinitrifosfaatti kompetitiivinen inhibiittori, jonka on todettu inhiboivan TYK2, JAK1 ja JAK2 proteiineja.

Molemmissa koejärjestelyissä IL-23 stimulaatio havaittiin laskemalla signaalien välinen erotus stimuloidun ja stimuloimattoman näytteen välillä. Virtaussytometriaa hyödyntävässä menetelmässä stimulaatio havaittiin käyttämällä fosforyloituneen STAT3 signaalin keskiarvoa näytteessä, yleisimmin käytetyn mediaani arvon sijasta. ELISA:an perustuvassa menetelmässä IL-22 signaalin voimakkuuteen vaikutti käytetty laimennoskerroin, jonka optimointi vaaditaan mahdollisia tulevia tarkasteluja varten. Samaa menetelmää käytettäessä havaittiin, että IL-2 sytokiinin lisääminen solujen kasvatusliuokseen samaan aikaan IL-23 stimulaation kanssa suurensi saatua IL-22 signaalia. Brepocitinib esti IL-23 signalointireitin etenemisen ja sen aikaan saama inhibitio ilmoitetaan konsentraationa, jolla puolet näytteen stimulaatiosta on inhiboitu.

Avainsanat: JAK/STAT signalointireitti, interleukiini 23, brepocitinib, virtaussytometria, ELISA

ABSTRACT

Veera Räkköläinen: Development of two methods for detection of IL-23 stimulation and inhibition
Bachelor's Thesis
Tampere University
Bachelor of Science, Biotechnology and Biomedical Engineering
February 2024

The study aimed to develop and compare two different methods for the detection of interleukin 23 (IL-23) stimulation and inhibition. The presence of IL-23 signaling can be determined from various points of the signaling pathway and the presented methods have two different parameters for detecting the stimulation and inhibition.

IL-23 is a proinflammatory cytokine that utilizes the JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway to transduce signals. JAK family includes JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2) proteins. The IL-23 signaling pathway begins with cytokine binding to its receptor on the cell surface. Intracellular TYK2 and JAK2 bind to the receptor and activate each other by phosphorylation. Activated TYK2 and JAK2 phosphorylate the tails of the receptor, forming docking sites for the STAT3 pair. STATs localize to the tail of the receptor where they are in turn phosphorylated by JAKs. Phosphorylated STAT3s dissociate from the receptor and dimerize before translocating to the nucleus to regulate the expression of cytokine-responsive genes.

Flow cytometric assay takes advantage of phospho flow technology and measures pSTAT3 signal levels from whole blood samples after stimulation with IL-23. ELISA-based assay detects the IL-23 induced production of interleukin 22 (IL-22) in peripheral blood mononuclear cells by measuring IL-22 concentration from cell culture supernatants. Inhibition of IL-23 signaling in these experiments is studied with brepocitinib. Brepocitinib is an adenosine triphosphate competitive inhibitor that shown effective inhibition against TYK2, JAK1, and JAK2.

IL-23 stimulation was detectable with both proposed methods when using fold change values between cytokine-stimulated and unstimulated samples as readout. In the flow cytometric assay, the stimulation was detected by using the mean values of the phosphorylated STAT3 signal. In the ELISA-based assay, the IL-22 signal level was affected by dilution ratios and it was observed that additional stimulation with interleukin 2 improved the IL-22 signal level in the sample. Brepocitinib inhibited IL-23 signaling in both experiments. Inhibition was calculated as the amount of brepocitinib that is needed for 50 % inhibition.

Keywords: JAK/STAT signaling pathway, IL-23, brepocitinib, flow cytometry, ELISA

PREFACE

This thesis is part of my Bachelor of Science -degree at the Faculty of Medicine and Health (MET) at Tampere University.

I am grateful to my supervisor PhD Anniina Virtanen for all the help and support during the experimental phase and guidance on the writing process. I would like to extend my sincere thanks to Prof. Olli Silvennoinen, the group leader of Molecular immunology research group. I very much appreciate the support and help from my family and friends.

Tampere, 15.2.2024

Veera Räkköläinen

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1. INTRODUCTION

1.1 JAK/STAT Pathway

JAK/STAT signaling pathway consists of cytokine receptors, Janus Kinases (JAKs), and signal transducers and activators of transcription (STATs). JAKs are non-receptor tyrosine kinases that transduce signals from outside the cell by binding intracellularly to cytokine receptors and activating their downstream effectors, STATs.

JAK family includes JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2) proteins. These proteins have four domains. JAKs bind to the receptor with the N-terminal FERM-domain (4.1 protein, ezrin, radixin, and moesin-domain) and following Scr homology 2 domain (George Abraham *et al.*, 2021). JAKs activity is regulated by the pseudokinase domain. The kinase domain is the active domain of the protein and is located in the C-terminus. It has the adenosine triphosphate (ATP) -binding pocket, which is slightly different between each JAK, and tyrosine residues that will be phosphorylated when JAK is active (Lin *et al.*, 2020; George Abraham *et al.*, 2021). Cytokine binding to its receptor results in receptor oligomerization which allows cytoplasmic receptor-bound JAKs to transphosphorylate each other and become activated. Activated JAKs further phosphorylate the tails of the cytokine receptor, forming a docking site for the STAT pair (Lin *et al.,* 2020).

There are seven STATs which in combination with different JAKs mediate specific signaling pathways. STATs localize to the phosphorylated intracellular tail of the receptor where they are in turn phosphorylated by JAKs. This phosphorylation leads to STATs dissociation and dimerization before translocating to the nucleus where phosphorylated STATs (pSTATs) regulate the expression of cytokine-responsive genes (Morris *et al.,* 2018).

1.2 IL-23 signaling

Interleukin 23 (IL-23) is a heterodimeric cytokine that is composed of subunits p19 and p40 through which it shares structural similarity to interleukin 12 (IL-12) (Croxford *et al.*, 2012). IL-23 receptor is associated with JAK2 and TYK2 which proceeds to phosphorylate mainly STAT3 upon IL-23 binding. IL-23 receptor comprises of IL-12Rβ1 and IL-23Rα subunits (Korta *et al.*, 2023). IL-23 signaling induces cytokine production of interleukin 17 and interleukin 22 (IL-22) and stabilizes T helper type 17 cells (T_H17) (Teng *et al.*, 2015; Korta *et al.*, 2023). IL-22 production is a consequence

of the activation of STAT3 and the expression of transcription factor RORγt (retinoic acid-related orphan receptor γt) (Ross and Cantrell, 2018). Overexpression of IL-23 cytokine causes dysregulation in cellular pathways which may lead to inflammatory autoimmune diseases such as psoriasis and inflammatory bowel disease.

1.3 JAK inhibitor brepocitinib

Drugs that inhibit the activity of JAK family enzymes are called JAK inhibitors. Most of the JAK inhibitors function as ATP competitive inhibitors, which can be divided into two classes based on how selective the inhibitor is, type-II inhibitors are more selective than type-I inhibitors (Leroy and Constantinescu, 2017). There are also allosteric inhibitors and covalent inhibitors.

Brepocitinib is a dual small-molecule inhibitor of TYK2/JAK1. It is a type-I inhibitor and has been also shown to inhibit JAK2 in addition to previously mentioned JAKs (Virtanen *et al.*, 2023). As an ATP competitive inhibitor, brepocitinib recognizes and targets the ATP binding pocket on the active site of the kinase domain (Lin *et al.*, 2020; George Abraham *et al.*, 2021; Martin, 2023). Hence, leading to inhibition of further signal cascade.

1.4 Flow cytometric assay

Flow cytometry is a technology that is used to analyze single cells or particles in solution with lasers. When passing the laser cell or particle produces light scatter that is measured in two directions, forward scatter, and side scatter (McKinnon, 2018). More analyzed parameters can be added by measuring fluorescence. Fluorescence-activated cell sorting (FACS) is used to sort different cell populations in a sample by fluorescence intensity. Cells are stained with fluorescent dyes or fluorescent conjugated antibodies.

Immunophenotyping is an application for flow cytometry and FACS that takes advantage of analyzation of cell populations for multiple parameters. Immunophenotyping utilizes fluorochrome-conjugated antibodies that will bind to the corresponding antigen on the cell surface (McKinnon, 2018). Cluster of differentiation (CD) markers are specific for immune cells' cellular antigens and can be used to define specific cell populations.

Phospho flow cytometry measures the protein phosphorylation at the single-cell level. Phospho-proteins can be measured simultaneously with surface markers making it an effective way to observe stimulation and inhibition of specific intracellular signaling pathway. By combining phospho

flow with fluorescent cell barcoding (FCB) there is an efficient way to study phosphorylated proteins in signaling pathways in large sample sets. FCB allows the combining of different samples into one tube that will be then analyzed. In FCB, each sample has a unique fluorescence intensity that is achieved by treating samples with different concentrations of FCB fluorophores. With two fluorophores as FCB markers can up to 36 samples be analyzed together. (Krutzik and Nolan, 2006)

1.5 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) is an immunological assay that is based on the interaction between the antigen and the primary antibody for that antigen. Antibody is enzyme-linked, and enzyme activity is measured as absorbance from the substrate color change. There are different ELISA categories for different uses of antigens, antibodies, and experimental conditions, direct ELISA, indirect ELISA, sandwich ELISA, and competitive ELISA. In Sandwich ELISA capture antibody is coated on the plate and the target antigen binds to that. The detection antibody binds to the target antigen. Both capture and detection antibodies are primary antibodies. Labeled secondary antibody binds to the detection antibody and with the enzyme substrate the antigen-antibody interaction is detected. (Alhajj *et al.*, 2024)

1.6 Aim of the study

This study aims to propose and test two different assays for the detection of IL-23 stimulation and inhibition. Inhibition is presented in IC50 values which indicates the amount of drug that is needed to inhibit a biological process by half. One assay is a flow cytometric detection of pSTAT in cells and the other assay is ELISA-based detection of IL-22 from growth medium. In the flow cytometric assay of a human whole blood sample is stimulated with IL-23 and inhibited with brepocitinib. IL-23 signals through STAT3 and phosphorylated STAT3 levels are then measured with phospho flow cytometry to detect the relative amount of pSTAT3 in CD4-negative T-cells. ELISA assay measures the IL-23 signaling a couple of steps further in the signaling pathway. It detects the amount of produced IL-22 from the growth medium of peripheral blood mononuclear cells (PBMCs). IL-22 is a product of IL-23 signaling in T_H17 cells. IL-22 concentration is measured by sandwich ELISA and standardized samples are used to determine the amount of protein in the sample.

2. MATERIALS AND METHODS

2.1 Flow cytometric detection of pSTAT in cells

Experiment details for flow cytometric detection of pSTAT in cells were planned based on two reference articles (Dowty *et al.*, 2019; Traves *et al.*, 2021). Target of the experiment was to determine cytokine stimulation and inhibition.

Whole blood from a healthy donor was collected according to a protocol approved by the Tampere University Hospital and divided into FACS tubes with a volume of 100 μl. The first flow cytometry experiment had 5 different samples and the second experiment had 16 samples. Samples were pretreated with JAK inhibitor brepocitinib, or with dimethyl sulphoxide for uninhibited samples, for 1 h at 37°C. The first experiment had only one concentration of the inhibitor,1 μM. The second experiment had 6 ten-fold dilutions from 8 μM to 0,08 nM.

The first flow cytometry experiment had cytokines interleukin 6 (IL-6) (100 ng/ml) and IL-23 (100 ng/ml), the second experiment had only cytokine IL-23. For unstimulated samples phosphate buffered saline (PBS) was used. Cytokine stimulation was performed after inhibitor treatment for 15 minutes at 37°C. Cells were fixed and permeabilized with BD bioscience Lyse/Fix Buffer 5X and their proposed protocol for flow cytometer analysis was followed for fixation and permeabilization before freezing samples at -80°C (https://www.bdbiosciences.com/en-at/products/reagents/cell-preparation-separation-reagents/blood-lysis/staining-and-cell-preparation/lyse-fix-buffer-5x.558049 4.12.2023).

Cells were prepared for flow cytometer analysis by fluorescence barcoding of samples with two FCB markers Pacific Orange and Pacific Blue. The set of samples included 8 samples and to differentiate them, three concentrations of each dye (0 μg/ml, 0,1 μg/ml, 0,45 μg/ml) were used in 8 unique combinations. All samples in each set were labeled with different concentrations of FCB dyes and incubated for 30 min at 37°C. After incubation samples were washed twice with FACS Buffer (PBS, 1% bovine serum albumin, 0,1% NaN_3 sodium azide) and then sample sets were combined into one FACS tube for staining.

Sample sets were stained with 4 surface stains CD3-FITC, CD4-APCeFluo780, CD33 – PE-Cy7, and pSTAT3-PE. The same stains were used for separate bead compensation samples and FCB markers were used for cell compensation samples for flow cytometer analysis. The samples were analyzed with CytoFLEX S (Beckman Coulter). Data from CytoFLex was analyzed with FlowJo 10.9.0 software.

2.2 ELISA-based detection of IL-22 cytokine from growth medium

The proposed method for ELISA-based detection of IL-22 was applied from a reference article where IL-23 induced secretion of IL-22 was measured from the growth medium by ELISA (Fridman *et al.*, 2010). PBMCs were used as cell samples. Culture plates were coated with anti-Hu CD3, and cells were cultured with anti-Hu CD28 for T-cell activation for 2 days in complete cell medium (RPMI 1640 + 10% fetal bovine serum + 1% penicillin/streptomycin + 1% L-glutamine) in the incubator in 5% CO_2 at 37°C.

After the T-cell activation cells were washed and recultured in a complete cell medium into a 12-well culture plate for differentiating samples for cytokine stimulation and inhibition. The first ELISA experiment had cytokine stimulation with IL-23 (100 ng/ml) and inhibition with brepocitinib (1 μM). The second ELISA experiment had cytokine stimulation with IL-23 (100 ng/ml) as well as IL-23 (100 ng/ml) + IL-2 (10 ng/ml) and inhibition with three different concentrations of brepocitinib (1 μM, 100 nM, 10 nM). Both experiments had also unstimulated cultures. PBMCs were cultured for 4 days after which the supernatants were collected by centrifugation. Secretion of IL-22 was measured with RayBio Human IL-22 ELISA Kit. Producer's user manual for this kit was followed (Catalog #: ELH-IL22). Absorbance was measured at 450 nm with Tecan Spark.

3. RESULTS

3.1 Flow cytometry

The first flow cytometry assay was to determine if IL-23 signaling is possible to detect from whole blood samples and if brepocitinib inhibits its signaling. The analyzed cells are CD4-negative T-cells. T-cells are recognized with a CD3 surface marker. IL-6 was used as a positive control. In Figure 1 there are detected median and arithmetic mean pSTAT3 values. IL-6 signal is very high compared to IL-23 signal therefore Figures 1C and 1D present the same values without IL-6 stimulated samples. The significance of the stimulated signal level is calculated as fold change (FC) between cytokine-stimulated and unstimulated samples. The threshold for significant stimulation is FC > 1,5. FC values calculated from median pSTAT3 were 17,4 for IL-6 and 1,3 for IL-23 and from mean pSTAT3, FC values were 20,3 for IL-6 and 3,3 for IL-23.

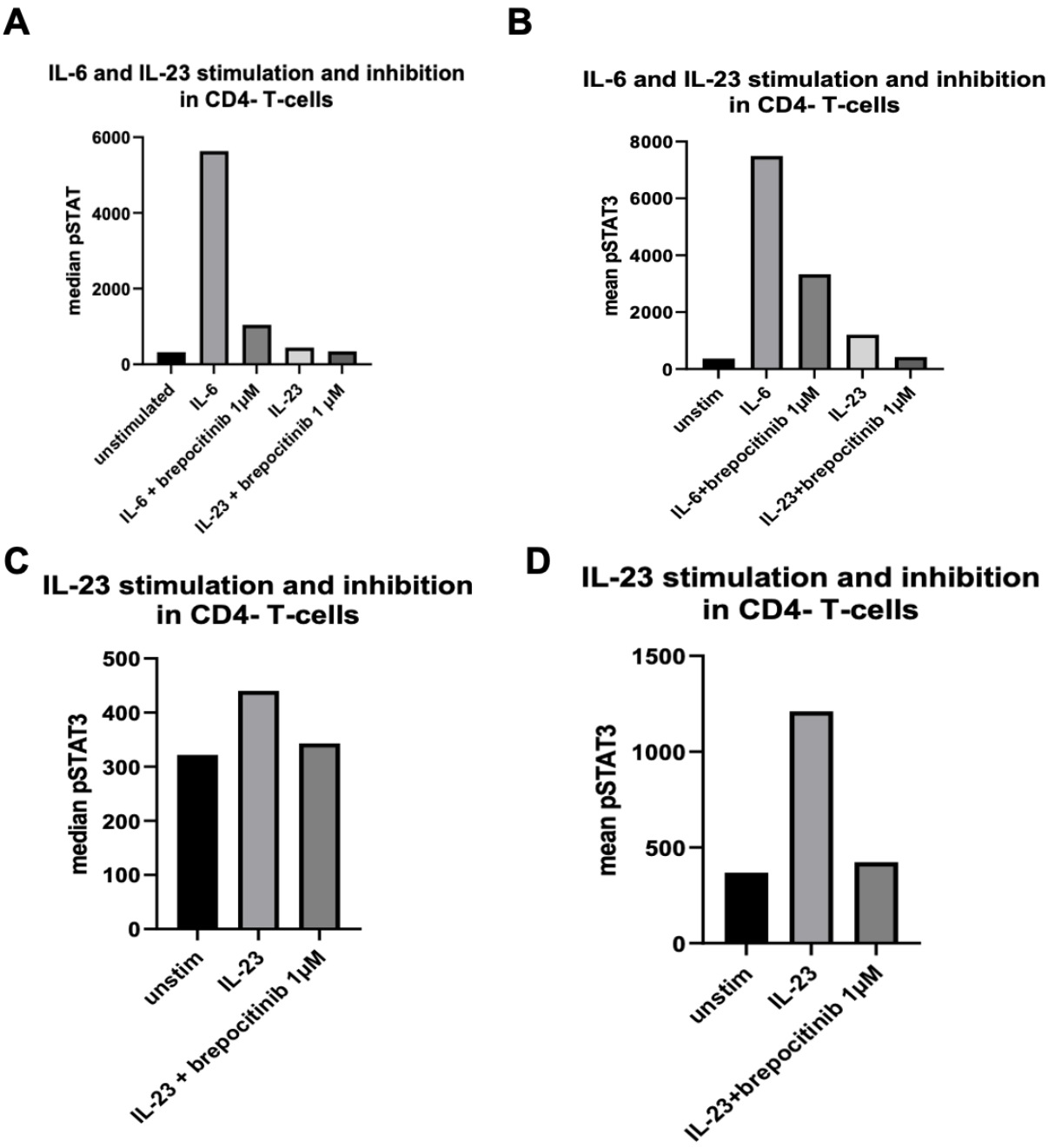

Figure 1. Bar graphs presenting median (A) and mean (B) pSTAT3 values for IL-6 and IL-23 stimulation and inhibition by brepocitinib in CD4 negative T-cells. IL-6 acts as a positive control. IL-23 stimulation and inhibition are also presented without IL-6 stimulation with median (C) and mean (D) pSTAT3 values. CD4, Cluster of differentiation marker 4; IL-6, interleukin 6; IL-23 interleukin 23; unstim, unstimulated sample.

The second flow cytometry assay aimed to determine IC50 value for brepocitinib from whole blood sample for TYK2 and JAK2 mediated IL-23/pSTAT3 signaling pathway. The inhibition curve was calculated from mean pSTAT3 values and is presented in Figure 2A. The determined IC50 value is 348,4 nM. Figure 2B shows a bar graph for IL-23 stimulation and its inhibition by brepocitinib in the same experiment.

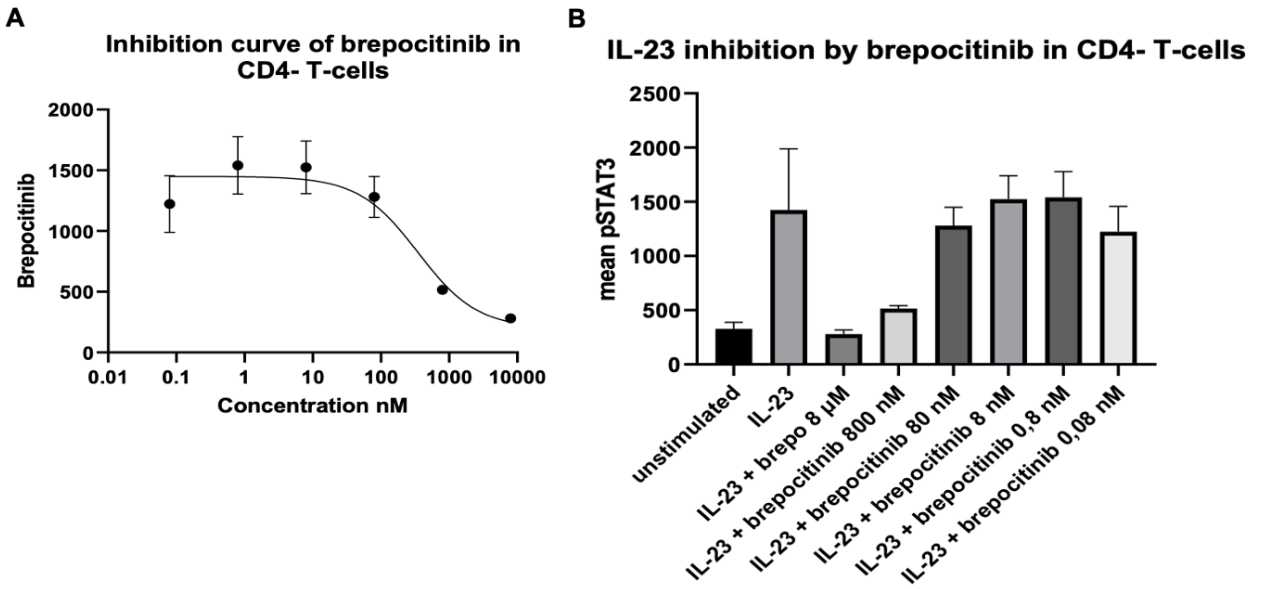

Figure 2. (A) The inhibition curve for brepocitinib in IL-23 stimulated CD4-negative T-cells from the whole blood sample. (B) Bar graph of IL-23 stimulation and inhibition by brepocitinib in CD4-negative T-cells from the whole blood sample. CD4, Cluster of differentiation marker 4; IL-23 interleukin 23.

3.2 ELISA

The purpose of the first ELISA experiment was to determine if IL-23 signaling in PBMCs is detectable from the growth medium's IL-22 concentration and if brepocitinib inhibits IL-22 production. In the first experiment, there are two sample sets non-diluted samples and 1:4 diluted samples. Samples were diluted with the ELISA kit's diluent buffer. Figure 3 presents IL-22 concentrations from non-diluted samples and IL-22 concentrations from 1:4 diluted samples. Fold change values for IL-23 stimulation were 1,2 for non-diluted samples and 2,8 for diluted samples.

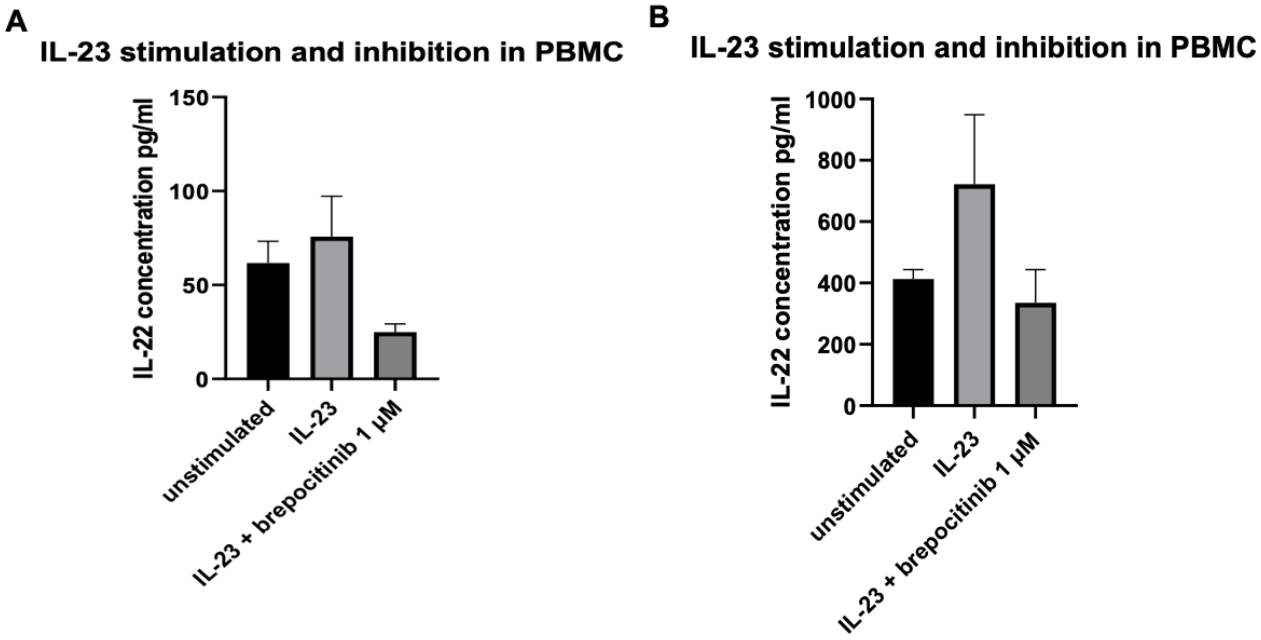

Figure 3. Bar graphs presenting IL-23 stimulation and inhibition by brepocitinib in PBMC measured in IL-22 production. (A) Samples are straight supernatant samples. (B) Supernatant samples were diluted in a 1:4 ratio for ELISA. IL-23, interleukin 23; PBMC, peripheral blood mononuclear cells.

The second ELISA experiment's purpose was to determine the IC50 value for brepocitinib from the PBMC sample. The inhibition curve in Figure 4A was calculated for IL-23 +IL-2 stimulated samples from three different inhibitor concentrations and the determined IC50 value is 22,95 nM. Figure 4B presents a bar graph for IL-23 + IL-2 stimulated samples. Stimulation with IL-23 + IL-2 had a fold change value of 5,8.

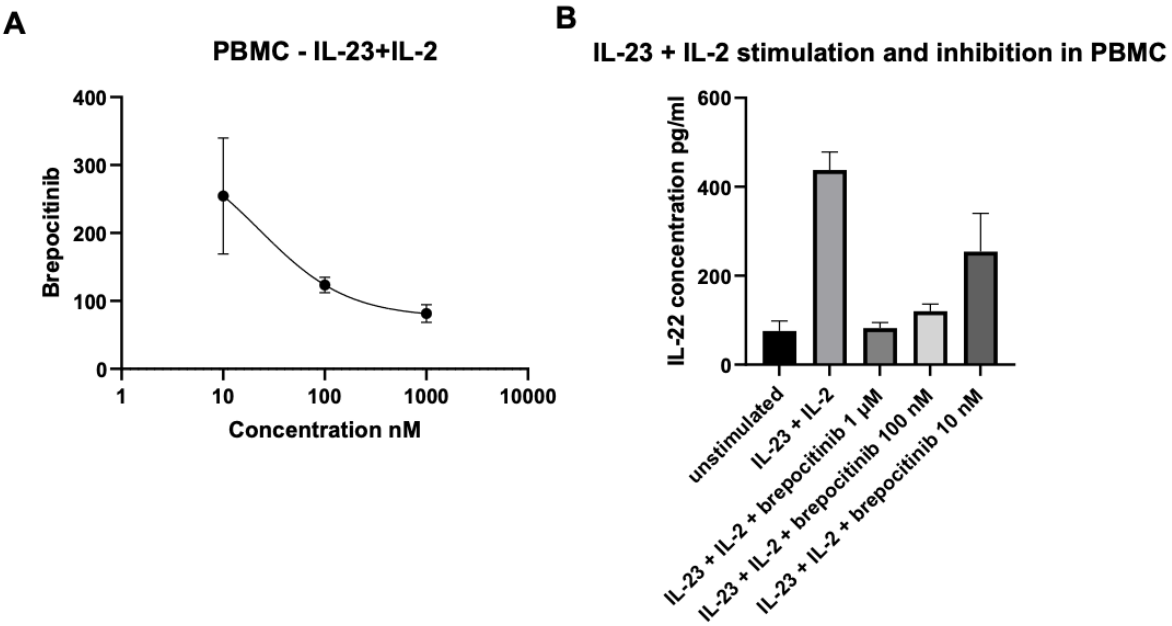

Figure 4. (A) Inhibition curve for brepocitinib in IL-23 and IL-2 stimulated PBMCs. (B) Bar graph presenting IL-22 concentration from IL-23 and IL-2 stimulated and brepocitinib inhibited PBMCs. IL-2, interleukin 2; IL-23, interleukin 23; PBMC, peripheral blood mononuclear cells.

Stimulation of PBMCs with only IL-23 did not result in clear dose-response inhibition for brepocitinib, in consequence, it was not possible to calculate the IC50 value from that experiment setup. Figure 5 presents these measured IL-22 concentrations from IL-23 stimulated and inhibited samples. The fold change value for IL-23 stimulation was 1,8.

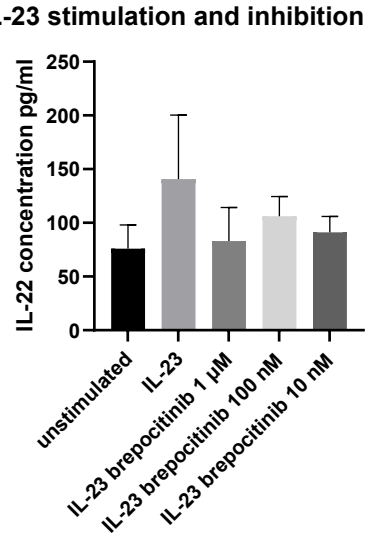

Figure 5. Bar graph of IL-22 concentration from IL-23 stimulated and brepocitinib inhibited samples. IL-23, interleukin 23; PBMC, peripheral blood mononuclear cells.

4. DISCUSSION

Two different assays were developed and proposed to detect IL-23 stimulation and inhibition. Both assays resulted in detected IL-23 stimulation and brepocitinib's ability to inhibit that. Stimulation is measured in fold change value between unstimulated and cytokine-stimulated samples. Based on a threshold value of 1,5, IL-23 stimulated CD4-negative T-cells when fold change was calculated with mean pSTAT3 values. In T_H17 cells IL-23 stimulation is calculated from the produced amount of IL-22. In the first ELISA assay, the IL-23 stimulation is detectable from diluted samples with a fold change value of 2,8. Since it was possible to measure IL-23 stimulation from samples and brepocitinib appeared to inhibit its signaling pathway, second experiments to discover the IC50 values were justified.

From both assays, it was possible to calculate the IC50 value for brepocitinib inhibition in the IL-23/STAT3 pathway. For flow cytometric assay this value was 348 nM and for ELISA-based assay,

it was 23,0 nM. Fensome et al. have measured 120 nM as the IC50 value for brepocitinib in this signaling pathway from human whole blood (Fensome *et al.*, 2018). The IC50 value from the flow cytometric assay is of the same order as the IC50 value from the literature and the IC50 value from the ELISA-based assay is distinctly smaller than either one. The literature value is also obtained from flow cytometric analysis of pSTAT, which could explain the difference between it and the IC50 value measured by ELISA. The assays measure different parameters from different cell types, which can all affect the IC50 value. In addition, the assays implemented in this study have only one-time measured results and repeats would be needed for more useful comparison.

In the flow cytometric assay, analysis of pSTAT3 values can be either median or mean values from the sample. Values differ from each other based on how many cells in the sample are responding to the stimulation by the cytokine. If the response to stimulation concerns a smaller part of the cells, then the mean value probably takes that better into consideration than the median value. Median value is a more commonly used parameter in flow cytometric assays. However, in our samples, IL-23 stimulation and inhibition are better detected when using mean values for pSTAT3. The inhibition curve and IC50 value are determined from mean values because clear dose-related inhibition can be seen from those.

ELISA-based assay was performed with two different kinds of cytokine stimulation. Stimulating cells together with IL-23 and IL-2 resulted in higher IL-22 signal levels and greater response to the inhibitor than when stimulating cells with only IL-23. IL-2 has a wide role in T_H cell differentiation and it increases the expression of the IL-12Rβ1 receptor that is bound by IL-23 (Liao *et al.*, 2011). Thus, cells that are stimulated with added IL-2 have more receptors in which IL-23 can bind and subsequently produce more IL-22.

In ELISA-based assay there were differences between experiments in IL-22 concentration. The differences were based on dilution ratios between samples in experiments. The first ELISA-based assay was carried out with two different dilution rations. Samples with bigger dilutions had higher IL-22 signal levels than non-diluted samples. Based on that the second ELISA-based assay had 1:1 dilution. In the second assay the sample with stimulation of IL-23 had a higher signal level than non-diluted sample in the first assay but not as high signal level as the 1:4 dilution in the first assay. Fold change values for them were not far apart, being 2,8 for the first assay with 1:4 dilution and 1,8 for the second assay. ELISA is a highly sensitive method, and the right dilution ratio will improve the sensitivity and specificity of the assay. In these assay settings, the best dilution ratio is not established and for further experiments, it would be necessary to find the best-fitting dilution ratio for the most accurate results.

5. CONCLUSIONS

In conclusion, two different assays for the detection of IL-23 stimulation and inhibition were both discovered to be accurate in terms of detecting IL-23 stimulation and inhibition by brepocitinib.
IL-23 stimulation was detectable in CD4 negative T-cells when the mean pSTAT3 values were used as readout from phospho flow analysis. IL-23 stimulation of PBMCs was observed and measured as IL-22 production by ELISA. Brepocitinib inhibits the TYK2/JAK2 mediated IL-23/pSTAT3 pathway and from both assays, it was possible to calculate the amount of brepocitinib that is needed for 50 % inhibition. For both assays, there is room for improvement. In flow cytometric assays the more common way to announce results is with median values and with the proposed method stimulation is only detectable with mean values. In the ELISA-based assay concentration of added IL-2 with IL-23 stimulation could require optimization and dilution ratios for samples in these experiments had not been optimized either. With good first-time results both methods can be further developed to suit research purposes.

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