UiT The Arctic University of Norway

Faculty of health science

Antigen-specific humoral immune responses in vitro

Developing a method for activating antigen-specific memory B-cells in vitro

Marie Gabler

Master's thesis in medicine MED-3950 June 2021

Foreword

I found myself interested in immunology and hoped to do a master project involving lab work. I therefore made contact with the leader of the immunological research group Tor Stuge, and asked if he had any ideas for a project. I was warmly welcomed, and a few meetings later we had a plan ready. The project started in August 2020 and finished May 2021. All lab resources were provided by the immunological research group. REK-approval was not required.

I would like to give a great thanks to my supervisor Tor Stuge for ideas and support throughout the project. Thanks for all the time spent preparing, doing lab experiments, analysing results and helping with writing this paper. I sincerely hope my work will help you in later research. Thanks to co-supervisor Maria Therese Ahlen for help with FACS, and the rest of immunological research group at UiT for the help in the lab and friendly faces in a time with little social activities.

26.05.21

Table of Contents

Abstract

Introduction: FNAIT is a condition were the immune system of a pregnant woman is activated and produces antibodies against an unborn child's blood platelets. The mechanisms behind the activation of the B-cells remain unknown. The purpose of this research was to look at antigen-specific humoral immune responses associated with FNAIT. Our goal was to activate antigen-specific B-cells in vitro.

Method: In order to establish a cell culture system to study activation of memory B-cells in vitro, PBMC from three donors were cultured with cytokines, antigen and EL-4-B5. B-cells were sorted with MACS, and flow cytometry was performed to analyse cell populations and to identify and isolate antibody-secreting plasmablasts. B-cell activation was confirmed by antibody production measured with ELISA, and/or detection of plasmablasts with ELISpot.

Results: Cultures showed increased number of plasmablasts producing IgG antibodies. EL-4-B5 was most important to activate and stimulate B-cells, and antigen had a synergic effect. Only a limited number of antigen-specific plasmablasts and antibodies were detected.

Conclusion: Isolated B-cells in culture with antigen, cytokines and $CD40L^+$ EL-4-B5 cells, differentiated to plasmablasts producing IgG, which were successfully identified with flow cytometry. Further research is needed to develop a method to activate antigen-specific B-cells.

Keywords: immunology, B-cell, TT, Tetanus toxoid, FNAIT, fetal neonatal alloimmune thrombocytopenia.

1 Abbreviations

ALP: Alkaline phosphatase

APC (cell): Antigen presenting cell

APC (fluorochrome): Allophycocyanin

BAFF: B-cell activating factor

B-cell: B lymphocyte

BCIP/NBT: Nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolyphosphate p-toluidine salt

BSA: Bovine serum albumin

EBV: Epstein-Barr virus

ELISA: Enzyme linked immunosorbent assay

FACS: Fluorescence activated cell sorting

FBS: Fetal bovine serum

FNAIT: Fetal neonatal alloimmune thrombocytopenia

FITC: Fluorescein isothiocyanate

HPA: Human platelet antigen

IL: Interleukin

IMDM: Iscove's modified Dulbecco's medium

IU: International units

MACS: Magnetic-activated cell sorting

MHC: Major histocompatibility complex

PBMC: Peripheral blood mononuclear cells

PBS: Phosphate buffered saline

PBSA: PBS + BSA

PE: Phycoerythrin

PE-A-F610: Phycoerythrin alexa fluor 610

PMN: Polymorphonuclear (-cells)

PNPP: P.Nitrophenylphosphate

RBC: Red blood cells

T-cell: T-lymphocyte

TNF: Tumor necrosis family

TT: Tetanus toxoid

2 Introduction

The immune system is essential for human life (1). We are surrounded by microbes every day and the immune system is our protector. It is simple and universal as well as complex and specific. The immune system is divided into the innate and adaptive system. The innate system consists of cells such as macrophages, monocytes and granulocytes, and many proteins and molecules, that respond quickly and faces all antigens the same way (1). The adaptive system reacts specifically to each antigen, this process takes time to react. The cells in the adaptive system are mainly the lymphocytes which are divided into the T-lymphocyte (or T-cell) and B-lymphocyte (B-cell). Memory cells in the adaptive system have the ability to remember an antigen and respond faster and greater upon repeated encounter. The immune system may also cause problems (1). In the condition fetal and neonatal alloimmune thrombocytopenia (FNAIT) the immune system of a pregnant woman may cause severe disease or death to an unborn child (2). In FNAIT, B-cells in the mother's immune system are activated and create antibodies against the foreign antigens of the fetal platelets (2). The purpose of this research was to investigate antigen-specific humoral immune responses connected to FNAIT.

The B-cell is activated by interacting with other cells and molecules (1, 3). The antigen presenting cell (APC) binds antigens in the tissues and present processed antigen-peptides on a molecule called major histocompatibility complex (MHC). Interaction between T-cell receptors and MHC is important for activation of T-cells. B-cells bind antigen and display antigen-peptides on MHC. The activated T-cell recognises the MHC on B-cell, and help activate B-cells through secreting cytokines and expressing a molecule called CD40 ligand (CD40L)(1). CD40L expressed on activated T-cells binds to a CD40 receptor on the B-cell and enhances B-cell survival and the production of cytokines. Cytokines are proteins produced by cells to affect other cells, and bind to specific receptors on the target cell to enhance a reaction (1). After activation, the B-cell differentiates into a plasma cell which produces antibodies, also known as immunoglobulins (Ig) (1). Antibodies are B-cell receptors that binds to antigens, e.g. bacteria. The antibodies can activate the immune system, inactivate the antigen (neutralization) or tag the antigen so that other cells destroy it (opsonization). Tetanus toxoid (TT) is an antigen derived from the bacteria *Clostridium tetani* (4). When infected, *C. tetani* produces the extremely potent tetanus toxin responsible for tetanus disease.

TT is the inactivated toxin, and TT activates the immune system resulting in production of antibodies against TT (anti-TT). TT is the basis for the tetanus vaccine (4).

The objective in this study is to develop a method for activating B-cells in vitro in order to further investigate the immunology of FNAIT. This will be done by multiple lab experiments connected to B-cell activation and production of antibodies. FNAIT is a not a common condition, and blood samples from affected patients are rare. Therefore, another antigen, tetanus toxoid (TT), was used as a test system. We predict that knowledge of B-cells activated by TT can directly be transferred to FNAIT. Our aim was to be able to use TT and T-cells expressing CD40L to activate B-cells. We looked at antigen-specific activation, and what combination of stimulating agents were the most beneficial. Our research started with blood from the blood bank and resulted in activation of immune cells and production of antibodies.

3 Relevant background information

The immune system is a collection of cells and proteins working together to prevent illness (1). Lymphocytes are responsible for the adaptive immune system, and the B- and T-cells are activated by specific antigens. Antigens activate naïve cells and they differentiate into effector cells and memory cells. Early after differentiation the cells are called blasts (e.g. plasmablast, lymphoblast) and later they become the mature cell (e.g. plasmacyte or plasma cell and lymphocyte). Effector B-cells are the antibody producing plasma cells responsible for humoral immunity (immunity due to antibodies)(1).

B-cells are activated by cell-to-cell contact and cytokines (1, 3), see figure 4.1. B-cells have B-cell receptors that interact with antigen and processed antigen-peptides are presented on MHC to be recognised by T-cells. Crosslinking of antigens are shown to increase antigens stimulating effects (5, 6). Antigen recognized by T-cell receptors activate T-helper cells to display CD40L. CD40 receptor on B-cells recognize CD40L on activated T-cells, and this T-cell to B-cell contact is found to be essential in B-cell activation (7, 8). The effect is strongest when both cells are activated by the same antigen, called linked recognition (3). Both B- and T-cells will release cytokines to help stimulate the opposite cell (1).

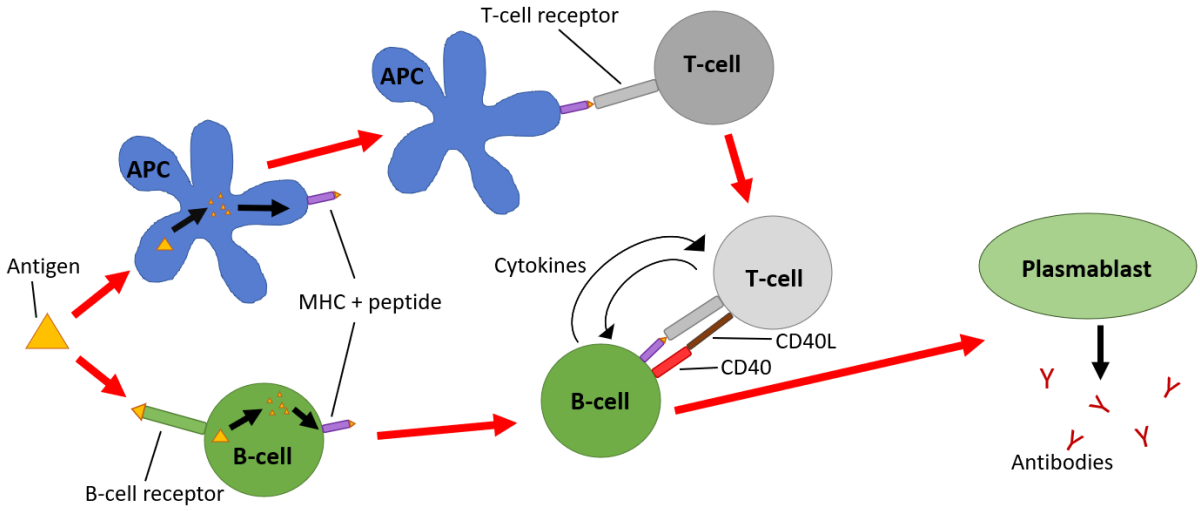


Figure 4.1: Simplified figure of B-cell activation.
Antigen is processed by B-cell and APC and presented on MHC. APC activates the T-cell to display CD40L. CD40L on activated T-cell interact with CD40 on B-cell. Cytokines such as BAFF and IL-21 stimulate to activation of B-cell. When activated, B-cell differentiates into the antibody-producing plasma cell. Activated T- and B-cells also differentiate into memory cells (not shown).

Cytokines bind specific surface receptors on target cells and enhance intracellular reactions, e.g. stimulation to enter cell cycle and proliferate (1). Cytokines produced by lymphocytes are called lymphokines or interleukins (IL) (1). Interleukin 21 (IL-21) is involved in innate and adaptive immune responses (9). It is produced by T-cells and has a central role in early B-cell responses, plasma cell differentiation and development of immunoglobulins (3, 9). BAFF (B-cell activating factor) is a cytokine in the TNF (tumor necrosis factor) family, and promotes B-cell survival through interaction with the BAFF-receptor (3). Cytokines can also have regulatory effects, e.g. IL-21 can enhance cell death (apoptosis) under some conditions (9). CpG are synthetic DNA strands mimicking DNA from bacteria, and are found to stimulate immune responses (5, 10-12).

Antibodies, or immunoglobulins, are cell surfaced or secreted B-cell receptors that bind to antigens (1). All antibodies consist of the same structure with Fab (Fragment antigen binding) and Fc (fragment crystallizable), see figure 4.2. The Fab binds to the antigen and consists of heavy- and light-chains. Fab has a constant region and a variable region with antigen-binding site specific for each antigen. The Fc fragment binds to Fc-receptors on immune cells, e.g. macrophages. It consists of two heavy chains which make the different classes of Ig´s (1).

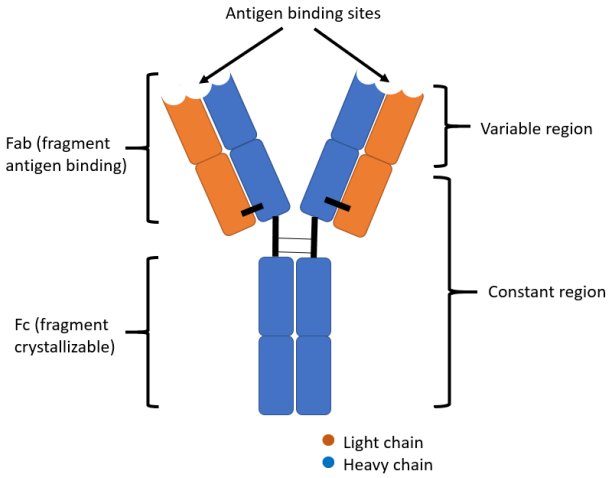


Figure 4.2: Structure of immunoglobulins. Immunoglobulins consist of Fab and Fc fragments. Fab is divided in a variable and constant region and contain light and heavy chains. The variable region has antibody-binding site specific for each antigen. Fc binds to immune cells. The figure is based on information from (1).

Immunoglobulins (Ig) are divided in 5 classes or isotypes; IgM, IgA, IgG, IgD and IgE (1). Young (naïve) plasma cells produce IgM, and IgM is therefore the first antibody to be produced in a response to a new antigen. IgM is attached to naïve B-cells in monomeric form or circulates in pentameric form. Later in the immune response there is an isotype shift from IgM to the other isotypes. IgA is the main antibody on the mucosa and there is some monomeric IgA in blood. IgG is produced by memory cells in the secondary immune response. IgG is divided into 4 subgroups (IgG1-IgG4) and is the most frequent immunoglobulin in blood. IgE is mostly bound by mast cells. The function of IgD is unknown (1). IgG is most important for secondary immune responses, as for FNAIT.

Immunoglobulins are antigen-specific (1). Memory B-cells will only be activated by the antigen matching its specific receptor. Interaction with the receptor cause the memory B-cell to differentiate and produce monoclonal antibodies (1). It is possible to activate B-cells without a specific antigen if the other stimulants are strong, but this activation will be unspecific and produce polyclonal antibodies (3). Because they are specific, antibodies can be used to detect and sort antigens from a compound. E.g. B-cells have antigens on the surface such as CD19 and CD22, and anti-CD19 and anti-CD22 can therefore be used to detect B-cells. Likewise are anti-CD38 and anti-CD27 used to detect plasmablasts (5).

FNAIT is a disorder caused by activation of B-cells and production of antibodies (2). Differences in maternal and fetal platelet antigens results in a maternal humoral immune response. The production and binding of IgG to fetal thrombocytes leads to thrombocytopenia and may cause severe bleeding and death. FNAIT is estimated in 0,5-1,5 per 1000 live births (2) and is a major cause of severe thrombocytopenia. >75% of FNAIT in the Caucasian population is caused by antibodies against human platelet antigen 1a (HPA-1a). More specifically, the antibodies bind to a glycoprotein called GPIIbIIIa (αIIbβ3 integrin), one of two major receptors on the fetal thrombocyte surface (2). There is no screening test and the condition is usually discovered when symptoms are severe. Preventing measures are only performed when the condition is known from a previous pregnancy. Treatment options are limited to general approaches (4). Details of the maternal immune response leading to FNAIT remains largely unknown*. There is no in vitro model well suited for further experimenting B- and T-cell interactions leading to antibody secretion in the context of FNAIT.

Experiments ex vivo mimic interactions in vivo. PBMC is a collection of the major immune cells such as lymphocytes (13), and will provide the B-cells for the experiments. EL-4-B5 is a mutant clone of T-cells from mouse (14) that continuously display CD40L (15). Cytokines such as BAFF and IL-21, and CpG will be used to stimulate cells. TT is used as antigen to stimulate B-cells to produce anti-TT specific antibodies. Tetanus is a well-documented antigen, and commonly used in immunological experiments. It is part of the standard vaccination program in many countries (4), and samples from donors with immunity to TT are therefore easy to acquire. Vaccination must be repeated to maintain a protective level of anti-TT antibodies in serum above 0,01 IU/mL (4). Tetagam is synthetic immunoglobulins against TT (anti-TT) and used to crosslink TT for increased effect.

4 Method

The experiments were performed at the immunological lab at the University of Tromsø (UiT) and the University Hospital of Northern Norway (UNN). Flow cytometry was performed at the Advanced Microscopy Core Facility (AMCF), UiT. Basic lab knowledge is expected for performing the experiments. A list of materials can be found in the appendix.

4.1 Isolating PBMC from buffy coat

PBMC was withdrawn from buffy coats received from the blood bank at UNN. The blood bags were chosen randomly, the donors are anonymous adults and expected healthy. All donors had given a general consent that their blood could be used for scientific purposes. The donors were marked donor 1, 2 and 3, this will also apply for the PBMC (e.g. PBMC1 from donor 1). Plasma was transferred from each donor in the same step as PBMC.

The contents of buffy coats were distributed in two 50 mL vials and diluted 1:1 in PBS (phosphate buffered saline). 12 mL 1.077 g/mL density gradient medium (Lymphoprep, Stemcell Technologies, Vancouver, Canada) was transferred to four 50 mL vials. ¼ of the diluted buffy coat solution was carefully layered on top of the density gradient medium in each vial to prevent mixing. The vials were centrifuged for 30 minutes at 800 x g, 24 degrees, without breaks. The interface containing the PBMC was extracted as shown in figure 5.1 using a plastic Pasteur pipette.

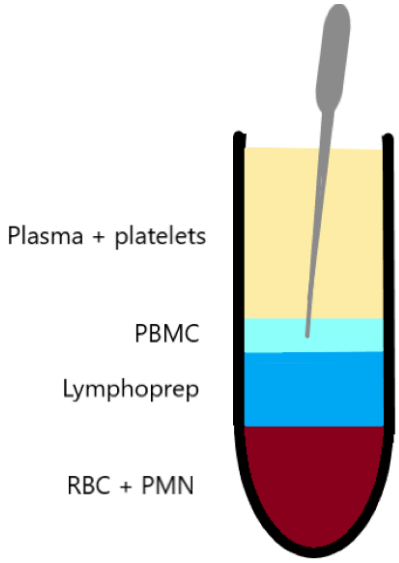


Figure 5.1: Layers of buffy coat after density gradient medium (Lymphoprep) is added and centrifuged.
Lymphoprep separate PBMC from RBC and PMN's.
The pipette point to the layer of PBMC extracted for further experimental use.
Plasma from top layer was also extracted.

The PBMC was washed three times using the following procedure: PBSA 0,2% (PBS + 0,2% BSA (bovine serum albumin)) was added to reach 45 mL, then centrifuged 10 minutes at 250 x g. Centrifugation forced the cells to the bottom of the vial, the supernatant was discarded by decantation into a bucket while the cells remained at the bottom of the vial. The cell pellet was detached from the bottom by flicking the vial. Cell culture medium (IMDM (Iscove's modified Dulbecco's medium) + 10% FBS (fetal bovine serum)) was added.

4.2 Counting cells using a hemocytometer

10 μL of cell sample was transferred to the hemocytometer using a micropipette. If the solution contained a high number of cells, the solution was diluted. For 1:10 dilution, 90 μL medium was transferred to a vial and 10 μL cell sample added. After mixing, 10 μL of diluted cell solution was transferred to the hemocytometer.

The number of cells were counted using a microscope. The Burker chamber consists of 9 big squares, divided in 16 medium squares, each divided in 16 small squares. One medium square is 1 mm^2, and the chamber is 0,1 mm deep. One medium square is therefore 1 mm^2 x 0,1 mm = 0,1 mm^3 = 0,1 μL. Multiplying by 10 000 gives the number of cells per mL. For example, a count of 40 cells in 16 small or one medium square: 40 cells/mm^2 = 40 cells/0,1μL = 400 000 cells/mL. If dilutions were made, this was accounted for, e.g. x 10 for 1:10 dilution. The count was multiplied with the number of mL cell sample to know the total cell count in the flask.

The hemocytometer is an easy method for estimating cell concentration. The method can however be incorrect because of uneven distribution of cells on the hemocytometer. To ensure a representative count, the mean value in 5-6 different squares was used for calculations. The cells can also be uneven distributed in the cell solution, although cells were detached and mixed before transferring to the hemocytometer. There might also be other cells in the solution giving a false high estimate, e.g. red blood cells, which can be difficult to distinguish from lymphocytes by 100 - 400 x magnification light microscopy.

4.3 Preparations for freezing cells

Cells were redistributed in a smaller volume by centrifuging 5 minutes at 350 x g and the surface solution discarded. Freezing medium (90% FBS, 10% IMDM) was added to the cell pellet and mixed before distributed in cryovials safe for freezing. The vials were put in a cooled box designed for freezing cells, with a separate chamber containing isopropanol. The box was then placed in freezer at -70°C. The cells could at any time be removed from freezer and used for experiments. The vials were defrosted quickly by vigorous shaking under warm water until partially thawed. The last visibly frozen content was thawed between the fingers

while inverting the vial. The content was immediately diluted at least 1:10 in cold cell culture medium, centrifuged 4 minutes at 400 x g, decanted, and the cells suspended in cell culture medium for over-night recovery in tissue culture flasks at 37°C.

4.4 Preparations for cell culture

Three different cultures were made for the research. Here follows a general description of the preparations. For more information of each culture, see results and the appendix. Sterile ELISA plates consisting of 96 wells were used for the cultures. The wells ensured good environment for cells and antigens to interact, and it was possible to remove surface solution for analysing without interrupting the cells. The supernatants could be stored in fridge at 4 °C until analysing.

Cells: Cells were counted and resuspended in medium. For PBMC/B-cells, calculations were made to gain approximately 20 000 B-cells per well. EL-4-B5 was irradiated to prevent further growth, and calculations made to gain 40 000 EL-4-B5 cells per well. Medium was added to gain 50 µL of cell suspension per well.

Antigen: The antigens were sterile filtered with a 0,2 µm filter before use. The tetanus toxoid (TT) concentration differed, for the first culture 50 µL of 4-64 ng/mL was added to wells. For the two last cultures 100 µL of 1,75 µg/mL TT was added to B-cells. The cells were placed on ice for 20 minutes. The leftover antigens were washed off using the following method: 10 mL PBSA 0,2% was added to each sample and then centrifuged 4 minutes at 400 x g. The supernatant was discarded, and the cell pellet detached by flicking the vial. If purified anti-TT antibody (tetagam) were used, TT was first added and washed off before tetagam was added. 50 µL of 2,5 IU/mL tetagam were added to B-cells, then placed on ice for 20 minutes. The leftover tetagam did not need to be washed off.

Cytokines: BAFF and IL-21 were added in a cytokine mix to easier distribute cytokines in wells. 50 µL of 100 ng/well of BAFF and 50 µL of 50 ng/well of IL-21 were used. The desired concentration of cytokines had to be multiplied by four because it would be diluted 1:4 in other solutions in culture. The calculated number of cytokines was added in medium to

gain 50 µL of cytokine mix per well. CpG was added in medium to gain 50 µL of 4 x 0,6 µg/mL per well and added directly to cultures.

Culture: 50 µL of each solution was added to each well. For control samples, each solution left out was replaced with medium so that each well contained a total of 200 µL. Figure 5.2 shows a general set up for two wells. The ELISA plates were then incubated at 37 °C, 7,5% CO_2, 6,6% O_2.

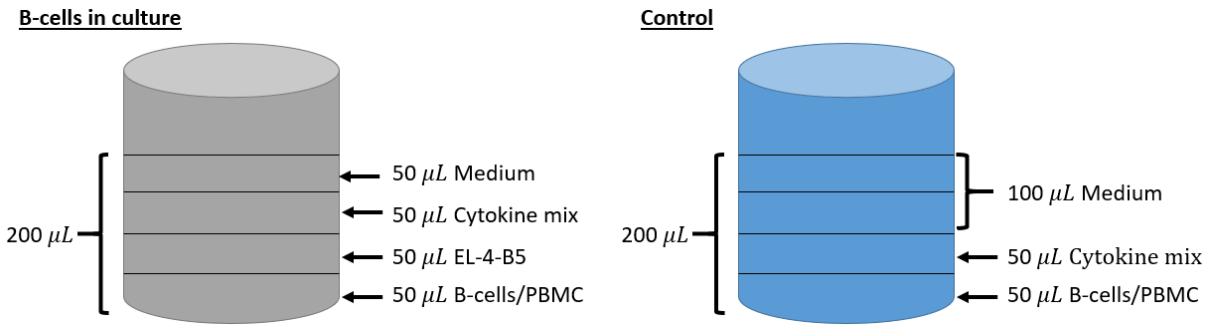


Figure 5.2: General set up of culture.
A: B-cells/PBMC in culture with EL-4-B5 and cytokines. B: Control sample without EL-4-B5. EL-4-B5 is replaced by 50 µL medium.

4.5 Sorting cells using a MACS-system

MACS (magnetic-activated cell sorting) is a system for sorting cells using columns with antibodies and a magnet. MACS was used to enrich the population of B-cells. Antibodies for CD22, an antigen found frequently on B-cells, were used. The magnet would hold on to the cells that attached to anti-CD22 (later called the column "adherent cell" population), and the adherent population will therefore mostly consist of B-cells. If the number of cells exceeded 50 million, the solution was divided in two and then run separately, to not exceed the column capacity.

For sorting, PBMC was transferred to vials and then centrifuged 4 minutes at 400 x g, supernatant was discarded. The cells were resuspended to 160 µL culture medium and left on ice for the rest of the experiment to prevent them from absorbing the antibodies. Antibody was added (40 µL anti-CD22/sample), and then left in fridge for 15 minutes. The cells were washed to remove any leftover antibodies as follows: 4 mL PBSA 0,2% was added to each

vial, then centrifuged for 4 minutes at 400 x g, 4°C. The supernatant was discarded, and cell pellet flicked to detach from vial. Medium was added to gain 3 mL cell suspension.

A MACS LS column and magnet (Miltenyi Biotec, Germany) was used for the cell sorting. The MACS column was placed in the magnet and rinsed with 3 mL PBSA 0,2%. The cells were added and the non-adherent cells (flow-through) collected in a vial. The column was then rinsed 3 times with 3 mL PBSA 0,2% to remove all non-adherent cells. The adherent cells (that attached to the magnet) were transferred to a vial by removing the column from the magnet and placing it in the vial, then adding 5 ml culture medium and vigorously flushing out the column adherent cells using a sterile plunger provided with the MACS column.

4.6 Flow cytometry/FACS

Flow cytometry is a commonly used procedure for detecting antigens and sorting cells. Flow cytometry was performed multiple times, here follows a general description of the preparations. For description on how to run the procedure, check the producer's manual and follow local guidelines. FlowJo software (Becton, Dickinson & Company, USA) was used for analysing the results. Fluorochrome-conjugated antibodies were used for flow cytometry and fluorescence-activated cell sorting (FACS). PE (Phycoerythrin) and FITC (Fluorescein isothiocyanate) were the main fluorochromes, but also APC (allophycocyanin) and PE-A-F610 (Phycoerythrin alexa fluor 610) were used. Figure 5.3 shows an example of vials used for detecting IgM and IgG in B-cells.

Vial	Cells	Antibodies	Use
1	Pre-sort	None	Compensation
2	Pre-sort	IgM (FITC)	
3	Pre-sort	CD19 (PE)	
4	Flowthrough	CD19 (PE)	Sample 1
5	Adherent	CD19 (PE) + IgM (FITC)	Sample 2
6	Adherent	CD19 (PE) + IgG (FITC)	Sample 3

Figure 5.3: Example of vials prepared for flow cytometry.
In this example there are 3 different cell samples after sorting PBMC with MACS (pre-sort, flow through and adherent). Vial 1-3 are compensating solutions for calibration. Vial 4-6 are solutions made to detect CD19 and IgG/IgM. PE and FITC are the conjugated fluorochromes.

Flow cytometry for detecting antigens: The cells were resuspended in medium to gain 50 μL per vial. A minimum of 100 000 cells were distributed per vial. Fluorochrome-conjugated antibodies were added to PBSA 0,2% to gain 50 μL/vial (see list of materials). If more than one fluorochrome was needed, compensating samples were made to calibrate the flow cytometer. All solutions were left on ice to prevent the cells from absorbing the antibodies. 50 μL antibody mixed with medium and 50 μL cell suspension was added to vials and left on ice for 30 minutes. The leftover antibodies were washed off by using the same method as described in removing antibodies for MACS. 200 μL PBSA 0,2% was added to each vial and flow cytometry was performed with BD LSRFortessa (Becton, Dickinson & Company, USA).

Flow cytometry for sorting cells (FACS): FACS was performed to sort cells based on the antigens. The preparations were mostly the same as above with a few adjustments. The cell count was larger, therefor a bigger volume of antibodies (twice the normal) and PBSA 0,2% (10 mL instead of 3 mL) were used. 400 μL PBSA 0,2% was added to compensating samples and 400 μL medium with 30% FBS was added to sorting samples so that the cells would survive for longer. Vials containing only medium were prepared to sort cells into. The compensating samples were used to calibrate and then the cell samples were sorted with BD FACSaria III (Becton, Dickinson & Company, USA).

4.7 ELISA

ELISA (enzyme-linked immunosorbent assay) was used to detect antibodies against TT. The method uses ELISA plates consisting of 96 wells that ensure a good environment for antigen-antibody interaction. An antibody conjugated to an enzyme binds to antigens and changes colour to a substrate (see figure 5.4). A standard solution was made to calculate concentration of anti-TT in samples.

A 1 μg/mL antigen solution was made from stock and coating buffer (carbonate/bicarbonate buffer). ELISA plates were coated with antigens by adding 100 μL to each well using a multi-channel pipette. The plate was covered in adhesive foil and left in fridge (at 4ºC) over night.

The coating solution was discarded and then washed 3 times as followed: 250 μL washing buffer (PBS 0,05% tween 20) was added per well, then discarded and tapped on paper towel

to remove remaining drops. 200 µL of PBSA 1% was then added to each well to block remaining antigens. The plate was covered in adhesive foil and left for 2 hours in room temperature. Dilutions for standard solution were made with PBSA 0,2% (see figure 5.5). Each tube was vortexed before performing the next step. The sample containing anti-TT was diluted in a similar way.

100 µL of each solution was placed in wells. The wells were covered in adhesive foil and left in room temperature for 1 hour, then washed 3 times with 250 µL washing buffer as described earlier. Tablets containing 5 mg p-nitrophenyl phosphate (pNPP; Pierce Biotechnology, USA) were diluted in 5 mL of the accompanying substrate buffer per tablet. 100 µL pNPP solution was added to each well and incubated in the dark for 20 minutes or until sufficient colour developed. 50 µL 3M NaOH was added to stop the substrate reaction. A spectrophotometer at 405 nm was used to measure optical density and Excel was used for analysing the results. The concentration and related absorbance of standard solution (tetagam) was used to make a standard curve. Linear regression was used to find a trend line, and the concentration of anti-TT was calculated.

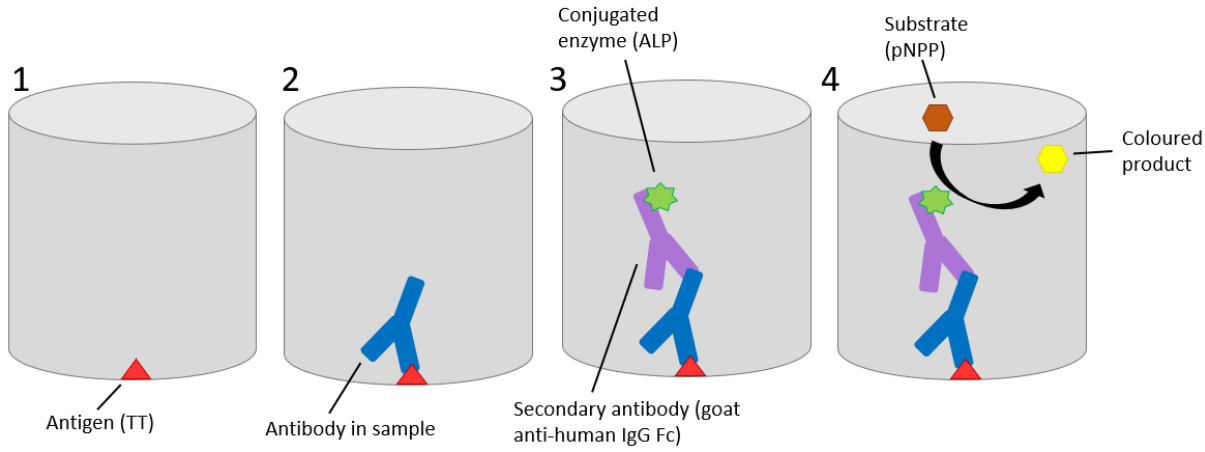


Figure 5.4: **Principles of ELISA for detecting anti-TT.**
1-4 show steps in performing ELISA. First wells are coated with antigens over-night. Sample is added and antibodies specific to antigen will attach. A secondary antibody conjugated to an enzyme is added and bind to primary antibodies if present. Substrate is added and changes colour in presence of enzyme. Coloured product is detected with a spectrophotometer.

Tube	Dilution	Final IU/mL (= concentration)	Volume antigen	Volume PBSA 0,2%
	Tetagam P	250		
S1	1:100	2,5	10 µL of stock tetagam P	990 µL
S2	1:10 000	0,025	10 µL of tube 1	990 µL
S3	1: 20 000	0,0125	500 µL of tube 2	500 µL
S4	1: 40 000	0,00625	500 µL of tube 3	500 µL
S5	1: 80 000	0,003125	500 µL of tube 4	500 µL
S6	1:160 000	0,00156	500 µL of tube 5	500 µL
S7	1: 320 000	0,000781	500 µL of tube 6	500 µL
S8	1: 640 000	0,000391	500 µL of tube 7	500 µL
S9	1: 1 280 000	0,000195	500 µL of tube 8	500 µL
S10	1: 2 560 000	0,0000977	500 µL of tube 9	500 µL

Figure 5.5: **Dilutions for standard solution.**
Standard solutions were made by transferring volume of tetagam stock or other tube (listed under "volume antigen") and adding PBSA 0,2% (listed under "volume PBSA 0,2%"). Only S3-S10 was used for measuring optical density. Concentration in IU/mL and corresponding measured absorbance were used to make a standard curve.

4.8 ELISpot

ELISpot is a method used for detecting antibody secreting cells. Plates were coated with TT antigen or goat anti-IgG capture antibody (see figure 5.6). TT would show cells producing specific anti-TT antibodies and goat anti-human IgG show all cells producing IgG antibodies. Each sample was made in 3 parallels.

The ELISpot plate was prepared by using the following method: 15 µL 35% ethanol was added to each well to soften the membrane. The plates were washed 4 times by adding 100 µL PBS per well and discarded. The remaining drops were removed by tapping the plate on paper towels. 50 µL of 10 µg/mL TT or goat anti-human IgG was added to respective wells. The plate was covered with adhesive foil and left in the fridge (at 4ºC) over night. The samples were washed 5 times using 100 µL PBS as described earlier. 120 µL blocking buffer (PBS + 2% FBS) was added to block remaining antigens. The plate was then left for 1 hour at room temperature.

The cell solution was prepared by resuspending cells to 100 µL per well. A positive control containing cells known to produce antibodies and a negative control of EL-4-B5 was prepared. Blocking buffer was discarded and 100 µL of cell suspension was added to the respective wells. The plate was left over night in a 7,5% CO_2 incubator (at 37°C).

The plate was washed 5 times with 120 µL washing buffer (PBS 0,01% tween 20) and then once with 120 µL PBS. The secondary antibody (goat anti-human IgG Fc alkaline phosphatase (ALP) conjugate) was prepared to gain 1 µg/mL. The antibody was sterile filtrated and 50 µL was added to each well. The plates were then left in the incubator for 2 hours and then washed again 5 times with 120 µL washing buffer and once with 120 µL PBS. Then 50 µL of filtrated substrate (nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolyphosphate p-toluidine salt (BCIP/NBT)) was added per well and left for 10 minutes in the dark. When spots appeared on the plate, it was rinsed for 3 minutes with tap water. The plastic film sealing the bottom of the plate was removed, and the plate was rinsed for another for 3-5 minutes. The plate was left to dry in room temperature over-night. Immunospot 3 (CTL Cellular Technology Limited, USA) was used for counting and image acquisition used for photographing the ELISpot plate.

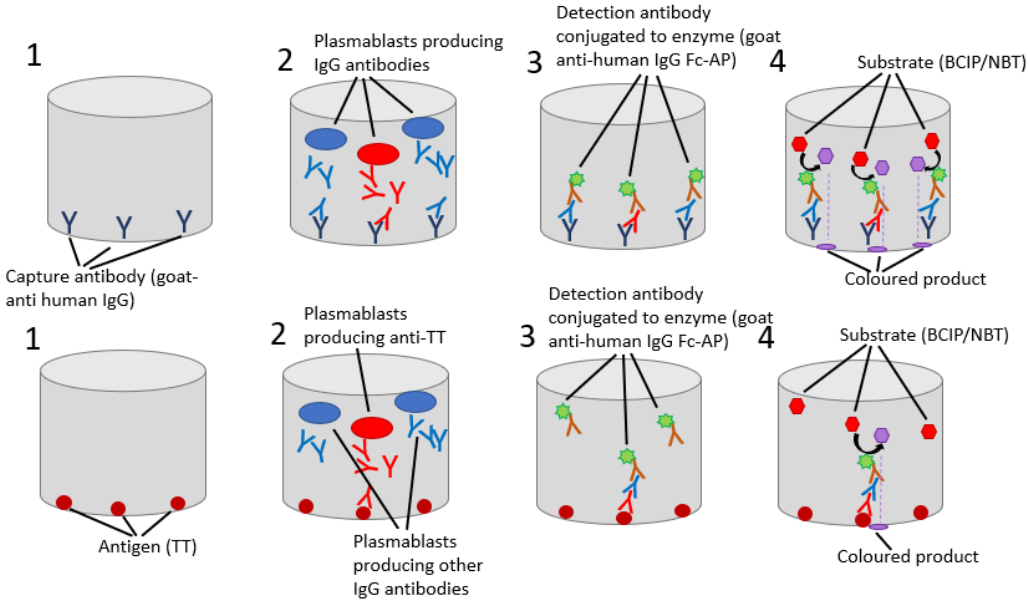


Figure 5.6: **Principles of ELISpot for detecting IgG and TT secreting cells.** Wells are coated with TT or goat anti-human IgG. Samples containing plasmablasts producing antibodies are added. Anti-TT will only bind TT, all other IgG antibodies will bind goat-anti human IgG. Cells and unbound antibodies are washed off. Detection antibody conjugated to enzyme is added and bind to antibody if present. Substrate changes colour by enzyme, and its product will fall to bottom of well seen as spots.

5 Results

To establish an in vitro cell culture system in which memory B-cells can be activated to differentiate into antibody-secreting cells, cultures with blood cells from donors and a selection of stimulating agents were made. Cell populations were analysed with flow cytometry. B-cell activation was confirmed with antibody production measured with ELISA, and/or detection of plasmablasts with ELISpot.

5.1 Stimulation of memory B-cells to produce anti-TT antibodies depends on blood cells from TT immunized donors and T-cells expressing CD40L

5.1.1 Do the EL-4-B5 cells express CD40L?

To find out if the EL-4-B5 cells expressed CD40L essential in activation of B-cells, flow cytometry was performed. Antibodies for CD40L in humans and mice were used to detect CD40L. PBMC and anti-CD19 were used for control samples.

The results show that EL-4-B5 cells express CD40L as expected (fig. 6.1). Sample F shows EL-4-B5 cells binding anti-CD40L from mouse and the large curve indicates that most cells express CD40L. Sample E shows that the human anti-CD40L cannot bind to the EL-4-B5. This indicates that the antibody is very specific for CD40L from humans only, and therefor will not bind the EL-4-B5 which originates from mice. Sample D is a positive control since CD19 is specific to B-cells. The PBMC contains other cells than B-cells, this is supported by a smaller curve of antibody binding cells than in sample F.

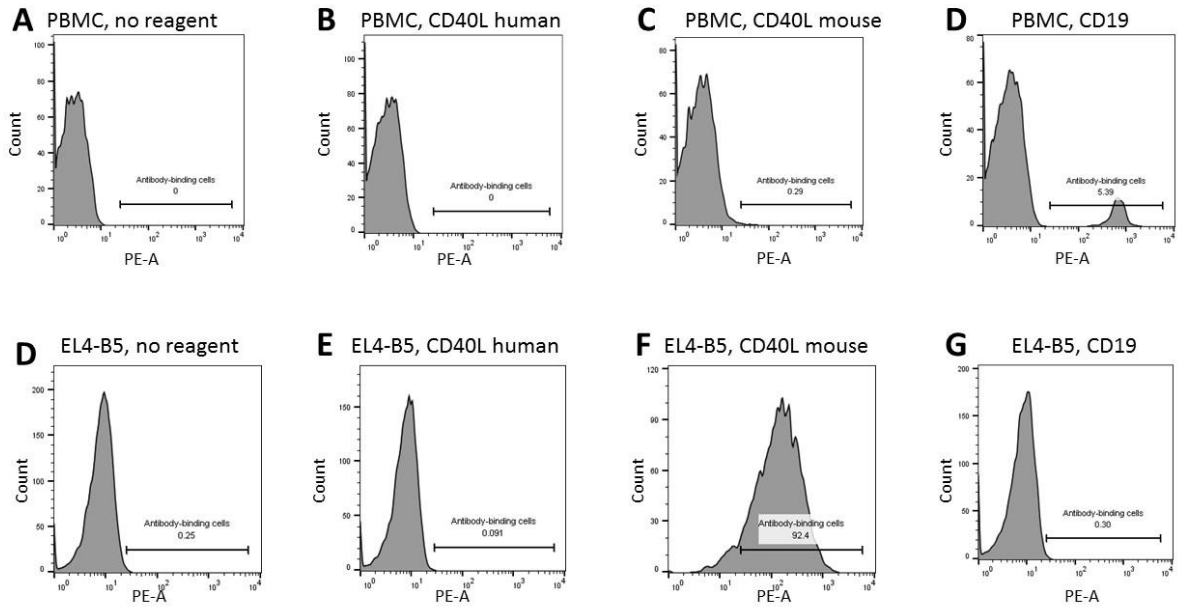


Figure 6.1: The EL-4-B5 cells express CD40L.
PBMC or EL-4-B5 cells were reacted with PE-conjugated anti-human or anti-mouse CD40L, or anti-human CD19 and analysed with flow cytometry.
The line indicates the area for antibody-binding cells. Samples D and F are positive, and the others are negative for the antibody tested. The figure shows lymphocyte population.

5.1.2 Does plasma from the blood donors contain anti-TT antibodies?

To confirm that the donors had been immunized with TT, ELISA was performed on plasma extracted from buffy coat. TT was used to coat plates, FBS was used for negative control and PBSA for blanc samples. The mean of each sample was calculated and the blanc subtracted. Standard solutions (Tetagam) were used to find the concentration of anti-TT in plasma. Plasma from donor 2 and 3 share standard solutions and therefore also standard curve. The results indicate that all donors were immunized with TT (fig. 6.2). Donor 1 had a high level of anti-TT and donor 3 a low level.

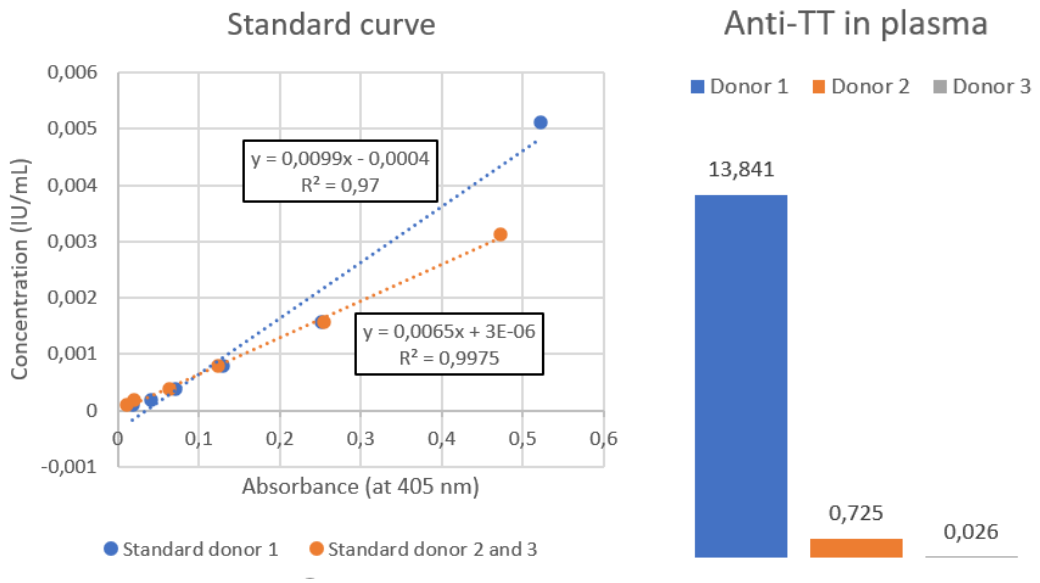


Figure 6.2: **All donors were immunized with TT.**
Plasma from all three donors were analysed for anti-TT by ELISA. The standard solutions were used to make a standard curve and linear regression to find a trendline. The diagram to the right shows mean values of anti-TT in plasma from donor 1, 2 and 3.

5.2 Can we stimulate B-cells from PBMC in cultures with EL-4-B5, CpG and antigen?

To investigate if B-cells from PBMC could be activated to antibody producing plasmablasts, cultures were made. PBMC from donor 1 and different combinations of EL-4-B5, CpG and antigen were cultured for 9 days. Antigen (TT) was added in 5 different concentrations (1, 2, 4, 8 and 16 ng/mL). EBV was used as a positive control sample. The surface solution from each sample was extracted on day 3, 6 and 9 and then analysed for anti-TT with ELISA.

The results show very low values of anti-TT indicating that B-cells were not activated (shown in the appendix). The standard solutions have appropriate values indicating that the ELISA method has been done correctly. For the EBV control samples, 1/3 of wells had a positive result of 0,831 in the undiluted day 9 result. This might indicate that there are too few B-cells in the other wells to be activated.

5.3 Can we further optimize B-cells?

5.3.1 Can we sort B-cells from PBMC using MACS?

To enrich B-cell populations for further experimenting MACS was performed. CD22 antibodies were used to detect and sort B-cells from PBMC. The pre-sort, flow through and adherent cell samples were analysed with flow cytometry. Anti-CD19 was used to detect B-cells.

The adherent population had a much higher proportion of CD19 binding cells than the pre-sort and flow through (fig. 6.3). There were still some B-cells in the flow through sample indicating that some B-cells were lost in the process. More than 90% of the adherent samples were B-cells for all donors, implying that the MACS was successful in enriching B-cell population for further research.

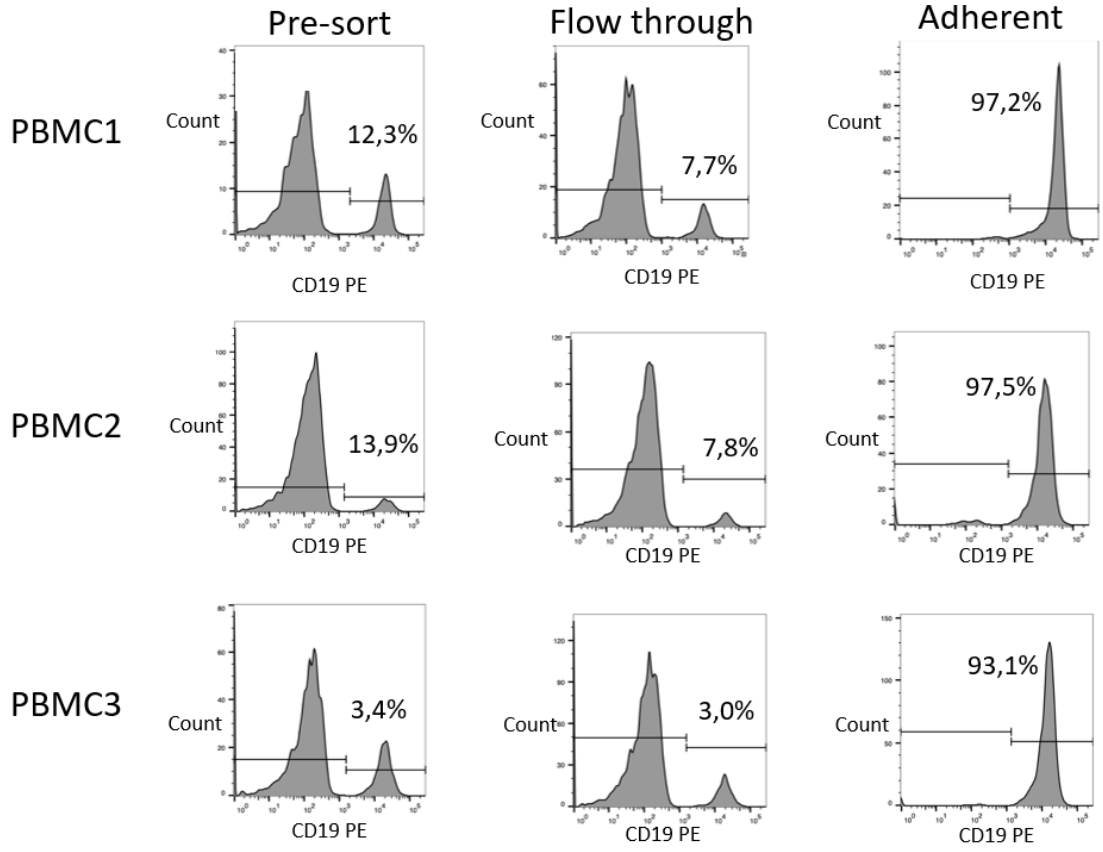


Figure 6.3: **MACS column adherent cells were highly enriched for B-cells (CD19+).** B-cells were sorted from PBMC by MACS using anti-CD22. A flow cytometry was performed to compare the adherent, flow through and pre-sort populations. PE-conjugated CD19 antibodies were used to detect B-cells.
The lines separate the population of CD19+ cells from CD19- cells. The number is the percentage of CD19 binding cells. The figure shows lymphocyte population.

5.3.2 Do all donors have B-cells expressing isotype switched immunoglobulins?

In the flow cytometry of adherent cells from donor 1, IgM and IgG antibodies were used to look for memory (IgG+) and naïve (IgM+) B-cells. The results show a big population of IgM positive cells (fig. 6.4). The IgG binding population should be in the area marked IgG+, but no clear population can be found. FlowJo calculated that IgM + cells were about 97-98%, and IgG + cells are 2-3%, but the numbers remain uncertain since there is no clear division between the populations. The results indicate that either the flow cytometry was not able to detect the IgG+ memory cells, or donor 1 had very few (if any). This would indicate that donor 1 was not suited for experimenting on humoral immune responses.

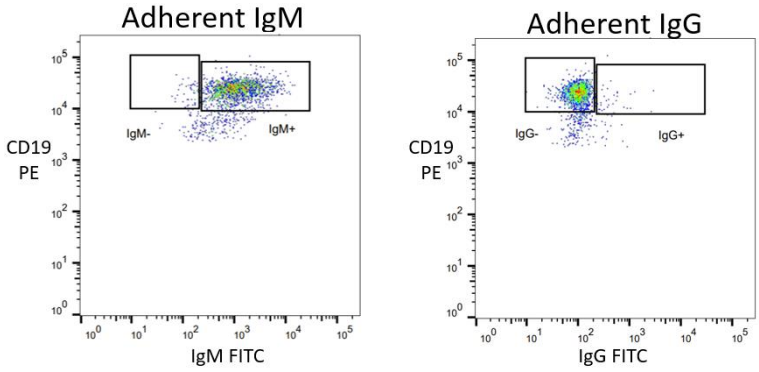


Figure 6.4: **Few B-cells express IgG in donor 1.**
Adherent cells from donor 1 were reacted with FITC-conjugated anti-IgM or anti-IgG and analysed with flow cytometry. The gates indicate the expected location of IgG and IgM + and - populations. The figure shows lymphocyte population.

Another flow cytometry was performed on all PBMC's (PBMC1, PBMC2 and PBMC3) to ensure the donors had memory cells. Antibodies for IgM, IgG and IgA were used for quantifying the different populations. Note that this experiment was performed on PBMC whereas the results displayed in figure 6.4 is performed on adherent cells and therefore missing the CD19 negative population.

Flow cytometry confirm IgG+ populations for all donors (fig. 6.5). FlowJo was used to quantify the population of CD19+ (B-cells) and IgG+ (memory B-cells) cells. 2,6% of B-cells in PBMC1 were IgG+, 6,3% for PBMC2 and 13,4% for PBMC3. Results for IgM and IgA can be found in the appendix. The results show that all donors had memory cells, but the size of the populations varies. Donor 1 had a smaller and less defined IgG+ population than the other donors.

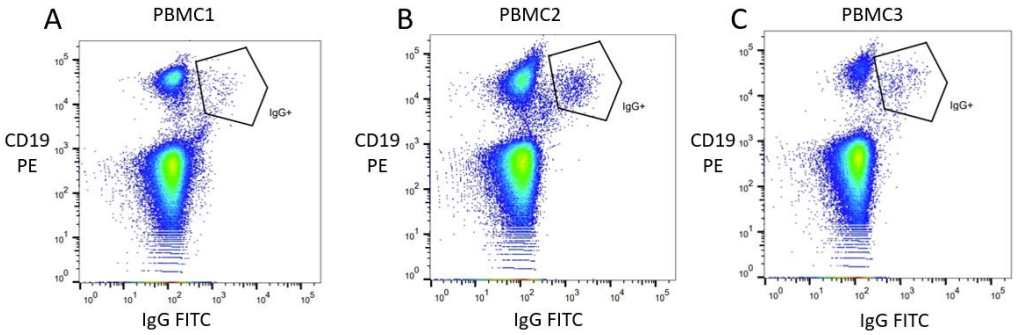


Figure 6.5: **All donors had B-cells expressing IgG**.
PBMC from all donors were reacted with PE-conjugated anti-CD19 and FITC-conjugated anti-IgM, anti-IgA or anti-IgG and analysed with flow cytometry. The gates indicate IgG+ populations. The figure shows lymphocyte population.

5.4 Can we stimulate isolated B-cells in cultures with EL-4-B5, cytokines and antigen in vitro?

5.4.1 Can we activate isolated B-cells in vitro to differentiate into antibody-secreting cells?

To activate B-cells, cultures with adherent cells from PBMC1 were combined with antigen (TT), tetagam, irradiated EL-4-B5 and cytokines (IL-21 and BAFF). EL-4-B5 and adherent cells alone were used for control samples. The cells were incubated for 1 week and then FACS was used to analyse and sort plasmablast populations. ELISpot was used to detect IgG and anti-TT secreting cells.

Microscopy of the cells after 1 week incubation indicate that the culture was successful in activating B-cells (fig. 6.6). Cells in culture are increased in number and size compared to adherent and EL-4-B5 cells alone.

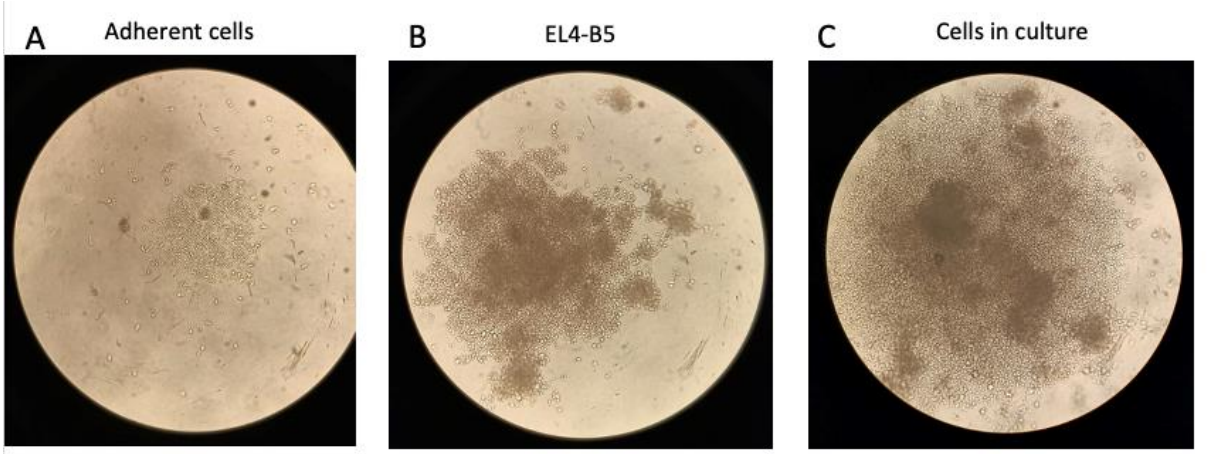


Figure 6.6: **Isolated B-cells cultured with irradiated EL-4-B5 cells increased in cell number compared to B-cells alone.**
Adherent cells from donor 1 were cultured 1 week with EL-4-B5, TT, tetagam and cytokines (IL-21 and BAFF). Adherent cells and EL-4-B5 cells were used for control samples. Photos from microscopy. Sample C did originally contain the same number of cells as A and B combined.

FACS was performed on cells from culture to analyse and sort cell populations. Anti-CD19 was used for detecting B-cells and anti-IgM to exclude naïve cells. Anti-CD27 and anti-CD38 were used to detect plasmablasts. PBMC was used as control sample. From flow cytometry it was hard to define IgM- and CD38- populations (fig. 6.7). There were more CD27+ cells in the culture than the control sample (PBMC), but a population of CD27++/CD38++ cells was not easy to distinguish (fig. 6.7C). Four populations were sorted by FACS (see figure 6.8).

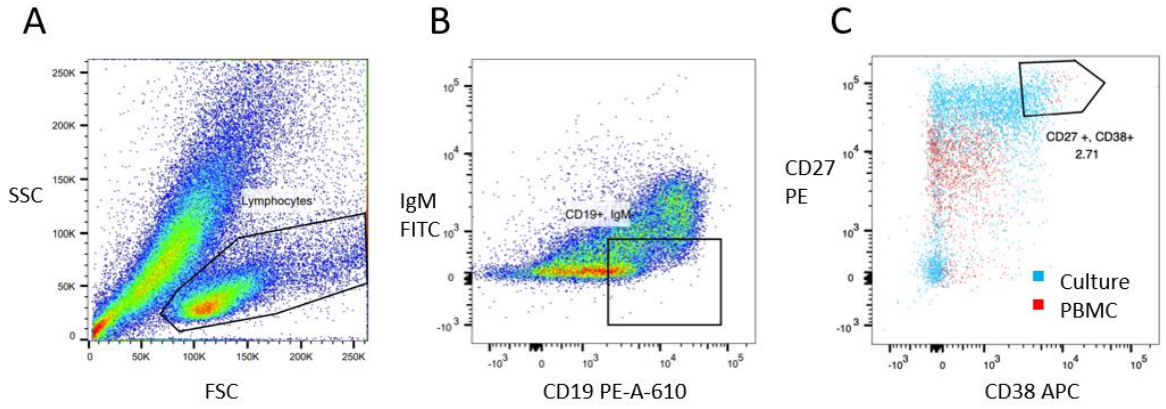


Figure 6.7: **There were more CD27+ cells in culture compared to control.**
The culture and control PBMC were added PE-A-F610-conjugated anti-CD19, FITC-conjugated anti-IgM, PE-conjugated anti-CD27 and APC-conjugated anti-CD38 and analysed with flow cytometry.
A: lymphocyte population in culture sample. B: CD19+ and IgM- (memory cell) population in culture. C: CD27+ and CD38+ cells in culture compared to control (PBMC).

ELISpot was performed to detect antibody secreting cells. Row 1-3 were coated with IgG to detect cells secreting polyclonal IgG antibodies. Row 4-6 were coated with TT to detect cells secreting TT-specific antibodies. The positive control contained a PBMC sample known to produce IgG (not anti-TT) and the negative control contained EL-4-B5. Column a and b had more than 200 cells per well secreting IgG (fig. 6.8). The count decreased from a to d as expected, because there are fewer plasmablasts in the later sorts. There were only a few cells secreting TT-specific antibodies. The results show that the method managed to activate B-cells to antibody producing plasmablasts, but only a few secret anti-TT.

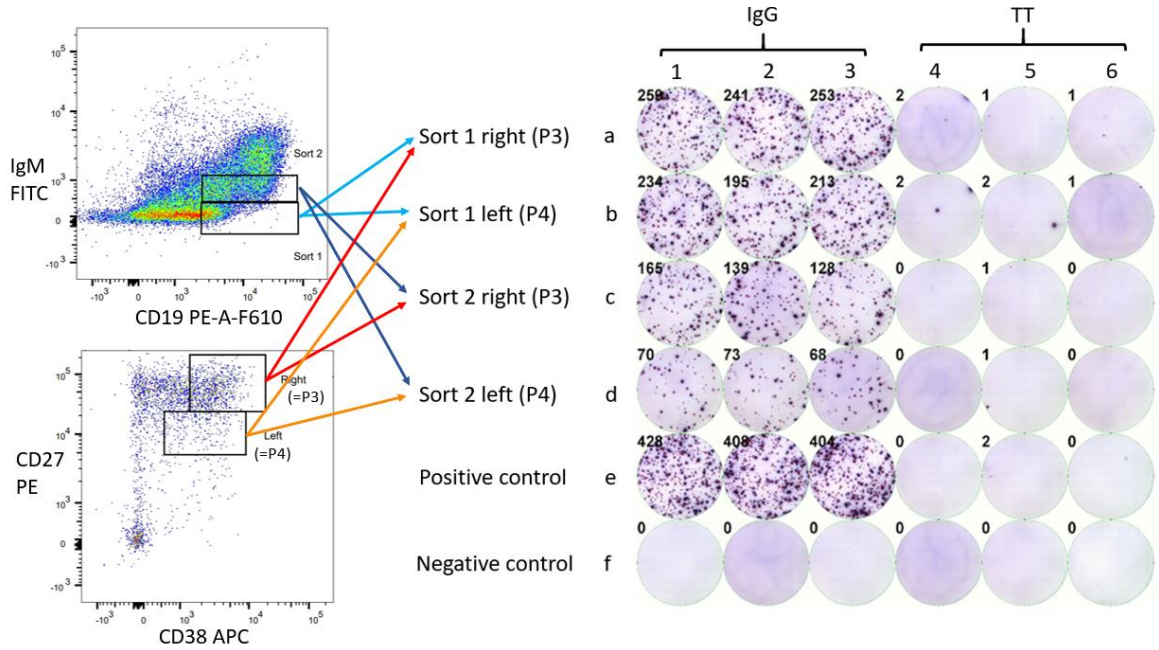


Figure 6.8: **The culture contained antibody-secreting cells.**
Plasmablasts sorted by FACS were added to ELISpot plates coated with IgG or TT. After incubation for 1 day, goat-anti human IgG Fc-ALP and BCIP/MBT substrate were used to detect cells secreting antibodies.
Each dot represents 1 antibody-secreting cell. Image acquisition was used for photographing wells. The number next to the wells are the number of cells counted by Immunospot 3. The figures to the left show the gates used for sorting. Sorted events are identified by consecutive gating of events in the upper and bottom plots. In the figure, only events from the lower "Sort 1" gate are shown in the bottom plot.

5.4.2 Is EL-4-B5 and antigen essential to activate B-cells?

Similar cultures were made to investigate if EL-4-B5 and antigen were essential for activating B-cells. The cultures consisted of isolated B-cells from PBMC2 or PBMC3 with either TT only, TT + tetagam or no antigen, and with or without EL-4-B5. All wells were added cytokines (IL-21 and BAFF) and left for 8 days in incubator. The cultures were analysed as in the previous experiment, supplemented with ELISA to detect anti-TT in the supernatant from culture. The microscopy showed increased number of cells in cultures with EL-4-B5 (fig. 6.9 for donor 3). There were no clear differences between wells with TT, TT + tetagam and no antigen. Cultures of cells from donor 2 showed a similar pattern (not shown).

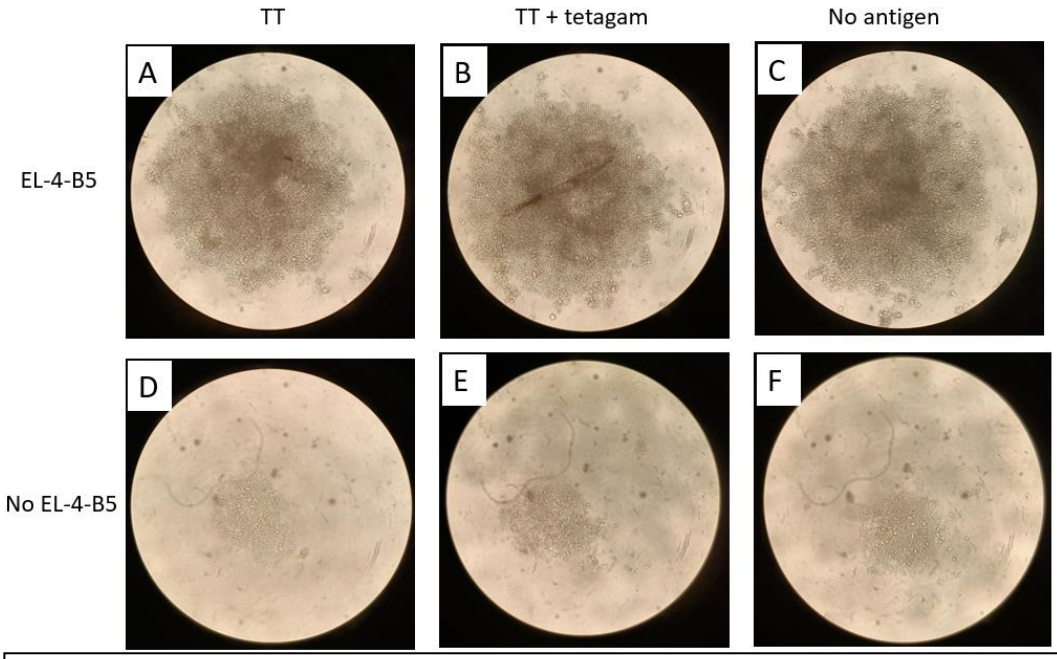


Figure 6.9: **Isolated B-cells in culture with irradiated EL-4-B5 had a higher number of cells compared to cultures without EL-4-B5.**
Adherent cells were cultured 8 days with cytokines (IL-21 and BAFF) and/or irradiated EL-4-B5 and/or TT and/or tetagam. Photos from microscopy of samples from donor 3.

FACS was performed as in the previous experiment to analyse the culture and sort plasmablasts. Samples from donor 3 had the greatest number of cells and will be discussed in detail. Results from donor 2 are found in the appendix. Plasmablasts were expected to be found in the CD27++/CD38++ population, and this population was sorted (P3). A population of CD27+/CD38- was sorted for comparison (P4).

The results show differences in the CD27++/CD38++ populations (fig. 6.10). Samples containing EL-4-B5 and EL-4-B5 + TT seemed to have greater populations than samples without EL-4-B5, indicating that EL-4-B5 is important for activation of B-cells. Note that the samples contain different numbers of cells and cannot be compared directly. The results are analysed later in combination with results from ELISpot (fig. 6.12).

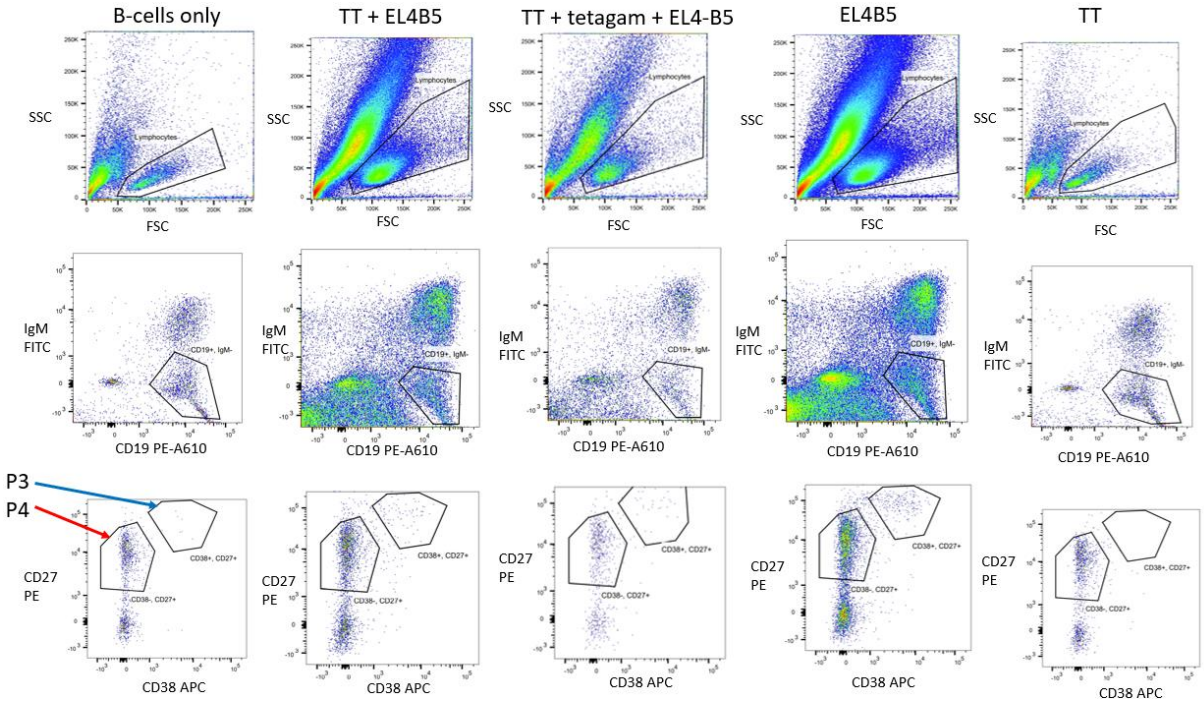
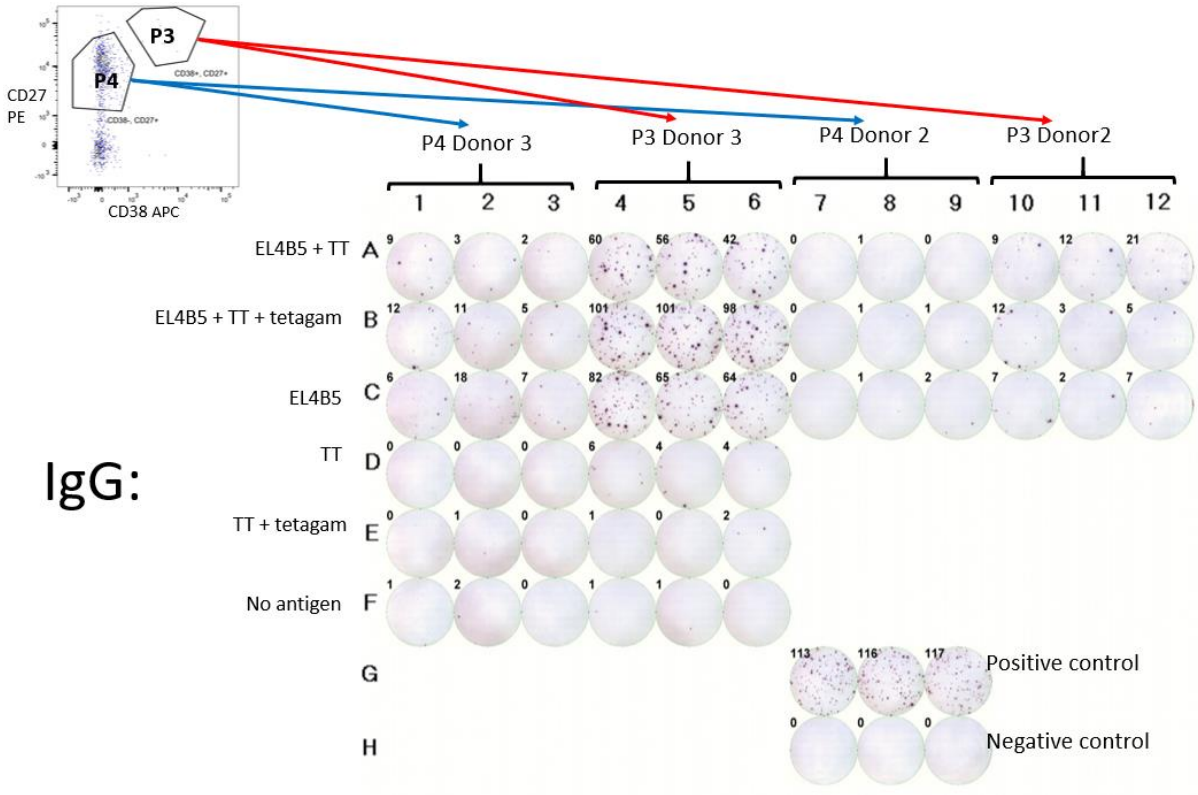


Figure 6.10: **There were more CD27++/CD38++ cells in samples with EL-4-B5.**
Cultures of isolated B-cells from donor 2 and 3 were added PE-A-F610 conjugated anti-CD19, FITC-conjugated anti-IgM, PE-conjugated anti-CD27 and APC-conjugated anti-CD38 and analysed with flow cytometry. The figure shows results of adherent cells from donor 3.
The gates indicate CD27+/CD38- population (left) and CD27++/CD38++ population (right). Arrows in figure A indicate the P3 (CD27++/CD38++) and P4 (CD27+/CD38-) populations sorted with FACS.

ELISpot was performed with P3 (CD27++/CD38++) and P4 (CD27+/CD38-) populations. For donor 2 the samples without EL-4-B5 were not sorted because of low number of cells. Samples were added to plates coated with IgG or TT to differentiate cells secreting antibodies specific to TT and polyclonal IgG antibodies.

The results show many plasmablasts producing anti-IgG, but few producing anti-TT (fig. 6.11). Twice as many wells contained cells from donor 3, therefore the cell count is higher than for donor 2. The wells were added different numbers of cells so the cell count must be analysed further, but it provides an indication of in which samples B-cell were activated. Wells containing EL-4-B5 had more IgG secreting cells compared to wells without EL-4-B5. The highest numbers were for samples containing TT + tetagam, indicating that this was the best combination for B-cell activation. There were more cells in P3 than P4 indicating that the FACS effectively sorted the plasmablast populations based on CD27 and CD38. There are a few artifacts in the plate coated with TT, and the cell count cannot be trusted. There are few anti-TT plasmablasts detected and no conclusions can be made.

The results suggest that B-cells were activated to plasmablasts secreting unspecific IgG antibodies. EL-4-B5 is essential and EL-4-B5 in combination with TT + tetagam seems the most beneficial for activating B-cells.



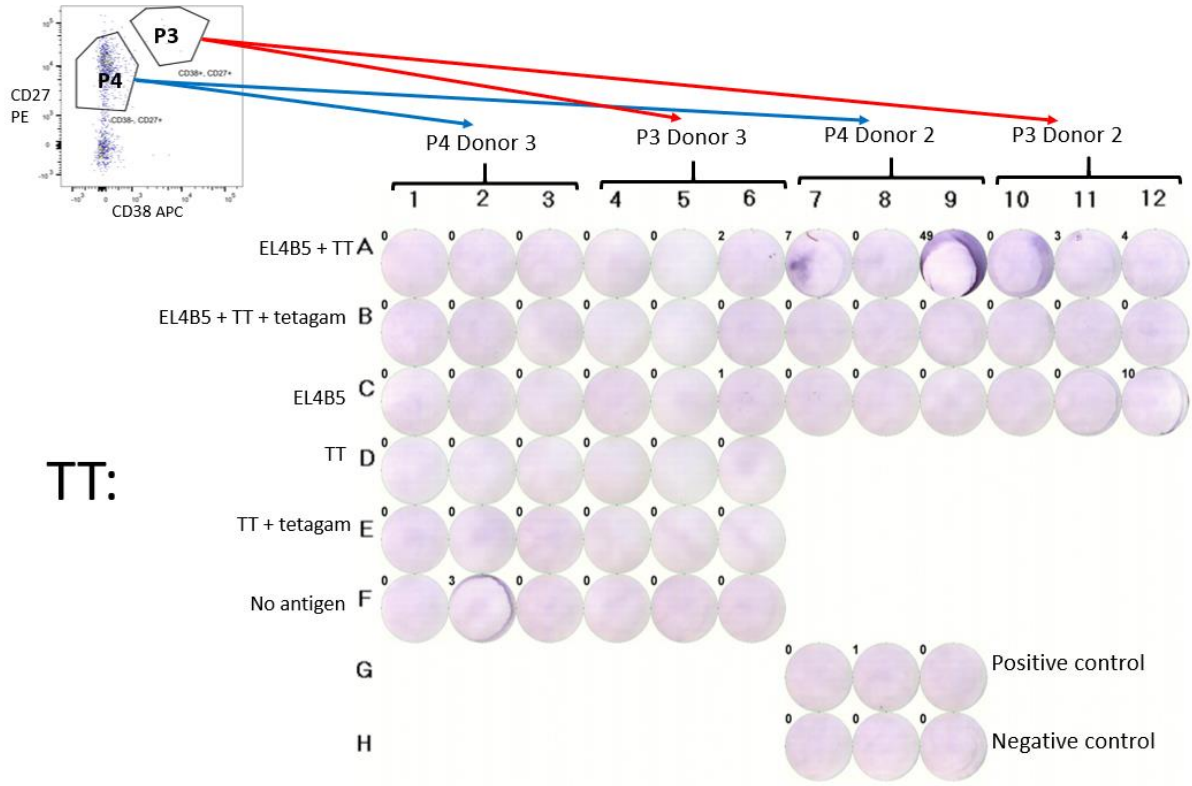


Figure 6.11: **EL-4-B5 is essential for activating B-cells to IgG secreting cells, TT + tetagam seems the most beneficial combination of antigens. Very few cells secreting anti-TT was detectable.**
Cultures of P3 and P4 from donor 2 and 3 were added to ELISpot plate coated with IgG or TT. After 1 day incubation, goat-anti-human IgG Fc-ALP and substate was used to detect IgG or anti-TT producing cells.
Figures at the top indicate gates used for sorting P3 and P4.
Each dot represents 1 antibody-secreting cell. The numbers next to the wells are the number of cells counted by Immunospot 3. Image acquisition was used for photographing wells.

Comparing the number of cells in P4 with P3 ensures a relative and more reliable cell count. The P3/P4 ratio shows that there are in fact most plasmablasts in samples with EL-4-B5 (fig. 6.12). TT + tetagam has slightly more plasmablasts than TT and EL-4-B5 only (0,66 vs. 0,54/0,48). All samples without EL-4-B5 have lower ratios.

From FACS the exact cell count in each sample is known, and therefore how many cells were added to ELISpot. These numbers can be used to find how many of the CD27++/CD38++ cells (plasmablasts) were detected as antibody-secreting cells. Approximately 15% of sorted plasmablasts in P3 with EL-4-B5, TT + tetagam were secreting IgG (fig. 6.12). The other samples with EL-4-B5 were also high compared to samples without EL-4-B5. Samples with

only TT had the highest percentage of plasmablasts secreting IgG, possibly an effect due to low count. Similar measurements for plasmablasts secreting anti-TT cannot be done because of few cells. The results support previous findings that EL-4-B5 is essential for activating B-cells, and that antigen increases the activation, especially when crosslinked. Results for donor 2 shows similar results, however % of IgG secreting cells for EL-4-B5 and TT + tetagam are lower than those of EL-4-B5 alone and EL-4-B5 + TT (shown in the appendix).

Sample	**Cells sorted in P3**	**Cells sorted in P4**	**Ratio of cells P3/P4**	**% of cells sorted in P3 secreting IgG**
EL-4-B5 + TT	1204	2194	0,54	13,1
EL-4-B5 + TT + tetagam	2020	3042	0,66	14,9
EL-4-B5	1696	3565	0,48	12,4
TT	53	922	0,06	26,4
TT + tetagam	67	1214	0,06	4,5
B-cells only	41	774	0,05	4,9

Figure 6.12: **EL-4-B5 in combination with TT and tetagam is most efficient for activating B-cells.**
CD27++/CD38++ (P3) and CD27+/CD38- (P4) populations were sorted with FACS. Both samples were analysed with ELISpot for detection of antibody secreting cells. Numbers are derived from FACS and ELISpot. The figure shows results of B-cells from donor 3.

5.4.3 Are these results confirmed by differences in antibody-production?

To confirm the results from the ELISpot, an ELISA was performed to detect anti-TT in the supernatant from the cultures. The mean blanc value (0,087) was subtracted from each value and 0,01 was used as cut off for positive results. The results for samples from donor 3 are compiled in figure 6.13. The result for donor 2 and the untreated data can be found in the appendix. Differences in absorbance indicate that EL-4-B5 induced B-cell activation. For wells containing EL-4-B5; 8/16 wells containing TT and 9/16 wells containing no antigens, were positive for anti-TT. For wells without EL-4-B5 all samples were negative.

All wells containing TT + tetagam were positive indicating that there is some background absorbance due to reaction with tetagam. The values were divided in “low OD” and “high OD” in attempt to divide samples containing anti-TT and samples with background absorbance only. 0,150 was used as cut-off between low and high OD. The cut-off was chosen because it seemed to fit; all values in “high OD” were more than 0,013 higher than the values in “low OD” and the variance in the low group was 0,023. The variance in the “high OD” was grater indicating absorbance + anti-TT. 10/16 wells containing EL-4-B5 were in the “high OD” group versus 1/16 of wells without EL-4-B5. The results support that EL-4-B5 is most important for activating B-cells. The results show no significant difference between samples containing TT + tetagam, TT only and no antigen.

Donor 3		TT				TT + tetagam				No antigen			
		1	2	3	4	5	6	7	8	9	10	11	12
EL-4-B5	A	+	+	+	+	High	High	High	High	+	+	+	+
	B			+		Low	High	Low	Low		+		+
	C	+	+	+	+	High	High	High	High	+	+	+	+
	D					High	Low	Low	Low				
No EL-4-B5	E					Low	Low	Low	Low				
	F					Low	Low	Low	Low				
	G					Low	High	Low	Low				
	H					Low	Low	Low	Low				

Figure 6.13: **ELISA confirms that EL-4-B5 is important for activating B-cells to produce anti-TT.**
Supernatant from cultures were added to plates coated with TT. Goat anti-human IgG Fc conjugated to ALP and pNPP were used to detect anti-TT. The absorbance for each value was found with a spectrophotometer at 405 nm. The mean blanc value was subtracted from all values. Figure shows results from donor 3.
Positive values are defined as ≥0,01 (indicated “+”) and negative values < 0,01. For samples with tetagam, values ≥ 0,150 are defined as high OD (marked “High”) and values < 0,150 are defined low OD (marked “Low”).

6 Discussion

The goal for our research was to activate B-cells in vitro to develop a method for further research on humoral immune responses. Memory B-cell responses against the antigen TT was measured. TT is well suited for testing methods since most people have been vaccinated against TT, and blood samples can be received from anonymous donors. No patient information was required for the research.

To activate and stimulate memory cells, B-cells were combined with T-cells expressing CD40L (EL-4-B5 cells) and cytokines (IL-21 and BAFF) or CpG. A disadvantage with the method is that all B-cells receive the same stimuli. In vivo, antigen-specific T-cells will search for B-cells specific to the same antigen, and only this cell will receive stimulatory signals and differentiate. This is harder to mimic in an in vitro experiment.

6.1 EL-4-B5 cells were essential for B-cell activation in our cultures

The results show that EL-4-B5 cells are important for B-cell activation. The flow cytometry from cultures with EL-4-B5 shows bigger populations of plasmablasts, and ELISpot confirms a higher number of antibody-secreting cells. ELISA also confirms that anti-TT is more often detected in wells containing EL-4-B5. EL-4-B5 are mutated T-cells, and it has been shown that CD40L in the EL-4-B5 is responsible for activating B-cells (15). Flow cytometry confirmed that the EL-4-B5 cells used for our experiments expressed CD40L. CD40L activate CD40 on B-cells and, along with cytokines, enhances B-cell survival and differentiation to plasma and memory cells (3). Other studies support our finding that EL-4-B5/CD40 is essential for activating B-cells (5, 7, 8, 15, 16). It is found that continuous CD40 activation can inhibit B-cells from differentiating into plasmablasts (5). Our EL-4-B5 cells continuously display CD40L, but still induced B-cells. A few B-cells were activated without the CD40L signal as shown in the results. This is supported by the fact that people with T-cell deficiencies are still able to produce certain antibodies (3).

6.2 Antigen enhanced general B-cell activation

In vivo, antigens are essential for activating B-cells (1). Antigens stimulate B-cells through B-cell receptor and stimulate T-cells to express CD40L. Only B-cells that bind to antigen and CD40L will be activated (1). In our experiments, all B-cells were stimulated by CD40L (and cytokines), resulting in B-cell activation regardless of antigen-specificity. Therefore, we examined antigens stimulating effect and if crosslinking the antigen enhanced B-cell activation.

The experiments show that cultures containing antigen in addition to EL-4-B5 are more likely to activate B-cells. Tetagam was added to crosslink the TT to increase its function (6). According to the results, TT + tetagam are slightly more likely to stimulate B-cells than TT alone. Other studies support our findings that antigen enhances activation of B-cells (6, 8, 11). It is suggested that antigen primarily lowers the threshold for activation of specific B-cells (6). This is supported by our findings since antigen is not enough to activate B-cells alone, but induces activation in combination with CD40L and cytokines.

The results show a general activation of B-cells, and multiple memory B-cells are stimulated. Antigen is important for activation of antigen-specific memory cells and production of antigen-specific antibodies (6, 8). In vivo, TT would have induced TT-specific memory B-cells to become anti-TT producing plasmablasts. TT-specific memory B-cells are only a small percentage of all memory cells, and because of the general activation, other memory cells were also activated. It was expected an increase in TT-specific plasmablasts in comparison to other memory cells, but this increase was most likely too small to be measured. In the culture of B-cells from donor 1, there are some anti-TT secreting cells supporting that some anti-TT-specific memory cells have been activated, possibly induced by TT. For this experiment all samples contained antigen, so we cannot distinguish if antigen was necessary to get anti-TT secretion or not. In the culture with B-cells from donor 2 and 3, almost no anti-TT secreting cells are detectable, and no conclusion could be made.

The ELISA detects anti-TT from supernatant, indicating that there are some anti-TT secreting cells present. The results show increased anti-TT production in cultures with EL-4-B5, indicating that EL-4-B5 stimulated anti-TT specific memory B-cells. Cultures with TT, TT + tetagam and no antigen show the same number of positive samples. The conclusion is that

antigen seem to induce EL-4-B5 stimulated activation of B-cells, but we cannot decide its function in antigen-specific activation.

Nonoyama et al. managed to stimulate B-cells to produce antibody-specific antibodies in culture with antigens, direct B-T-cell contact via CD40 and cytokines (IL-10)(8). Possible reasons for the results in our study are that there were few TT-memory cells, or anti-TT plasmablasts could simply not be detected. Donor 2 and especially donor 3, had lower levels of anti-TT in serum, and therefore perhaps fewer TT-specific memory cells. This could explain why anti-TT secreting cells could not be found in these samples. This is supported by the fact that donor 1 had more anti-TT secreting cells and a higher level of anti-TT in serum. Since anti-TT was detected with ELISA, this show that there are some anti-TT secreting cells present in the cultures with EL-4-B5. This indicates that we did not manage to detect these cells with ELISpot, perhaps because there were too few or because ELISA had a higher sensitivity.

6.3 Activated B-cells were not detected from the first culture

The method used in the first culture failed to measure antibody production in the supernatant, indicating that B-cells were not activated. Our suggestion was that there were too few B-cells to be activated because of the results of the control samples. EBV is well known to stimulate activation and expansion of B-cells in vitro (17). Alternatively, if all control samples were negative, this could indicate that something went wrong before or during incubation. If all samples with EBV were positive, it would indicate that stimulation in the other samples were not enough to activate B-cells. Our results show that only one sample of EBV was positive, therefore we suggested that there were too few B-cells to be activated in the other samples. There could also be something else wrong with the B-cells from donor 1 which was investigate further.

Another possible explanation is that the positive result was random, and that B-cells were not stimulated. We had already proven that the EL-4-B5 expressed CD40L necessary to stimulate B-cells. CpG is known to stimulate activation of B-cells in vitro (5, 10-12). CpG contains DNA strands and stimulate cells by enhancing production and secretion of cytokines (12). Marasco et al. found that CD40L in combination with CpG inhibits CpG induced plasmablast

differentiation and antibody secretion (5). In the further experiments, cytokines such as IL-21 and BAFF was used instead of CpG, and this alteration could be why we managed to activate B-cells.

Only anti-TT-specific antibodies were measured in the first culture, so other antibodies could have been present. In later experiments, ELISpot shows plasmablasts secreting antibodies other than anti-TT. Unspecific memory B-cells could also be activated in the first culture. However, in the later experiments ELISA was successful in detecting anti-TT from cultures, indicating that the later cultures had a better combination of stimulating agents for activation of B-cells.

6.4 Inter-donor variability

The preparations revealed different levels of anti-TT in serum between donors. All donors had levels above the protective level of 0,01 IU/mL (4). For donor 1, ELISA measured a high level (13,8 IU/mL), but after looking at the experiment, we predict the actual antibody concentration was lower because of the dilution already made when isolating plasma (from buffy coat). Our estimate is therefor that the level of anti-TT for donor 1 is ≈ 9,2 IU/mL. When isolating PBMC for donor 2 and 3 this was accounted for. The value is still higher than for donor 2 (0,73 IU/mL) and 3 (0,025 IU/mL). Farzad et al. has measured levels of 0,06 – 1,0 IU/mL in a population before a booster vaccine and 16-32 IU/mL 14 days after (18). Compared to our results, this indicate that donor 1 has recently been immunized with TT through vaccination. For donor 2 and 3 the immunization happened longer ago. Even though the level for donor 3 is higher than 0,01 IU/mL, there could be fewer TT-specific memory cells, explaining why ELISA could not detect significant numbers of cells secreting anti-TT specific antibodies.

The results also showed various levels of anti-Ig binding cells in PBMC between donors. IgG was most important for our research since memory cells are essential for producing plasmablasts. Donor 3 had the highest level of IgG+ B-cells. 13,4% of immunoglobulin binding cells were IgG+, more than twice the percentage for donor 2 and more than 5 times for donor 1 (fig. 10.8). The results indicate that PBMC3 had the larger portion of memory

cells of the 3, and therefore perhaps most suitable for experiments on antigen-specific humoral immune responses.

Donor 1 had few IgA and IgG binding cells and a high number of IgM binding cells compared to the other two. This pattern is found in an inherited immune deficiency disorder called hyper-IgM syndrome (1, 19). The cause is a defect in CD40L leading to impairment in the isotype shift from IgM to the other groups of immunoglobulins, resulting in high or normal levels of IgM and low or absent levels of IgA, IgG and IgD. The varying levels of Ig+ cells found in hyper IgM syndrome could help explain some of our results. The low level of IgG+ cells could help explain why we had difficulties activating B-cells in the first culture. In the next culture plasmablasts were detected, but the flow cytometry showed abnormal results. Comparing flow cytometry from the first and second FACS, more defined populations can be seen of both IgM+ and IgM- cells, and CD27++/CD38++ cells in samples from donor 2 and 3 (see figures 6.7 and 6.10). For donor 1 there is one big population of cells with different amounts of IgM, and IgM+ and – populations cannot be distinguished. A CD27+ population can be found, but CD38+ and - populations are blended. The high level of IgM and low level of IgG and IgA could help explain these results for donor 1.

The conclusion is that our research suggests that donor 1 had the best basis for analysing TT-specific immune responses, but few memory cells. Donor 3 had many memory cells to examine humoral responses, but possibly few TT-specific. In further experiments we suggest choosing a recently immunized (high anti-TT in serum) donor with high or normal levels of IgG+ cells.

6.5 Other methods for activating B-cells

We managed to activate isolated B-cells in combination with EL-4-B5, IL-21 and BAFF, but there could be other cells and cytokines to consider in developing a method. Some sources indicate that B-cells in culture with other non-B-cells are more likely to differentiate to plasmablasts (5). We found that there were not enough memory B-cells in PBMC, but when a method is in place this could be investigated further. Cytokines such as IL-21 (5), IL-10 (8), IL-2 (7) are previously used to stimulate B-cells, and different cytokines and stimulating

agents could be compared. CpG in addition to these cytokines should also be considered, although one should be aware of the CD40L inhibiting effect explained earlier (5).

Analysing methods should also be considered. Flow cytometry was suitable for detecting CD40L in EL-4-B5 cells. ELISA has been found to be sensitive for detecting anti-TT in serum (18), and we predict that the divergent levels that were measured are due to actual differences in anti-TT. Flow cytometry confirmed that the MACS did enrich B-cell population using anti-CD22 antibodies.

The flow cytometry and FACS showed well defined populations of plasmablasts in samples from donor 2 and 3. Plasmablasts were defined as B-cells (CD19+ lymphocytes), IgM- and CD27++ and CD38++. We confirmed that B-cells were stimulated to differentiate to plasmablasts, and identified defined plasmablast populations from culture. ELISpot was later used to confirm antibody-secreting cells in the sorted samples. We conclude that CD38 and CD27 are suited for distinguishing plasmablasts, also shown in other studies (5). It is possible to estimate how many of the CD27++/CD38++ cells sorted by FACS were antibody secreting cells, and this technology will be important for further research. Comparing number of cells secreting antigen-specific antibodies and antigen-unspecific antibodies will be of interest. For our study, a low percentage of antibody-secreting cells from sorted plasmablasts were found. The highest ratio of plasmablasts secreting IgG is 14,9% in culture with isolated B-cells from donor 3, EL-4-B5 and TT + tetagam (see table 6.1). Some cells are expected to be lost in the process of ELISpot and some produce other immunoglobulins than IgG. The low percentage could however indicate that cells do not survive or that ELISpot is not able to detect all plasmablasts. ELISpot could therefore be less suited for confirming B-cell activation. ELISpot in combination with ELISA analyses both cells and supernatant from culture, and we predict that these methods combined give a good estimate.

6.6 Further research

The purpose of our research was to look at antigen-specific humoral immune responses associated with FNAIT. We have shown that B-cell activation is possible in culture with CD40L, antigen and cytokines. CD40L is most important for B-cell activation and combination with antigen enhances the stimulation of B-cells. Most of the activated B-cells

were producing IgG antibodies but not anti-TT. In order to look at the antigen-specific immune responses, we suggest isolating antigen-specific B-cells. We predict that our method was efficient but had too few antigen-specific B-cells to prove antigen-specific activation. If performed on a population of TT-specific memory B-cells, we would be able to detect more anti-TT secreting cells.

B-cells are only one part of the humoral immune responses. Once a method for activating B-cells is in place, the next step should be to look at T-cell activation. A method for making T-cells from PBMC express CD40L would be of interest. Our research on B-cells will then form an important basis for further studies, and TT could be used to look at antigen-specific responses. In our experiments the EL-4-B5 cells were constantly expressing CD40L, precluding linked recognition. By introducing the same antigen to both B- and T-cells, activation could be easier. A challenge will be to activate antigen-specific memory T-cells in vitro.

The purpose for developing methods for activating B- and T-cells, is to look at the humoral immune responses behind FNAIT. In FNAIT, B- and T-cells in the mother's circulation is activated and stimulated to produce antibodies against HPA-1a. The immunological responses behind FNAIT are more complex than those of TT, but when the methods are in place, activating B- and T-cells in blood from HPA-1a positive donors could provide essential knowledge of the maternal immune response that causes FNAIT. Hopefully we will someday be able to understand the condition fully and make changes to save lives.

7 Conclusion

The purpose of this study was to look at antigen-specific humoral immune responses associated with FNAIT. We specifically looked at the B-cell, and the goal was to find a method for activating antigen-specific B-cells in vitro and to identify and isolate activated B-cells secreting antibodies. Our research showed that we managed to stimulate isolated B-cells in culture with cytokines, antigen and CD40L from EL-4-B5 to differentiate to antibody-secreting plasmablasts. Plasmablasts were identified as CD19+, CD27+, CD38+ cells and live cells were isolated by FACS to confirm antibody-secretion. CD40L was most important in the activation, and antigen had a synergic effect. However, the antibodies produced were mostly unspecific IgG, indicating an antigen-unspecific activation of B-cells. Further research is needed to find a method for activation of antigen-specific B-cells. We suggest isolating antigen-specific memory B-cells, and then integrating the method explained in this paper. Tetanus toxoid can be used as antigen for further development of methods, but donors should be chosen wisely.

8 References

1. Parham P, Janeway CA. The immune system. 3rd ed. ed. London: Garland Science; 2009.
2. Zdravic D, Yougbare I, Vadasz B, Li C, Marshall AH, Chen P, et al. Fetal and neonatal alloimmune thrombocytopenia. Semin Fetal Neonatal Med. 2015;21(1):19-27.
3. Murphy K, Weaver C. Janeway's immunobiology: Garland science; 2016.
4. WHO. The immunological basis for immunization series: module 3: tetanus: update 2018. 2018.
5. Marasco E, Farroni C, Cascioli S, Marcellini V, Scarsella M, Giorda E, et al. B‐cell activation with CD40L or CpG measures the function of B‐cell subsets and identifies specific defects in immunodeficient patients. Eur J Immunol. 2017;47(1):131-43.
6. Ingvarsson S, Lagerkvist AC, Mårtensson C, Granberg U, Ifversen P, Borrebaeck CA, et al. Antigen-specific activation of B cells in vitro after recruitment of T cell help with superantigen. Immunotechnology. 1995;1(1):29-39.
7. Grabstein KH, Maliszewski CR, Shanebeck K, Sato TA, Spriggs MK, Fanslow WC, et al. The regulation of T cell-dependent antibody formation in vitro by CD40 ligand and IL-2. J Immunol. 1993;150(8):3141-7.
8. Nonoyama S, Hollenbaugh D, Aruffo A, Ledbetter JA, Ochs HD. B cell activation via CD40 is required for specific antibody production by antigen-stimulated human B cells. J Exp Med. 1993;178(3):1097-102.
9. Spolski R, Leonard WJ. Interleukin-21: a double-edged sword with therapeutic potential. Nat Rev Drug Discov. 2014;13(5):379-95.
10. Eastcott JW, Holmberg CJ, Dewhirst FE, Esch TR, Smith DJ, Taubman MA. Oligonucleotide containing CpG motifs enhances immune response to mucosally or systemically administered tetanus toxoid. Vaccine. 2001;19(13):1636-42.
11. Bishop GA, Yi A-K, Teasdale R, Waldschmidt TJ, Koretzky GA, Krieg AM, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature. 1995;374(6522):546-9.
12. Krieg AM. Lymphocyte activation by CpG dinucleotide motifs in prokaryotic DNA. Trends Microbiol. 1996;4(2):73-7.

13. Pourahmad J, Salimi A. Isolated Human Peripheral Blood Mononuclear Cell (PBMC), a Cost Effective Tool for Predicting Immunosuppressive Effects of Drugs and Xenobiotics. Iran J Pharm Res. 2015;14(4):979-.
14. Zubler RH, Erard F, Lees RK, Van Laer M, Mingari C, Moretta L, et al. Mutant EL-4 thymoma cells polyclonally activate murine and human B cells via direct cell interaction. J Immunol. 1985;134(6):3662.
15. Kwekkeboom J, De Boer M, Tager JM, De Groot C. CD40 plays an essential role in the activation of human B cells by murine EL4B5 cells. Immunology. 1993;79(3):439-44.
16. Werner-Favre C, Gauchat JF, Mazzei G, Aubry JP, Bonnefoy JY, Zubler RH. Similar CD40 ligand expression on EL-4 thymoma cell lines with widely different helper activity for B lymphocytes. Immunology. 1994;81(1):111-4.
17. Maurer M, Müller AC, Parapatics K, Pickl WF, Wagner C, Rudashevskaya EL, et al. Comprehensive Comparative and Semiquantitative Proteome of a Very Low Number of Native and Matched Epstein–Barr-Virus-Transformed B Lymphocytes Infiltrating Human Melanoma. J Proteome Res. 2014;13(6):2830-45.
18. Farzad Z, James K, McClelland DBL. Measurement of human and mouse anti-tetanus antibodies and isotype analysis by ELISA. J Immunol Methods. 1986;87(1):119-25.
19. Qamar N, Qamar N, Fuleihan RL, Fuleihan RL. The Hyper IgM Syndromes. Clin Rev Allergy Immunol. 2014;46(2):120-30.

9 Appendix

List of materials:

	Material	Use	Information	Origin
Regular lab resources	Cell culture medium	Cell culture	90% IMDM (iscove's modified Dulbecco's medium) + 10% FBS	Gibco
	Coating buffer	ELISA, ELISpot	Carbonate/bicarbonate buffer (0,80g Na_2CO_3 1,465g Na_2HCO_3) Molarity: 20mM, pH: 9,2	Immunological research group at UiT
	Freezing medium	Freezing cells	90% FBS (fetal bovine serum) + 10% DMSO (dimethyl sulfoxide)	WAK – chernie Medical GmbH
	PBS (phosphate buffered saline)	Buffer	0,14M NaCl, 2,7mM KCl, 10mM phosphate buffer, pH 7,2	Medicago AB (09-9499-100)
	PBS + 10% FBS	Blocking buffer ELISpot	90% PBS + 10 % FBS	Immunological research group at UiT
	PBSA 0,2%		98,8% PBS + 0,2% BSA (bovine serum albumin)	Immunological research group at UiT
	PBSA 1%	Blocking buffer ELISA	99% PBS + 1% BSA	Immunological research group at UiT
	Sterile syringe filter	Sterile filtrating antigens	0,2µm PES (polyethersulfone)	Avantor
	Washing buffer	ELISA, ELIspot	PBS 0,05% tween 20	Sigma -aldrich, Immunological research group at UiT
Analysing equipment	BD FACSAria III	FACS		Beck Dickingson and company
	BD LSRFortessa	Flow cytometry		Beck Dickinson and company
	Centrifuge (Rotina 420 R)	Centrifuging components		Hettich
	Hemocytometer	Estimate cell count	Burker chamber	Karl Hecht
	MACS (Magnetic-activated cell sorting)	Sorting cells		Militenyi biotec
Special resources	BAFF (B-cell activating factor)	Stimulate cells	Ref: PHC1674	Invitrogen
	BCIP/NBT (plus)	Substrate for ELISpot	Alkaline phosphatase (ALP) substrate	MOSS inc
	Buffy coat	Isolate PBMC	From random, anonymous adult donors. Consented for scientific purposes	The blood bank of the University hospital of Northern Norway.
	CpG (ODN 2006)	Stimulate cells	N. 74301513	Integrated DNA technologies
	EBV (Epstein Barr virus)	Positive control in culture	Human gamma herpesvirus 4	ATCC
	EL-4-B5	Display CD40L	Mutated murine thymoma cells	Cell line based on R. H. Zubler's research (14)
	Ethanol 35%	ELISpot	Wetting the membranes	From UiT stock

	IL-21 (Interleukin 21)	Stimulate cells	Ref: PHC0215	Gibco
	Lymphoprep	Isolating PBMC	Density gradient: 1.077g/mL.	Serumwerk
	pNPP (p-nitrophenyl phosphate)	ELISA	Substrate for ELISA	Pierce biotechnology
	Tetagam P	Crosslinking TT, Standard curve in ELISA	Antibody against TT. Desired concentration: 2,5 IU/mL	Anti-TT from the university hospital of Northern Norway
	TT (tetanus toxoid)	Antigen	Concentration: differs. See method/results	Sarstedt
Antibodies	CD8 FITC (human CD8 fluorescein isothiocyanate conjugated)	Compensating sample in flowcytometry	Ref: MHCD0801	Invitrogen
	CD19 APC (Mouse anti-human CD19 allophycocyanin conjugated)	Detecting B-cells in flow cytometry	SJ25C1 Ref: 345791	Beckson, Dickinson and company
	CD19 PE (human CD19 phycoerythrin conjugated)	Detecting B-cells in flowcytometry	Ref: MHCD1904	Invitrogen
	CD19 PE-A-F610 (Human CD19 phycoerythrin alexa fluor 610 conjugated)	Detecting B-cells in flow cytometry	Ref: MHCD1922	Invitrogen
	CD22 (human CD22)	Isolating B-cells by MACS	Mat. No: 120-000-257	Miltenyi biotec
	CD27 PE (human CD27 phycoerythrin conjugated)	Detecting plasmablasts in flowcytometry	Cat: MHCD2704	Invitrogen
	CD38 APC (Mouse anti-human CD38 allophycocyanin conjugated)	Detecting plasmablasts in flowcytometry	Used for 1. FACS Cat: 5555462	Beckson, Dickinson and company pharmingen
	CD38 APC (human CD38 allophycocyanin conjugated))	Detecting plasmablasts in flowcytometry	Used for 2. FACS Ref: MHCD3805	Invitrogen
	CD40L PE (human CD40L phycoerythrin conjugated)	Detecting CD40L in flow cytometry	Cat: 555700	Beckson, Dickinson and company pharmingen
	CD40L PE (mouse CD40L phycoerythrin conjugated)	Detecting CD40L in flowcytometry	Cat: 561719	Beckson, Dickinson and company pharmingen
	Goat anti-human IgG (Fc specific)	Capture antibody for ELISpot	I2136-1ML	Sigma
	Goat anti-human IgG Fc-ALP conjugate	Detection antibody for ELISpot	Ref: A18832	Invitrogen
	IgA FITC (polyclonal rabbit anti-human IgA fluorescein isothiocyanate conjugated)	Detecting IgA+ cells in flowcytometry	Ref: F0057	Dako
	IgG FITC (polyclonal rabbit anti-human IgG fluorescein isothiocyanate conjugated)	Detecting IgG+ cells in flowcytometry	Ref: F0185	Dako
	IgM FITC (polyclonal rabbit, anti-human IgM fluorescein isothiocyanate conjugated)	Detecting IgM+ cells in flow cytometry	Ref: F0058	Dako

Additional results

Table 10.1: Untreated data from ELISA for detecting levels of anti-TT in plasma from donor 1

	1	2	3	4	5	6
A	1,391	1,363	1,382	2,516	2,282	2,139
B	0,868	0,872	0,865	2,133	1,901	1,927
C	0,581	0,58	0,62	1,781	1,673	1,575
D	0,323	0,324	0,324	1,296	1,2	1,185
E	0,2	0,202	0,204	0,889	0,775	0,804
F	0,144	0,141	0,145	0,574	0,537	0,542
G	0,116	0,108	0,116	0,34	0,318	0,332
H	0,09	0,073	0,071	0,071	0,072	0,072

Explanation to table 10.1:

	Standard solution			Plasma		
	1	2	3	4	5	6
A	1:20 000			1:100		
B	1:40 000			1:200		
C	1:80 000			1:400		
D	1:160 000			1:800		
E	1:320 000			1:1600		
F	1:640 000			1:3200		
G	1:1 280 000			1:6400		
H	1: 2 256 000	Neg. control (FBS)		Blanc		

Table 10.2: Untreated data from ELISA for detecting levels of anti-TT in plasma from donors 2 and 3.

	7	8	9	10	11	12
A	1,403	1,403	0,23	0,216	0,087	0,091
B	0,889	0,888	0,131	0,146	0,071	0,083
C	0,551	0,569	0,136	0,127	0,092	0,105
D	0,34	0,342	0,09	0,095	0,092	0,122
E	0,208	0,213	0,095	0,088	0,081	0,111
F	0,148	0,154	0,092	0,082	0,073	0,081
G	0,107	0,106	0,088	0,102	0,073	0,083
H	0,1	0,097	0,08	0,075	0,066	0,081

Explanation to table 10.2:

	Standard solution		Plasma		No antigen	
	7	8	9	10	11	12
A	1:20 000		Plasma donor 2 1:800			
B	1:40 000		Plasma donor 2 1:1600			
C	1:80 000		Plasma donor 2 1:3200			
D	1:160 000		Plasma donor 2 1:6400			
E	1:320 000		Plasma donor 3 1:800			
F	1:640 000		Plasma donor 3 1:1600			
G	1:1 280 000		Plasma donor 3 1:3200			
H	1:2 256 000		Plasma donor 3 1:6400	Blanc		

Tables 10.3, 10.4, 10.5 and 10.6: Untreated data from ELISA of supernatant from culture of PBMC 1

10.3: Day 3 (diluted 1:10)

	1	2	3	4	5	6	7	8	9	10	11	12
A	**0,099**	**0,096**	**0,087**	**0,094**	**0,107**	**0,091**	**0,091**	**0,088**	**0,106**	0,038	0,034	0,035
B	**0,096**	**0,083**	**0,082**	**0,084**	**0,079**	**0,079**	**0,085**	**0,078**	**0,078**	0,033	0,033	0,034
C	**0,105**	**0,099**	**0,099**	**0,092**	**0,106**	**0,1**	**0,101**	**0,091**	**0,115**	0,032	0,034	0,033
D	**0,089**	**0,085**	**0,086**	**0,087**	**0,082**	**0,082**	**0,082**	**0,082**	**0,082**	0,035	0,034	0,033
E	**0,089**	**0,079**	**0,081**	**0,079**	**0,076**	**0,076**	**0,082**	**0,078**	**0,074**	0,033	0,033	0,033
F	0,086	0,087	0,081	0,082	0,082	0,077	**0,085**	**0,082**	**0,08**	0,035	0,037	0,033
G	0,085	0,09	0,085	0,086	0,088	0,091	0,084	0,082	0,084	0,033	0,034	0,035
H	0,089	0,085	0,085	0,086	0,085	0,093	**0,091**	**0,084**	**0,089**	0,035	0,032	0,031

10.4: Day 6 (Diluted 1:10)

	1	2	3	4	5	6	7	8	9	10	11	12
A	**0,102**	**0,091**	**0,088**	**0,089**	**0,102**	**0,098**	**0,091**	**0,096**	**0,113**	0,031	0,032	0,031
B	**0,083**	**0,084**	**0,082**	**0,081**	**0,085**	**0,084**	**0,087**	**0,075**	**0,084**	0,032	0,03	0,03
C	**0,112**	**0,096**	**0,093**	**0,095**	**0,102**	**0,102**	**0,104**	**0,097**	**0,116**	0,032	0,03	0,03
D	**0,079**	**0,08**	**0,076**	**0,08**	**0,084**	**0,095**	**0,081**	**0,075**	**0,083**	0,031	0,033	0,034
E	**0,083**	**0,078**	**0,078**	**0,082**	**0,078**	**0,08**	**0,08**	**0,074**	**0,083**	0,033	0,033	0,032
F	0,09	0,079	0,083	0,083	0,077	0,084	**0,083**	**0,089**	**0,084**	0,033	0,037	0,033
G	0,089	0,086	0,111	0,08	0,081	0,083	0,089	0,08	0,095	0,033	0,033	0,035
H	0,107	0,087	0,089	0,086	0,081	0,082	**0,084**	**0,083**	**0,085**	0,032	0,033	0,031

10.5: Day 9 (diluted 1:10)

	1	2	3	4	5	6	7	8	9	10	11	12
A	**0,099**	**0,098**	**0,091**	**0,112**	**0,102**	**0,106**	**0,109**	**0,1**	**0,101**	0,029	0,033	0,044
B	**0,106**	**0,112**	**0,085**	**0,099**	**0,102**	**0,091**	**0,123**	**0,092**	**0,106**	0,029	0,03	0,028
C	**0,121**	**0,126**	**0,114**	**0,133**	**0,127**	**0,125**	**0,129**	**0,136**	**0,131**	0,029	0,028	0,031
D	**0,109**	**0,106**	**0,104**	**0,1**	**0,12**	**0,11**	**0,112**	**0,118**	**0,123**	0,03	0,029	0,031
E	**0,097**	**0,094**	**0,083**	**0,102**	**0,113**	**0,104**	**0,108**	**0,108**	**0,103**	0,032	0,03	0,029
F	0,098	0,114	0,099	0,108	0,109	0,093	**0,112**	**0,119**	**0,103**	0,032	0,027	0,029
G	0,097	0,109	0,11	0,104	0,105	0,105	0,111	0,108	0,104	0,031	0,033	0,028
H	0,102	0,099	0,089	0,101	0,103	0,095	**0,109**	**0,158**	**0,097**	0,027	0,031	0,032

10.6: Day 9 (undiluted)

	1	2	3	4	5	6	7	8	9	10	11	12
A	**0,097**	**0,098**	**0,101**	**0,102**	**0,158**	**0,145**	**0,098**	**0,097**	**0,093**	**3,255**	**3,282**	**3,884**
B	**0,102**	**0,1**	**0,1**	**0,102**	**0,134**	**0,112**	**0,118**	**0,106**	**0,113**	**3,399**	**3,117**	**3,248**
C	**0,121**	**0,121**	**0,122**	**0,142**	**0,129**	**0,14**	**0,134**	**0,137**	**0,122**	**2,941**	**2,941**	**3,242**
D	**0,121**	**0,098**	**0,105**	**0,123**	**0,11**	**0,117**	**0,101**	**0,123**	**0,126**	**2,255**	**2,264**	**2,292**
E	**0,094**	**0,084**	**0,093**	**0,108**	**0,105**	**0,117**	**0,085**	**0,081**	**0,085**	**1,258**	**1,353**	**1,311**
F	**1,527**	**1,479**	**1,525**	0,085	0,082	0,079	**0,086**	**0,089**	**0,083**	**0,736**	**0,784**	**0,783**
G	0,09	0,107	0,098	0,095	0,081	0,087	0,098	0,083	0,084	**0,435**	**0,463**	**0,452**
H	**0,098**	**0,096**	**0,084**	**0,095**	**0,08**	**0,085**	**0,098**	**0,921**	**0,103**	**0,269**	**0,282**	**0,27**

Explanation for tables 10.3, 10.4, 10.5 and 10.6

	1	2	3	4	5	6	7	8	9	10	11	12
A	1, 2, 3 (1 ng/mL), 5			1, 2, 3 (1 ng/mL), 4			1, 3 (16 ng/mL), 5x2			Standard: 1:20 000*		
B	1, 2, 3 (2 ng/mL), 5			1, 2, 3 (2 ng/mL), 4			1, 3 (16 ng/mL), 4, 5			1:40 000*		
C	1, 2, 3 (4 ng/mL), 5			1, 2, 3 (4 ng/mL), 4			1, 4, 5x2			1:80 000*		
D	1, 2, 3 (8 ng/mL), 5			1, 2, 3 (8 ng/mL), 4			1, 2, 4, 5			1:160 000*		
E	1, 2, 3 (16 ng/mL), 5			1, 2, 3 (16 ng/mL), 4			1, 2, 5x2			1:320 000*		
F	Serum donor 1 1:3200*						1, 5x3			1:640 00*		
G										1:1 280 000*		
H	Blanc*			Blanc*			1, 4, 5, 6			1:2 560 000*		

1: PBMC1, 2: EL-4-B5, 3: TT with concentration, 4: CpG, 5: medium, 6: EBV.

*only in table 10.6 (day 9 undiluted)

Figure 10.7: Flowcytometry of IgA- and IgM-binding cells in PBMC1, PBMC2 and PBMC3

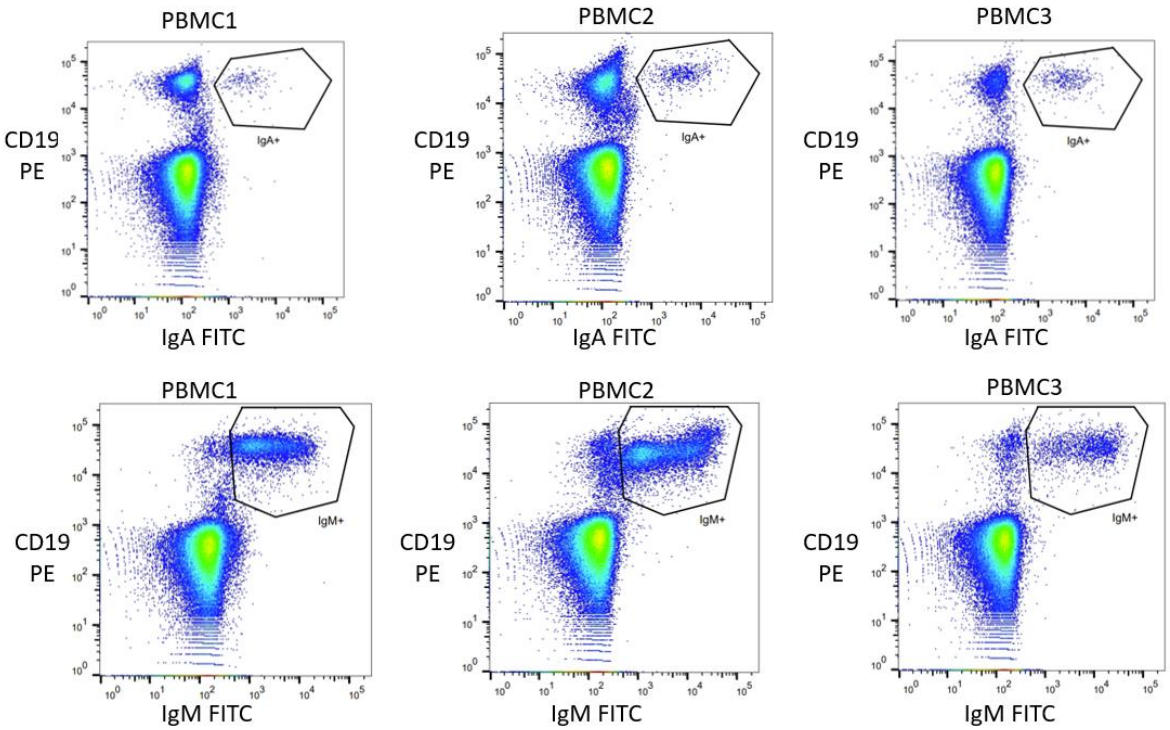


Figure 10.8: Percentages of IgM, IgA and IgG positive B-cells in PBMC1, PBMC2 and PBMC3.

	PBMC1	PBMC2	PBMC3
IgG	2,6 %	6,3 %	13,4 %
IgM	87,7 %	80,3 %	65,4 %
IgA	2,7 %	5,6 %	14,8 %

Comment: FlowJo is used for estimating a count of B-cells (CD19 PE) and IgM, IgA and IgG + populations (FITC). Percentages of B-cells binding IgA, IgM and IgG are calculated.

Figure 10.9: Results from FACS of B-cells from donor 2 in culture with cytokines and different combinations of EL-4-B5, TT and tetagam.

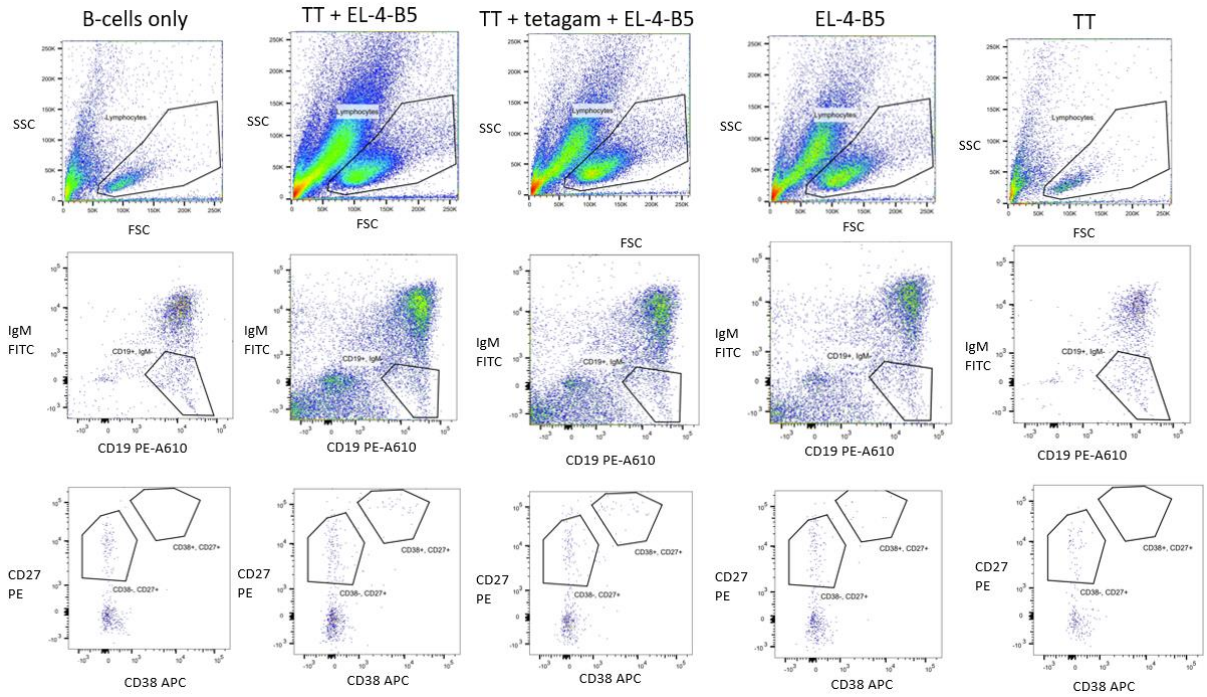


Figure 10.10: Results from ELISpot and FACS for donor 2.

Sample	Cells sorted in P3	Cells sorted in P4	Ratio of cells P3/P4	% of cells sorted in P3 secreting IgG
EL-4-B5 + TT	261	242	1,08	16,1
EL-4-B5 + TT + tetagam	271	192	1,41	7,4
EL-4-B5	137	169	0,81	11,7

Figure 10.11: Untreated data from ELISA of anti-TT in culture from donor 3.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,101	0,122	0,102	0,112	0,269	0,254	0,247	0,253	0,106	0,106	0,218	0,134
B	0,083	0,08	0,46	0,08	0,216	0,241	0,21	0,219	0,083	0,302	0,096	0,484
C	0,107	0,123	0,11	0,103	0,264	0,252	0,244	0,245	0,116	0,106	0,111	0,402
D	0,081	0,079	0,078	0,08	0,306	0,215	0,219	0,222	0,091	0,079	0,081	0,08
E	0,077	0,076	0,077	0,077	0,216	0,211	0,205	0,211	0,083	0,077	0,081	0,076
F	0,081	0,083	0,083	0,081	0,22	0,217	0,214	0,22	0,082	0,079	0,09	0,083
G	0,085	0,096	0,086	0,084	0,224	0,246	0,22	0,228	0,086	0,085	0,09	0,088
H	0,085	0,083	0,081	0,081	0,217	0,214	0,219	0,224	0,082	0,078	0,088	0,087

Explanation to figure 10.11

	1	2	3	4	5	6	7	8	9	10	11	12
A	EL-4-B5 TT				EL-4-B5 TT + tetagam				EL-4-B5			
B												
C												
D												
E	TT				TT + tetagam				B-cells only			
F												
G												
H												

Figure 10.12: Untreated data from ELISA of anti-TT in culture from donor 2.

	1	2	3	4	5	6
A	0,088	0,08	0,406	0,441	0,082	0,09
B	0,075	0,072	0,375	0,389	0,072	0,071
C	0,097	0,089	0,432	0,426	0,089	0,1
D	0,079	0,075	0,405	0,415	0,079	0,073
E	0,088	0,078	0,399	0,419	0,08	0,073
F	0,084	0,087	0,393	0,398	0,085	0,085
G	0,091	0,085	0,317	0,317	0,103	0,09
H	0,102	0,094	0,191	0,19	0,102	0,091

Explanation to figure 10.12

	1	2	3	4	5	6
A	EL-4-B5 TT		EL-4-B5 TT + tetagam		EL-4-B5	
B						
C						
D						
E	TT		TT + tetagam		B-cells only	
F						
G						
H						

Figure 10.13: Results from ELISA of anti-TT in culture from donor 2.

		TT		**TT + tetagam**		**No antigen**	
		1	2	3	4	5	6
EL-4-B5	A			High	High		
	B			High	High		
	C	+		High	High		+
	D			High	High		
No EL-4-B5	E			High	High		
	F			High	High		
	G			High	High	+	
	H	+		Low	Low	+	

Comment: Positive values are >0,01 (indicated with +) and negative values < 0,01.
Values for tetagam a cut-off at 0,150 is used to divide into a high OD group (High) and low OD group (Low).

GRADE

Referanse:	Design: Diagnostisk studie	
B-cell activation with CD40L or CpG measures the function of B-cell subsets and identifies specific defects in immunodeficient patient. (Marasco E, Farroni C, Cascioli S, Marcellini V, Scarsella M, Giorda E, et al. B - cell activation with CD40L or CpG measures the function of B - cell subsets and identifies specific defects in immunodeficient patients. Eur J Immunol. 2017;47(1):131-43.)	Dokumentasjonsnivå	
	GRADE	Middels - høy

Formål	***Materiale og metode***	***Resultater***	***Diskusjon/kommentarer***
-Find method for performing and evaluating functional tests? -Study B-cell functions in in infancy and throughout childhood? - Test the use of anti - CD40L?	PBMC isolated from blood or buffy coat. Stimulated with CpG, CD40L, IL-21, anti IgA/IgM/IgG. Culture for 7 days. Cultures are analysed with flow cytometry, ELISA, activated B-cells are lysed and analysed with real-time PCR. «In order to study the functions of the B-cell compartment in vitro, we chose to activate B cells with either TI and/or TD signals. CpG ODN-2006, a synthetic oligonucleotide that binds to TLR9, was chosen as a representative TI stimulus; whereas the TD signal was mimicked by engaging CD40 on B cells. When PBMCs were stimulated with CpG ODN2006 (from now on referred to simply as CpG) for seven days, B cells proliferated, differentiated into plasmablasts (identified as CD19low CD27++CD38++) and secreted immunoglobulins. … CD40L, binding to CD40 expressed on B cells, is considered the prototypical TD signal»	**Hovedfunn:** «Here we report our experience on the study of B-cell functions in infancy and throughout childhood and show how the proliferative potential, plasmablast differentiation and immunoglobulin secretion of B-cell subsets can be reliably assessed in children through TI activation by the toll-like receptor 9 agonist CpG. We also demonstrate how B cells respond to T-dependent co-stimulation. Finally, we show that in vitro functional tests can support physicians in assessing the state of the humoral immune system of patients with immune defects» **Bifunn:** - Time is essential: «plasmablasts started to appear in the second cell division, peaking after five cell divisions and plateauing thereafter. … Accordingly, the amount of immunoglobulins secreted was significantly higher after seven days of culture than after four day.» - Anti-CD40L does not activate B-cells: «rhCD40L …, in the presence of IL-21, induced the proliferation of B cells, strong homotypic interactions and clump formation (data not shown), but it did not promote either differentiation of B cells into plasmablasts or significant immunoglobulin secretion.» - CpG + CD40L inhibits plasmablasts: «When B cells were activated with both CpG and CD40L, CD40L inhibited CpG-induced plasmablast differentiation, immunoglobulins secretion, and the expression of PRDM1» - Non-B-cells stimulate: «memory and naive B-cell responses are enhanced by the presence of non-B cells in culture» - CpG not essential: «presence of non-B cells, a small fraction of CD27+ memory B cells differentiated into plasmablasts even without CpG» - Crosslinking: «CD40L together with BCR crosslinking induces mainly naive B cells to proliferate». - B-cell subsets and B-cell response to CpG change with age - Results connected to testing B-cell functions in patients with SIgAD (selective IgA deficiency). - Continuous activation of CD40 prevents B cells from differentiating into plasma cells	**Sjekkliste:** - Er formålet klart formulert? Ikke klart formulert, men klart hva de ønsker å vise i artikkelen. - Er metoden klart beskrevet? Ja - Er analyseringsmetoden velegnet? Ja - Er resultatene signifikante? Ja - Er det gode kontroller? Ja – forsøk med/uten stimulanter, forsøk i ulik alder, blod fra friske/syke pasienter i samme alder - Tatt høyde for bias? Ja, for IgM/IgG og isotype shifte. Ellers ikke nevnt. - Ble testene utført ihht protokoll for utførelse? Ja, det virker slik - Kan resultatene overføres til praksis? Jmf. Populasjon/protokoll/ kostnader/ekspertise tolkning res. Ja. - Vil kunnskapen fra testresultatet forbedre pasienthåndteringen/pas-ientens velvære/prognose Den er med å kaste lys på diagnostikk av immundefekter. - Hva diskuterer forfatterne som: - Styrke: Ikke diskutert - Svakhet: Måler kun CD 40 effekt med B-celle aktivering «valuating proliferation alone narrows the focus of the assay to only one of the many pathways induced by T-cell help through CD40» - Viser forfatterne til annen litteratur som styrker/svekker resultatene? Ja, både negative og positive resultater.
Konklusjon «Here, we report our experience on the study of B-cell functions in infancy and throughout childhood. We show that T-independent stimulation with CpG measures proliferation and differentiation potential of memory B cells. Switched memory B cells respond better than IgM memory B cells. On the other hand, CD40L, a T-dependent stimulus, does not induce plasma cell differentiation, but causes proliferation of naive and memory B cells.»			
Land Italy			
År publisert 2017			

Referanse: Antigen-specific activation of B cells in vitro after recruitment of T cell help with superantigen (Ingvarsson S, Lagerkvist AC, Mårtensson C, Granberg U, Ifversen P, Borrebaeck CA, et al. Antigen-specific activation of B cells in vitro after recruitment of T cell help with superantigen. Immunotechnology. 1995;1(1):29-39.)	Design: Diagnostisk studie
	Dokumentasjonsnivå
	GRADE: Middels - Høy

Formål	***Materiale og metode***	***Resultater***	***Diskusjon/kommentarer***
«To investigate whether superantigen could be used to direct polyclonal T cell help to human B cells stimulated by antigen in a restricted manner resulting in production of antigen-specific antibodies in vitro.» ***Konklusjon*** «The results obtained demonstrate that the superantigen SEA can recruit T cell help to human B cells specifically stimulated by antigens, resulting in production of antigen reactive antibodies in vitro.» → «This report shows that it is possible to induce a primary antigen-specific response when stimulating B cells via sIg and using SEA as a T cell mitogen.» ***Land*** Sweden ***År publisert*** 1995	Isolate PBMC from buffy coat, then isolating B- and T-cells from PBMC. Put in culture with anti-IgM, different concentrations with CEA, other antibodies 7 days. TT used as recall antigen. Analysed with ELISA and FACS «Purified B cells were preincubated with the antigen in manners allowing crosslinking of surface-Ig. The antigen exposed B cells were then cultured together with autologous CD4+ helper T cells and in the presence of various concentrations of SEA (staphylococcal enterotoxin A). Antibody production was measured by ELISA after 7-12 days of culture.»	**Hovedresultater:** «Antigen-specific activation of B cells could be obtained after stimulating the B cells with antigen or anti-surface-Ig antibodies in the presence of T helper cells and SEA. The degree of B cell activation (proliferation as well as antibody production) depended on the dose of antigen as well as on the dose of SEA used. Increased crosslinking of surface-Ig on antigen-specific B cells enhanced Ig production. Specific antibody production to a secondary recall antigen (tetanus toxoid) and to primary antigens (DNP and GM2) were obtained. The specific B cell response was dependent on contact between T and B cells.» **Bifunn:** - Crosslinking is important: «SEA by itself, only induced production of low amounts of immunoglobulin as did stimulation with anti-p chain antibodies. However, combinations of SEA and anti-Ig antibodies, synergistically induced signifiant amounts of human immunoglobulin … encreasing the degree of crosslinking resulted in enhanced production of specific IgG antibodies». - T-B-cell contact is important: «T-B cell contact is needed for efficient activation of specific B cells» - Antigen specific activation: «Specific antibody production was only observed when both antigen and SEA were present in the cultures. … Stimulation with TT by itself only gave rise to low amounts of anti-TT antibodies, but preincubation with TT followed by murine anti-TT increased the specific response several-fold and addition of MACS particles resulted in a further 30-35% increase of specific antibody production» - «Recent experiments have, indeed, demonstrated that human B cells can be activated antigen-specifically in vitro when supplied with signals via CD40, either in the form of soluble recombinant CD40L, or CD40 antibodies and lymphokines [27,28]. This suggests that the role of the antigen in that system was merely to lower the threshold for activation of the specific B cells. However, in this report we demonstrate that intimate contact between T and B cells is required for an efficient antigen-specific activation of the B cells»	**Sjekkliste:** - Er formålet klart formulert? Ja. - Er metoden klart beskrevet? Ja - Er analyseringsmetoden velegnet? Ja - Er resultatene signifikante? Ja - Er det gode kontroller? Ja – med/uten T-celler/antigen, ulike konsentrasjon av antigen/antistoff - Ble testene utført ihht protokoll for utførelse? Ja - Kan resultatene overføres til praksis? Jmf. Populasjon/protokoll/ kostnader/ekspertise tolkning res. Resultatene kan overføres til andre immunologiske eksperimenter/forsøk - Vil kunnskapen fra testresultatet forbedre pasienthåndteringen/pasientens velvære/prognose Muligheter for å bedre kunne utvikle monoklonale antistoffer som kan brukes i klinikken. - Hva diskuterer forfatterne som. - Styrke - Svakhet Ikke diskutert. - Viser forfatterne til annen litteratur som styrker/svekker resultatene? Ja, både artikler som støtter/motsier resultatene.

Referanse: Measurement of human and mouse anti-tetanus antibodies and isotype analysis by ELISA (Farzad Z, James K, McClelland DBL. Measurement of human and mouse anti-tetanus antibodies and isotype analysis by ELISA. J Immunol Methods. 1986;87(1):119-25.)	Design: Diagnostisk studie
	Dokumentasjonsnivå
	GRADE: Middels - høy

Formål	***Materiale og metode***	***Resultater***	***Diskusjon/kommentarer***
Want to develop a method for detecting anti-TT. «In this paper we describe an enzyme-linked immunosorbent assay (ELISA) which was developed to meet the above mentioned purposes (assessing the immune status of individuals at risk of tetanus infection, monitoring the efficiency of vaccination programs, selection of potential donors for the production of anti-tetanus Ig for clinical use)»	Examine different methods for ELISA in order to detect anti-TT. Also test SEI (sandwich enzyme immunoassay), Haemagglutination assay (HA) and protein A-Sepharose chromatography. Test anti-TT in 20 donors. Also immunize mice and measure monoclonal anti-TT in ascites.	**Hovedresultater:** «The ELISA and SEI procedures to be described are simple, safe, cheap, rapid and sensitive assays for the quantitative determination of total anti-tetanus and specific Ig isotype antibodies respectively. The ELISA can detect human anti-tetanus antibody levels as low as 5×10^4 IU/ml. Both assays can be performed in 4-5 h and antigen coated plates can be stored for routine use for at least 1 year» **Bifunn:** - Durability: «The most valuable findings were that microtitre plates coated with tetanus toxoid had a shelf life of at least 1 year with no reduced sensitivity.» - About antibody responses: «antibody responses varied among the individuals but the peak of response was generally day 7 or 14 after booster immunization.» - Durability antibodies: «The high level of antibody was maintained in the donor's serum for up to 6 months.» - ELISA is better: «ELISA is substantially more sensitive than HA, especially late in the response. The most likely explanation for the difference between the assays is that ELISA detects all classes of antibodies whereas HA preferentially detects IgM antibody. Late in the response IgG might be expected to predominate.» - Exact levels of anti-TT: «Anti-tetanus antibody levels were between 0.06-1.0 IU/ml before booster immunization, and increased gradually up to day 14 after the boost. By this time the individuals achieved a significant rise in antibody titre (16-32 IU/ml). This high level of antibody was maintained in the donor's serum for up to 6 months when tested by ELISA but this was not shown by the less sensitive HA technique.»	**Sjekkliste:** - Er formålet klart formulert? Formål med artikkelen er formulert, men ikke studien/eksperimentet i seg selv. - Er metoden klart beskrevet? Ja - Er analyseringsmetoden velegnet? Ja - Er resultatene signifikante? Ja, kunne nok hatt større antall. - Er det gode kontroller? Kommer frem at de beskriver den beste metoden de har funnet, det antas at de andre metodene er gode kontroller selv om det ikke blir beskrevet direkte. - Ble testene utført ihht protokoll for utførelse? Det fremstår slik - Kan resultatene overføres til praksis? Jmf. Populasjon/protokoll/ kostnader/ekspertise tolkning res. Ja - Vil kunnskapen fra testresultatet forbedre pasienthåndteringen/pas-ientens velvære/prognose Ja, beste metode kan benyttes i klinisk praksis for screening/utvikle monoklonalt antistoff - Hva diskuterer forfatterne som. - Styrke - Svakhet Ikke diskutert. - Viser forfatterne til annen litteratur som styrker/svekker resultatene? Ja.
Konklusjon «A rapid and sensitive enzyme immunoassay (ELISA) was developed for the quantitation of anti-tetanus antibodies. This technique was used to measure antibody levels in the plasma of immunized donors, in human anti-tetanus IgG preparations and in human and mouse hybridomas producing monoclonal antibodies to tetanus toxoid.»			
Land U.K.			
År publisert 1985			

Referanse:	Design: Diagnostisk studie	
B-Cell Activation via CD40 Is Required for Specific Antibody Production by Antigen-stimulated Human B Cells (Nonoyama S, Hollenbaugh D, Aruffo A, Ledbetter JA, Ochs HD. B cell activation via CD40 is required for specific antibody production by antigen-stimulated human B cells. J Exp Med. 1993;178(3):1097-102.)	Dokumentasjonsnivå	
	GRADE	Middels

Formål	***Materiale og metode***	***Resultater***	***Diskusjon/kommentarer***
«…However, the role of CD40 and these cytokines in the Ag-specific Ab production is not well understood. To investigate this, we used the T cell-dependent Ag, bacteriophage 174 (phage) (8-10), which allows us to study the induction and regulation of phage-specific Ab synthesis.» ***Konklusjon*** «The establishment of a B cell culture system producing Ag-specific Ab has allowed us to study molecules involved in activating Ag-primed memory B cells. Ag-specific Ab production by primed B cells depends on three signals: Ag, anti-CD40, and IL-10. In the absence of any one of these reagents, B cells fail to produce specific Ab, suggesting that each component provides a distinct and synergistic signal to B cells. ***Land*** USA ***År publisert*** 1993	IgD+ cells from purified B and T-cells were sorted by FACS. Cultured with anti-CD40, phage and other antibodies. Some wells with autologous T-cells for comparison. After 12 days supernatants were tested for Ig by ELISA and phage neutralizing antibody activity. For immunedeficiency experiments: «Human volunteers (12) and four patients from three families with x-linked hyper IgM syndrome were injected intravenously twice, 6 wk apart … Serum was collected before and at weekly intervals after immunizations and antiphage Ab activity determined by a sensitive phage neutralization assay»	**Hovedresultater:** «In this study, we demonstrate that if cultured in the presence of anti-CD40, interleukin 10 (IL-10), and Ag, purified B cells can produce antiphage Ab in quantities comparable to those synthesized by B cells cocultured with Ag and T cells.» **Bifunn:** - Antigen is important: «In the absence of Ag, anti-CD40/IL-10-stimulated B cells produce only minute amounts of antiphage Ab, indicating that Ag stimulation is indispensable and provides a signal that is synergistic with anti-CD40 and IL-10.» … «cells cultured with autologous T cells in the absence of Ag failed to produce detectable antiphage Ab. … addition of Ag to B cells cultured in the presence of anti-CD40 and IL-10 resulted in the production of high titers of phage-neutralizing a. - «activated T cells from patients with x-linked hyper IgM syndrome express functionally defective gp39 and respond with depressed Ab titers and fail to switch from IgM to IgG after multiple phage immunizations» - B-cells alone is not enough: «Purified B cells obtained from human subjects 6 wk or later after the last immunization with phage failed to produce spontaneous antiphage Ab in the absence of Ag, indicating that they were in a resting stage. Addition of Ag (phage) to cultured B cells failed to induce phage-specific Ab production.» - T-cells are important: «Autologous T cells efficiently initiated the production of antiphage Ab by Ag-stimulated B cells, demonstrating that T cell help is necessary for the induction of phage-specific Ab production.» - CD40 + IL10 is necessary: «Neither anti-CD40 nor IL10 alone induced specific Ab production by Ag-stimulated B cells, indicating that combination of anti-CD40 and IL-10 is required for specific Ab production.» - IL10 is essential: "B cells cultured with Ag and anti-CD40 produce phage-specific Ab only if IL-IO is added to the system. … IL-4 failed to induce antiphage Ab synthesis in vitro» - Can use anti-CD40: «Addition of a soluble form of the CD40 ligand (sgp39) to the culture system has a similar effect on specific Ab synthesis as anti-CD40»	**Sjekkliste:** - Er formålet klart formulert? Ja, relativt sett - Er metoden klart beskrevet? Ja - Er analyseringsmetoden velegnet? Ja, kunne hatt større antall i immundefekt experimentene. - Er resultatene signifikante? Ja - Er det gode kontroller? Ja – med/uten alle stimulanter, friske/syke donorer. - Ble testene utført ihht protokoll for utførelse? Ja, det antas. - Kan resultatene overføres til praksis? Jmf. Populasjon/protokoll/ kostnader/ekspertise tolkning res. Kan overføres til annen forskningspraksis ja. - Vil kunnskapen fra testresultatet forbedre pasienthåndteringen/pas-ientens velvære/prognose Bidrar til videre forskning - Hva diskuterer forfatterne som. - Styrke - Svakhet Ikke diskutert. - Viser forfatterne til annen litteratur som styrker/svekker resultatene? Noe, kun støttende litteratur.

Referanse: Fetal and neonatal alloimmune thrombocytopenia (Zdravic D, Yougbare I, Vadasz B, Li C, Marshall AH, Chen P, et al. Fetal and neonatal alloimmune thrombocytopenia. Semin Fetal Neonatal Med. 2015;21(1):19-27.)	Design: Review article
	Dokumentasjonsnivå
	GRADE: Høy

Formål	***Materiale og metode***	***Resultater***	***Diskusjon/kommentarer***
«In this review, we introduce the pathogenesis of FNAIT, particularly those new discoveries from animal models, and discuss possible improvements for the diagnosis, therapy, and prevention of this devastating disease» ***Konklusjon*** Lists practice points and research directions at end of article as conclusion. ***Land*** Canada ***År publisert*** 2015	No method is described. It is a review article of relevant literature regarding FNAIT.	**Hovedresultater:** Investigate: - Pathogenesis of FNAIT - Lessons learned from animal models of FNAIT - Management and prevention strategies for FNAIT **Bifunn:** - A lot about relevant background information, collected information and studies. - Statistics: «FNAIT is the most frequent cause of severe thrombocytopenia in liveborn neonates [30,31], with an estimated frequency of 0.5-1.5 per 1000. This number, however, does not include miscarriages, since the incidence of mortality of FNAIT fetuses has not been adequately studied.» «FNAIT cases may occur during the first pregnancy», «In the Caucasian population, 75-85% of maternally derived antibodies reported target HPA-1a on integrin b3» «women who are HLA DRB3*01:01 positive have an approximately 25 times higher risk of HPA-1a-immunization when compared to women who lack this allele» - Consequences: "…FNAIT is a severe and potentially life-threatening disease.» - A lot is still unknown: «Among the questions which remain unanswered: …» - Diagnostics: «Most cases of FNAIT are discovered when a child is born at term with petechiae or other signs of bleeding in the absence of any other condition known to be associated with neonatal thrombocytopenia», «The monoclonal antibody immobilization of platelet antigen (MAIPA) assay has long been considered as the gold standard for detection of platelet antibodies.», gene testing, platelet count, detection of antibodies… are also discussed - Treatment: Discusses treatment such as IVIG, glucocorticoids, thrombocyte infusion, cesarian section… "To date, the maternal immune response to fetal platelet antigens is still not well understood and it is unclear why bleeding is more severe in FNAIT than in ITP.»	Sjekkliste: - Er formålet klart formulert? Ja - Er metoden klart beskrevet? Nei - Er det tatt høyde for stor nok mengde litteratur? Ja, 99 artikler er inkludert I litteraturliste. - Er det store sprik I resultatene? Det fremstår ikke slik. - Kan resultatene overføres til praksis? Jmf. Populasjon/protokoll/ kostnader/ekspertise tolkning res. Ja, - Vil kunnskapen fra testresultatet forbedre pasienthåndteringen/pas-ientens velvære/prognose Samler eksisterende forskning og foreslår ny forskning. - Hva diskuterer forfatterne som. - Styrke - Svakhet Ikke diskutert. - Viser forfatterne til annen litteratur som styrker/svekker resultatene? Ja.