The Investigation of Autoimmunity and B Cell Characteristics in Common Variable Immunodeficiency Patients

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STATEMENT OF ORIGINALITY

I, Jennifer Wanders, confirm that the work presented in this thesis is my own. Any work done in collaborations has been acknowledged. This work has not been submitted for any other degree or other qualification of the University College London or any other University or Institute of learning.

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Abstract

Common Variable Immune Deficiency (CVID) is characterized by hypogammaglobulinemia and impaired antibody responses to vaccination. Ten percent of CVID patients have low B cell counts and 20-30 % develop autoimmune conditions, mainly idiopathic thrombocytopenic purpura (ITP), autoimmune haemolytic anaemia (AIHA) and thyroiditis. Eight to ten percent of CVID patients carry mutations in TACI while the majority of these patients have autoimmune manifestations.

The aim of this study was to investigate why some CVID patients have low B cell numbers. One hypothesis is that they have auto-antibodies against B cells, which would make CVID a primary autoimmune condition that could explain the hypogammaglobulinaemia through autoimmunity. Alternatively, they might have reduced levels of B cell survival factors.

To detect auto-antibodies in the serum of patients, a FACS-based assay was developed. B cells from healthy donors were incubated with patients' sera and stained with anti- human CD19, - IgM and –IgG antibodies. Thirty-six CVID patients with low B cell numbers, autoimmune manifestation or both were screened. None were found to have IgM or IgG anti-B cell auto-antibodies. To explore the second hypothesis, serum levels of the B cell activating factor BAFF were analyzed. One group of patients with extremely low BAFF levels and a second group with highly elevated BAFF levels were found. B cell phenotypes and BAFF serum levels were used to select patients for sequencing BAFF, or BAFF receptor (BAFF-R) for possible mutations. The coding regions of BAFF were sequenced in 19 patients with low BAFF levels and all showed wild type sequences. In 17 patients with high BAFF levels, coding regions of BAFF-R were sequenced. Five patients showed previously described rare

polymorphisms; four had a heterozygous P21R and one had a heterozygous G64V polymorphism. The novel heterozygous R106Q polymorphism was detected in one patient. The surface expression of BAFF-R on B cells from patients with rare polymorphisms showed no major impairment compared to healthy individuals.

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1 Introduction

1.1 Basic concepts of Immunology

The immune system contains cells and proteins, predominantly derived from the haematopoietic system to provide protection of the host from infection. Two main branches are relevant: the innate and adaptive immune systems which both contribute to immediate host defence against infection. The innate immune system has two main key components which are made up of antigen-presenting cells (APC) such as dendritic cells and macrophages and the other part being proteins such as complement and c reactive protein (CRP). APCs recognize dangerous pathogens and trigger the adaptive immune response, which can be divided into cell mediated and humoral immunity. A characteristic feature of adaptive immunity is clonal selection of antigen-specific cells to generate sufficient amount of cells necessary to control the infection and generate immunologic memory which plays an important role throughout life. T cells are a part of the cell-mediated immunity, which protects the host, either by direct killing of infected host cells or by secretion of cytokines to enhance immune responses. The concept of humoral immunity on the other hand, is composed of B cells secreting antibodies with high specificity to a wide variety of antigens after they have been activated. The function of antibodies is primarily to protect the host against extracellular pathogens. Antibodies can protect in three different ways: first, they can bind to pathogens leading to neutralization of the targeted antigen. Secondly, the opsonization of antigens and pathogens by antibodies can promote phagocytosis. And thirdly,

antibodies can activate the complement system which enhances the lysis of bacteria.

B cells of the humoral immune response are derived from common lymphoid progenitors and are produced in the bone marrow. Immature B cells express the B cell receptor (BCR) as well as B cell receptor co-receptors such as CD19:CD21:CD81. These immature B cells are transitional B cells in humans and circulate between the tissues, peripheral blood and lymphoid organs such as lymph nodes and spleen, to detect potential pathogens. Dependent on the appropriate signals received by the innate immune response, antigen is presented in the lymph node. The B cell response is initiated and B cells undergo proliferation in the germinal centres of lymphoid organs. Therefore the recognition of antigen activates B cells, followed by proliferation and differentiation into antibody-secreting plasma cells and long-lived memory B cells.

Two different humoral immune responses are known: the T-cell independent (TI) and T-cell dependent (TD) response. The TI response has the ability to respond rapidly and directly to many pathogens. This occurs through extensive cross-linking of antigen to the BCR or the binding of receptors from the innate immune system such as toll-like receptors (TLR). To initiate a TD response, antigen is recognized by the BCR and in addition to activating the B cell the antigen recognized is internalized and processed into peptides. These peptides are presented to T cells via MHC class II on the surface of APC such as B cells. The APC and Thelper (Th) cell need to recognize the same antigen, and this is known as linked recognition. Before linked recognition occurs, Th

cells must have been primed by other APCs. These APCs presenting antigenic peptides enable Th cells to trigger the B cell into producing antibodies against the antigen.

During this germinal centre reaction somatic hypermutations are induced. This refers to a process where point mutations are introduced and lead to a higher rate of specificity and affinity of the antibody. Another important interaction during the T-cell dependent response is between CD40 ligand (CD40L) on Th cells and CD40 on B cells. This induces isotype class switching of the antibodies to change the effector functions of the different antibody isotypes. Both somatic hypermutation and isotype class switching further increase the diversity and specificity of antibodies, thereby ensuring that T-cell dependent responses are highly specific.

Antibodies exist in five different isotypes: IgM, IgD, IgG, IgA and IgE, serving different functions. The antibody for a primary response in a TI response is IgM and is also mainly responsible of the activation of complement. IgG, IgA or IgE initiate a rapid and efficient protection against pathogens during the secondary antigen contact. IgG is also involved in the activation of complement, opsonisation and is an important component for successful vaccination. IgE is responsible for the defence of parasites and IgA is important in the defense and homeostasis of mucosal surfaces.

1.2 B cell survival

The tumor necrosis factor (TNF) family members ligands BAFF (B cell activating factor) and APRIL (a proliferation inducing ligand) mediate survival and proliferation in B cell homeostasis. Their corresponding receptors belong to the TNF-receptor family; BAFF-Receptor (BAFF-R), TACI (transmembrane activator and CAML interactor) and BCMA (B cell maturation protein) which are expressed differentially at various times during B cell differentiation. They are predominantly expressed on B cells.

BAFF is also known as BLyS, THANK, TALL-1 and zTNF4 and is expressed on the surface of monocytes, dendritic cells (DC), neutrophils, stromal cells, activated T cells, malignant B cells and epithelial cells. BAFF binds to all three receptors, BAFF-R, TACI and BCMA. The expression of BAFF is upregulated by lFNγ, IL-10 and G-CSF on myeloid cells (Scapini P *et al* 2003; Nardelli B *et al* 2001). Especially BAFF enhances long-term B cell survival primarily through the alternative NF-KB pathway by up-regulating anti-apoptotic proteins. In addition to being membrane bound, BAFF is cleaved from the cell surface and circulates as an active soluble homotrimer that only binds to BAFF-R and can further assemble to a 60-mer multimer. The multimers and cell surface bound BAFF bind to TACI (Bossen C *et al* 2007).

APRIL is a homologue of BAFF and is expressed on lymphocytes, monocytes and macrophages. It binds to TACI and BCMA, but not to BAFF-R. Through proteolytic cleavage, APRIL is also found as a soluble homotrimer, but not as a

multimer. B cell proliferation and plasma blast survival are enhanced by APRIL binding to BCMA. BCMA is therefore responsible for the survival of plasma cells. BCMA binds to APRIL with high affinity and to BAFF with a lower affinity.

TACI is expressed on marginal zone B cells, a subpopulation of naive B cells and is the predominant receptor for short lived plasma cells. It has been shown in vitro that a T-independent response by TLR binding leads to signalling of the maturation and survival of activated B cells, immunoglobulin secretion and plasma cell generation (Castigli E *et al* 2007; von Bulow GU *et al* 2001, Yan M *et al* 2001). From this data one can conclude that TLR ligation promotes the upregulation of TACI (Treml LS *et al* 2007). On the contrary, TACI-deficient mice have excessive numbers of B cells indicating that TACI negatively regulates the B cell compartment (Seshadayee D *et al* 2003*;* Mackay F *et al* 2008). It appears that TACI stimulates plasma cell differentiation while inhibiting prolonged B cell proliferation (Mantchev GT *et al* 2007). Therefore, TACI may have both B cell- stimulatory and -inhibitory functions.

BAFF and APRIL induce class switching to IgG or IgA through the upregulation of activation ligand deaminase (AID) (Litinskiy MB *et al* 2002) which is mainly triggered by TACI and to a lesser extent by BAFF-R (Castigil E *et al* 2005). Both TACI- and APRIL-deficient mice have low levels of serum IgA which supports this finding. However, CD40 ligation induces class switching to a much higher extent than through BAFF and APRIL.

Binding of BAFF to BAFF-R is essential for the survival of B cells with a minor contribution from TACI and none from APRIL or BCMA. BAFF-R is expressed

on transitional B cells and is therefore a crucial factor for further differentiation (Meyer-Bahlburg A *et al* 2008). During BCR activation the BAFF-R is up-regulated (Smith SH *et al* 2003). On the other hand, during plasma cell maturation the BAFF-R is down-regulated whereas BCMA is up-regulated and essential for plasma cell survival (Avery DT *et al* 2003).

1.3 Concepts of Autoimmunity in the B cell survival cascade

Central tolerance begins in the bone marrow where auto-reactive BCRs are randomly generated and are normally removed from the B cell repertoire. In the bone marrow this is dependent on the signal induced by self antigen that cross-links with the BCR. Strong signalling results in apoptosis and is called clonal deletion. Alternatively, auto-reactive B cells undergo a process called receptor editing. During this process a new random rearrangement of the variable region of the immunoglobulin takes place and the self reactive receptors are replaced with non-self-reactive ones. Low affinity auto-reactive B cells become anergic.

It has been reported that patients with various autoimmune conditions such as systemic lupus erythematosus (SLE) (Stohl W *et al* 2003), rheumatoid arthritis (RA) (Cheema GC *et al* 2001) and Sjögren's syndrome (Groom J *et al* 2002) have elevated BAFF serum levels. In order to further understand the mechanism of autoimmunity in the B cell survival cascade various mouse models have been generated: $BAFF^{-/-}$ -, A/WySnJ- BAFF-R mutant-, BAFF transgenic-, TACI-Ig transgenic- and $TACI^{-/-}$ mouse models. These mouse models are subsequently discussed in detail.

The BAFF$^{-/-}$ mouse model showed reduced peripheral B cells in lymph nodes and spleen. B cell development was blocked at the transitional B cell stage lacking mature and marginal zone B cells. In the bone marrow an absence of recirculating B cells was observed (Gross JA *et al* 2001, Schiemann B *et al* Science 2001). Therefore, BAFF in mice seems to be essential for B cells beyond the development of immature B cells. This supports the hypothesis that elevated BAFF serum levels favour the survival and escape of auto-reactive B cells during maturation and therefore elevated BAFF levels in SLE, RA and Sjögren's syndrome can lead to autoimmunity (Do R K *et al* 2002, Mackay F 2009).

The A/WySnJ- BAFF-R mutant mouse expresses a naturally occurring mutant form of BAFF-R in the intracellular signalling domain and leads to impaired BAFF-R. This mouse model showed a similar phenotype to the BAFF$^{-/-}$ mouse (Mackay F *et al* 1999). The reduced mature B cells in both BAFF mutant and BAFF$^{-/-}$ mouse can lead to the conclusion that BAFF explicitly binds to BAFF-R that is present on transitional B cells.

In contrast the BAFF transgenic mouse showed an increase of mature B cells, enlarged lymphoid organs and spleen. Hypergammglobulinemia and autoimmune-like manifestations, including anti-DNA antibodies, were found. Overall, an SLE-like phenotype was described. In the bone marrow no changes were observed, indicating that the role of BAFF is restricted to the peripheral B cell compartment (Mackey F *et al* 1999; Khare SD *et al* 2000).

Interestingly, the TACI-Ig transgenic mouse blocks BAFF and therefore has a similar but milder phenotype than the $BAFF^{-/-}$ mouse (Gross JA *et al* 2001). This is due to the fact of less BAFF available for proliferation and again this supports the hypothesis that BAFF is important for B cell proliferation (Schneider P *et al* Immunol Lett. 2003).

The $TACI^{-/-}$ mouse model shows marked increased numbers of circulating B cells, splenomegaly, development of lymphomas and autoimmunity in a lupus-like phenotype (Yan M *et al* 2001; von Bulow GU *et al* 2001). The observed impaired immune response to T cell independent specific antibody led to the conclusion, that TACI plays an important role for a stable TI immune response (Yan M *et al* 2001). In addition, elevated BAFF serum levels were detected that suggests TACI regulates circulating BAFF. These elevated BAFF levels possibly lead to the survival of auto-reactive B cells which would otherwise die, and therefore to the observed autoimmunity. In conclusion, the absence of TACI can lead to increased BAFF-R signalling resulting in B cell hyperplasia and activation of auto-reactive B cells. Therefore, the elevated B cell numbers indicate a negative regulation of B cell numbers in periphery through TACI (Seshadayee D *et al* 2003*;* Mackay F *et al* 2008).

In conclusion an increased competition for BAFF results in a more stringent deletion of auto-reactive B cells. On the other hand, decreased competition for BAFF in the context of B cell lymphopenia or increased levels of circulating BAFF results in relaxation of B cell selection and the release of more auto-reactive naive B cells, showing the importance of a proper balance between BAFF and B cells (Mackay F *et al* 2009; Mackay F 2010; Le Pottier L *et al*).

This is supported by the TACI$^{-/-}$ mouse developing autoimmunity and lymphoproliferation showing elevated BAFF levels (Lee J *et al*).

1.4 CVID

CVID is the most frequently diagnosed primary immunodeficiency and the most common symptomatic congenital deficiency of the immune system with an estimated incidence of 1:25.000. CVID usually presents in the second and third decade of life (Chapel H *et al* 2008). It is characterized by a marked decrease of two major serum immunoglobulins, IgG and IgA and/or IgM. Diagnosis is based on impaired ability to produce specific antibodies after vaccination or exposure to pathogens and exclusion of secondary or other primary antibody deficiencies. In addition to recurrent upper respiratory tract infections, systemic granulomatosis, lymphoproliferation, autoimmune manifestations, and gastrointestinal diseases are quite frequently observed. Further diagnostic criteria's are issued by the European Society for Immunodeficiencies (ESID; www.esid.org). Both T and B cell defects have been described in CVID patients (Cunningham-Rundles C *et al* 1999; Warnatz *et al* 2002; Giovanetti A *et al* 2007; Chapel H *et al* 2008; Wehr C *et al* 2008).

The treatment of choice for CVID is either intravenous or subcutaneous immunoglobulin substitution. In addition to the substitution of immunoglobulins an antibiotic prophylactic therapy for bacterial infections and further treatment of other complications may be needed.

1.4.1 Clinical features of CVID

Recurrent sinusitis, otitis, bronchitis and pneumonia are usually due to Streptococcus pneumonia, Moraxella catarrhalis and Haemophilus influenzae in CVID patients (Cunningham-Rundles C *et al* 1999). Bronchiectasis and chronic lung disease evolve over time as a result of bacterial infections. In 5% of patients mycoplasma infection can be found in the urinary tract and joints (Bonilla HF *et al* 1997). Gastrointestinal manifestations caused by Giardia lamblia, Salmonella, Shigella and Campylobacter have been described in 60% of patients before starting intravenous immunoglobulin therapy (Washington K *et al* 1996). Additionally inflammatory bowel disease is common in CVID patients (Lai Ping So A *et al* 1997). It is important to distinguish between inflammatory or infectious gastrointestinal complications.

30% of CVID patients show splenomegaly and lymphadenopathy. Similar to other primary immunodeficiencies an increased risk of lymphoproliferative diseases is present. In 8-22% a granulomatous syndrome has been described affecting the lungs, skin, liver, bone marrow and lymph nodes (Mechanic L *et al* 1997). This is correlated with the presence of splenomegaly and autoimmunity. CVID patients have a higher risk of developing malignancies such as lymphomas and stomach cancer.

1.4.2 Autoimmunity in CVID

Autoimmune conditions, such as idiopathic thrombocytopenia (ITP), autoimmune hemolytic anemia (AIHA) (Cunningham-Rundles C *et al* 2002, Wang J *et al* 2005, Knight AK *et al* 2006), thyroiditis, SLE (Fernandez-Castro

M *et al* 2007), rheumatoid arthritis (Uluhan A *et al* 1998) and vitiligo have been reported in 20-30% of CVID patients. The most common autoimmune conditions in CVID patients are ITP and AIHA which can show prior to the diagnosis of the CVID. Why this predisposition occurs is still very poorly understood.

1.4.3 B cell phenotyping in CVID patients

CVID has been shown to have phenotypic and functional defects in the humoral immune system. Not only is the search for pathogenic mechanisms important, but also the identification of immune parameters and markers for the classification, diagnosis and prognosis of CVID (Bergbreiter A *et al* 2009). The investigation of CVID on a cellular level by flow cytometry was introduced nearly 10 years ago (Warnatz K *et al* 2002; Piquers B *et al* 2003). Since then B cell markers have been evaluated for the diagnosis and classification of CVID. Three different B cell subsets have emerged to be important for the classification of CVID: naïve B cells ($CD19^+CD27^-IgM^+IgD^+$), IgM memory, also known as marginal zone B cells ($CD19^+CD27^+IgM^+IgD^+$) and switched memory B cells ($CD19^+CD27^+IgM^-IgD^-$). Based on the B cell subset markers three different classifications have been suggested; the Freiburg classification (Warnatz K *et al* 2002), the Paris classification (Piquers B *et al* 2003) and the EuroClass classification (Wehr C et al 2008).

A majority of patients show reduced switch memory B cells. In another group you can find both IgM memory and switched memory B cells reduced. In 10% of CVID patients an expansion of transitional ($CD19^+$ $CD21^{int}$ $CD38^{++}IgM^{++}$)

and $CD21^{lo}$ ($CD19^{++}CD21^{low}CD38^{low}IgM^{+}$) B cells was found (Warnatz K *et al* 2002).

1.4.4 T cell defects in CVID patients

T cell defects have also been described in CVID patients and a classification based on the number of naive $CD4^{+}$ T cells has been suggested (Giovanetti A *et al* 2007). The classification is divided into three groups: The first group is characterized by a significant reduction of $CD4^{+}$ naive T cells and a very high T cell activation, proliferation and increased apoptosis. Furthermore disruption of the normal T cell receptor repertoire which is associated with splenomegaly and a more severe immunodeficiency. The second group showed the same features as the first, but were less distinct. The third group had normal naive $CD4^{+}$ T cell, however, the $CD8^{+}$ fraction showed predominant alterations. Splenomegaly was less frequent and a milder clinical phenotype was presented.

1.4.5 Genetic studies of CVID

CVID is a very heterogeneous disorder and the genetic cause of this disease is unknown for the majority of cases. The genetic study of CVID is further complicated by a number of observations which demonstrate that the phenotype associated with mutations in certain known genes can be very variable. Frequently CVID is sporadic, however approximately 10-20% are familial with both autosomal dominant (AD-CVID) and autosomal recessive trait (AR-CVID) (Vorechovsky I *et al* 1995).

Monogenetic defects have been reported in several families with AR-CVID with *ICOS* (Grimbacher *et al* 2003, Salzer U *et al* 2004) mutated in five families, *CD19* (Van Zelm M *et al* 2006; Kanegane H *et al* 2007) in at least four families, and *TNFRSF13C (*BAFF-R) in one family (Warnatz K *et al* 2005). *TNFRSF13B* (TACI) (Salzer *et al* 2005; Castigli E *et al* 2005) mutations are associated with AD-CVID; however, it has also been reported in AR-CVID. Most recently mutations in LRBA (lipopolysaccharide responsive beige-like anchor protein) have been described in a cohort of CVID patients with early onset (Lopez-Herrera G *et al* 2012).

Together, these defects account for about 10-15% of all cases of CVID. Single cases with mutations in *CD20* (Kuijpers TW *et al* 2010), *CD21* (Thiel J et al 2011), *MSH5* (Sekine H *et al* 2007) and *CD81* (Van Zelm MC *et al* 2010) have also been reported.

Since heterozygous mutations in the TACI gene also occur in around 1% of healthy individuals, it is believed that in addition to TACI mutations modifier genes are contributing to the disease as well.

This study aimed to find out:

Why some CVID patients have low B cell numbers, asking:

a. Whether they have auto-antibodies against B cells, and/or
b. Whether they have impaired BAFF-mediated B cell survival signals

2 Material and Methods

2.1 Patients

In total 45 patients diagnosed with CVID took part in the study.

Patient samples were collected under ethical approval at the Department of Immunology Royal Free Hospital London. All patients gave informed consent prior to enrolment into the study.

2.2 DNA extraction from whole blood

Genomic DNA was extracted from peripheral blood using self made red blood cell lysis (RBC) buffer (10mM NH_4HCO3, 0.14M NH_4Cl) and a Gentra® Puregene® purification kit (Qiagen, West Sussex, UK). Ten ml EDTA blood was added to 30 ml RBC buffer and incubated on ice for 10 minutes. After centrifugation at 1500 rpm for 5 minutes the supernatant was discarded and the pellet was vortexed. An additional 30 ml of RBC buffer, mixed with the pellet and incubated on ice for 10 minutes and then centrifuged as before. The supernatant was discarded and 5 ml of cell lysis solution (Gentra® Puregene®, Qiagen, West Sussex, UK) was added to the cell pellet and mixed thoroughly. One ml of protein precipitation solution (Gentra® Puregene® Qiagen, West Sussex, UK) was added to the cell lysis solution and vortexed vigorously. The sample was centrifuged at 3500 rpm for 20 minutes at 4°C. The supernatant containing the DNA was transferred to a clean 50 ml tube (TPP, Trasadingen, Switzerland) while the brownish-white pellet containing total protein was discarded. An equal volume of ice cold isopropanol was added. The tube was inverted 10-20 times as white DNA strands appeared. The precipitated DNA

was centrifuged at 3500 rpm for 10 minutes at 4°C, the supernatant was discarded and the pellet was washed with 70% ethanol and centrifuged as above. After discarding the supernatant, the pellet was dried at room temperature for approximately 1 hour and rehydrated in appropriate amount of DNA hydration solution (Gentra® Puregene®, Qiagen, West Sussex, UK) depending on pellet size.

2.3 RNA extraction

Patients' RNA was extracted from frozen Peripheral Blood Mononuclear Cells (PBMCs) (section 2.10 and 2.11) by using the RNeasy Kit (Qiagen, United Kingdom). All buffers are included in the kit.

Frozen PBMCs were thawed, washed with warm complete RPMI Medium (10% FCS, 1% Penicillin/Streptomycin and 1% L-Glutamin) and centrifuged at 1500 rpm for 5 min. The cell pellet was disrupted by adding 600 µl RLT buffer and thoroughly mixing by pipetting up and down. The lysate was passed at least 5 times through a blunt 20-gauge needle fitted to an RNase-free syringe. The lysate was directly pipetted into a QIAshredder spin column to homogenize the lysate. The spin column was placed in a 2 ml collection tube, and centrifuged for 2 min at 13000 rpm. An equal volume of 70% ethanol was added to the homogenized lysate, and mixed well by pipetting. Up to 700 µl of the sample, including any precipitate that may have formed was transferred to an RNeasy spin column which was placed over a 2 ml collection tube. This step was repeated until all lysate had been processed through the spin column. The spin column was centrifuged for 15 s at 13000 rpm. RNA is now located in the membrane of the column. The flow-through was discarded and

700 µl of Buffer RW1 was added to the RNeasy spin column. Again, the tube was centrifuged for 15 s at 13000 rpm to wash the spin column membrane. Flow-through was discarded and 500 µl buffer RPE was added to the RNeasy spin column to wash the spin column membrane. The column was centrifuged for 15 s at 13000 rpm. The flow-through was discarded, this procedure was repeated and centrifuged for 2 min at 13000 rpm to dry the spin column membrane. The RNeasy spin column was placed in a new 1.5 ml collection tube. 30–50 µl RNase-free water was directly added to the spin column membrane and centrifuged for 1 min at 13000 rpm to elute the RNA.
RNA concentration was determined by spectrophotometer (Ultrospec 1100 Pro UV Vis Spectrophotometer, GE). RNA is very unstable and therefore needs to be stored long term at -80°C.

2.4 cDNA transcription

The cDNA was prepared by using the QuantiTect reverse transcriptase kit (Qiagen). All buffers contained in the kit were thawed on ice. Frozen RNA was thawed and then kept on ice at all times during this procedure.
To prevent a gDNA contamination of the RNA, a gDNA elimination reaction (gDNA wipeout) was performed prior to transcription.
A total volume of 14 µl gDNA wipeout mix was prepared, including 2 µl of gDNA Wipeout Buffer and up to 1 µg RNA template. The remaining volume was made up of RNase free water. This was incubated for 2 min at 42°C and then immediately placed on ice. This was added to 1 µl Quantiscript Reverse Transcriptase, 4 µl 5x Quantiscript RT Buffer,

1 µl RT Primer Mix, to give a total volume of 20 µl.The reverse transcript mix was incubated for 15 min at 42°C following 3 min at 95°C to inactivate Quantiscript Reverse Transcriptase. This step was performed in a PCR machine. An appropriate amount of cDNA was amplified for *BAFF* with BAFF cDNA primers.

2.5 PCR reaction on DNA and cDNA

Two genes, BAFF (exon 1-5) and BAFFR (exon 1-3) were amplified by Polymerase Chain Reaction (PCR). A H_2O control was added to each PCR reaction to exclude contamination.

For the PCR reaction a master mix of a total volume of 20 µl was prepared as shown below.

2 µl 10x PCR reaction buffer
2 µl 1mM dNTP-mix
0.5 µl 25 µM forward primer
0.5 µl 25 µM reverse primer
0.2 µl Taq polymerase (1U)
4 µl Q solution
9.8 µl dH2O
to1 µl of DNA (50ng)

The PCR was run with the following programme (Table 1):

	Temperature in °C	Time	Number of cycles
Denaturation	95	5 min	1
Denaturation Annealing Extension	95 53-63 72	15 s 30 s 50s	30
Extension	68	10 min	1

Table 1: PCR conditions

The annealing temperature varied for the different genes. BAFF PCR reactions had an annealing temperature of 53°C whereas BAFF-R PCR reactions had an annealing temperature of 63°C. Following primer pairs were used PCR's for BAFF (Table 2), BAFF-R (Table 3) and cDNA primers (Table 4).

BAFF	primers
Exon 1 forward	5_CCC TTG ACT GTG CCA ATC CAA ACT_3
Exon 1 reverse	5_GCG AGG GAG AGG CAG CAG TGT A_3
Exon 2 forward	5_CAA GGC CGG CCT GGA GGA AG_3
Exon 2 reverse	5_CAG CGC TGG GGC TTT GCT CTA A_3
Exon 3 forward	5_AGA GTG GGT TTC TAG CTT TGT GTT_3
Exon 3 reverse	5_GTG TGA TGT TCC CCT TCC TGT A_3
Exon 4/5 forward	5_AGT GAT GGC AAA GAA TCC AGT G_3
Exon 4/5 reverse	5_GGG CAC CAT CCT TTC ATT TT_3
Exon 6 forward	5_TTC TTT TGG GGA AGT CCA TT_3
Exon 6 reverse	5_TAC AGA GAA AGG GAG GAA AAT AGC_3

Table 2: PCR primers for the amplification of exons one to six of the BAFF gene

BAFFR	primers
Exon 1 forward	5_AGC CTC AGT CCC CGC AGC T_3
Exon 1 reverse	5_GTT CTC CCC GCA GCT GCC G_3
Exon 2 forward	5_AGA CCG TCC CGA CAC CCC_3
Exon 2 reverse	5_CCT GGC CTC CCT CCC TGT_3
Exon 3 forward	5_AGC CCC AGC TCC CCA ACT CC_3
Exon 3 reverse	5_AGC CCC AGC TCC CCA ACT CC_3

Table 3: PCR primers for the amplification of exons one to three of the BAFFR gene

BAFF cDNA	primers
P1 forward	5_GAT GCA GAA AGG CAG AAA GG_3
P1 reverse	5_CTG AAC GGC ACG CTT ATT TC_3
P2 forward	5_CCT CAC GGT GGT GTC TTT CT_3
P2 reverse	5_TGG TAT TTT CAG TTA GAT TCT TTC TTC_3

Table 4: PCR primers for the amplification of exons one to three of the BAFFR gene

2.6 PCR analysis

All amplified PCR products were visualized with gel electrophoresis in a 1% agarose gel. Ethidium bromide (0.5µg/ml) was added to enable the detection of double stranded DNA fragments under ultraviolet light. The agarose gel was loaded with 2 µl PCR product and 4 µl 6x loading buffer (Sigma-Aldrich, UK). The molecular weight marker Hyperladder I (200bp- 10.000bp) (Bioline, UK) was used to verify PCR fragment size. The DNA fragments were visualized with a gel documentation system with UV light (295 nm).

2.7 Sequencing Reactions

PCR products from section 2.5 were purified before proceeding to the sequencing reaction according to a purification protocol provided by Applied Biosystems (ABI). PCR products were digested with 1µl Shrimp alkaline phosphatase (Promega, Madison, MI) and 0.5µl Exonuclease I (Thermo Scientific, Waltham,MA) added to 20 µl PCR reaction. The enzyme digest was conducted in a PCR machine, for 15 minutes at 37°C followed by 15 minutes at 80°C. The purified PCR products were then used in the sequencing

reactions using ABI PRISM BigDye Terminator cycle ready reaction kit V3.1 (Applied Biosystems, Foster City, CA).

Sequencing was performed using a 20 µl master mix shown below:

1 µl Big Dye V3.1
4 µl 5x sequencing buffer
1.3 µl 2.5µM PCR amplification primer
12.7 µl dH2O
1 µl PCR product

Please note that for the sequencing reaction the amplification primer is either forward or reverse primer of the primers used for the PCR reaction. PCR conditions were used as in Table 5.

	Temperature in °C	Time	Number of cycles
Denaturation	96	1 min	1
Denaturation Annealing Extension	96 53-63 60	10 s 5 s 4 min	30

Table 5: The following table shows the sequencing programme in PCR machine

The same annealing temperatures and primer sequences were used as for the previous PCR reaction. Care was taken that the reactions were kept in the dark as Big Dye is labelled with a fluorescent dye and is therefore sensitive to light.

2.8 Sequencing

Sequencing reactions from 2.7 were precipitated loading onto the sequencing machine. For the precipitation, 2 µl 125 mM EDTA, 2 µl 3M sodium acetate and 50 µl 100% ethanol were added to the sequencing reactions. The samples were incubated at room temperature for 15 minutes in the dark and then centrifuged at 3500rpm for 30 minutes at 4°C.The supernatant was discarded carefully without disrupting the pellet. The pellet was washed with 120 µl 70% ethanol and again centrifuged at 3500rpm for 15 minutes at 4°C. As previously, the supernatant was discarded and the pellet was dried at 96°C for 3 minutes to eliminate any ethanol that would interfere with the sequencing. The pellet was dissolved in 30 µl H_2O before loading onto the sequencer. Sequencing was performed on the 3130xl Applied Biosystems Genetic Analyzer and the data was analyzed with Sequencing Analysis software, version 5.2 (Applied Biosystems, Foster City, CA) and Sequencher^{TM}, version 4.8 (Gene Codes Corporation, Ann Arbor, USA). Reference sequences were imported from the genbank “Ensemble” (http://www.ensembl.org/index.html) to align sequences to detect mutations.

2.9 BAFF-R surface staining

Patients’ frozen PBMCs were thawed. 10^6 PBMCs were stained with anti-human CD19 APC (BD), anti-human CD24 PE (BD), anti-human CD38 FITC (BD) and anti-human BAFFR clone HuBR9.1 biotin (Caltag). Cells were incubated for 10 minutes at room temperature and washed with 2 ml of FACS wash (PBS, 2% BSA) by centrifugation at 1500 rpm for 5 minutes. Supernatant was discarded and 300µl of 4% paraformaldehyde were added to fix the cells.

Cells were acquired by flowcytometry LSR II (BD Biosciences, Oxford UK) and analyzed with FlowJo 7.5.

2.10 Isolation of Peripheral Blood Mononuclear Cells (PBMC)

PBMCs were isolated from whole blood using a density gradient.
One volume of Lymphoprep (Axis-Shield;Oslo,Norway) was placed at the bottom of a conical tube. Whole blood was diluted 1:2 with medium without supplements. Diluted whole blood was slowly layered on top of the gradient avoiding mixing of the two solutions. The gradient is then centrifuged at 1600 rpm for 20 min with no break applied to avoid disruption of the interface. The mononuclear cells were visible at the interface. Plasma and other components were located above the interface and the gradient (Lymphoprep) below the interface. Erythrocytes and granulocytes were found in the pellet of the tube. The interface was carefully taken off with a Pasteur pipette and transfer to a new conical tube. PBMCs were washed with PBS and centrifuged at 1500 rpm for 5 min with the break applied. The supernatant was discarded and the cell pellet resuspended in 5 ml PBS. This step was repeated before counting the cells. A sample of the resuspended cells was diluted 1:50 in trypan blue (Sigma; Suffolk, UK). All dead cells appeared blue enabling to count the viable cells that are not blue. The cells were now ready for use or, alternatively for freezing (section 2.11).

2.11 Freezing of PBMCs

PBMCs were frozen as soon as the PBMC isolation was completed.
The cells pellet was resuspended in freezing medium, (40% complete RPMI medium, 50% FCS and 10% DMSO). The cells were adjusted to 5-10 x 10^6 per ml in freezing medium after counting the cells. The cells were then transferred to cryovials (NUNC; UK) and stored at -80C° over night in a polystyrene box or Mr. Freezy and transferred to liquid nitrogen (LN2) the following day for storage.

2.12 CD19 and IgM positive B cell isolation

Either $CD19^+$ or IgM^+ B cells were isolated (purified) using the Macs® magnetic MicroBeads separation system (Miltenyi Biotec Ltd; Surrey, UK). PBMCs were prepared and counted. They were washed with 10 ml of buffer (PBS, 0.5% bovine serum albumin and 2mM EDTA) by centrifugation at 1500 rpm for 10 min. Supernatant was discarded and the cell pellet was resuspended in 80 µl of buffer and 20 µl of either anti-IgM or anti-CD19 magnetic MicroBeads per 10^7 total cells. The tube was mixed well and incubated for 15 min at 4°C. Cells were washed with 1-2 ml of buffer and centrifuged at 1500 rpm for 10 min. Supernatant was discarded and the pellet was resuspended in 500 µl of buffer. A LS separation column was placed in the magnet and rinsed with 3 ml of buffer. The cell mix was applied to the column and washed 3 times with 3 ml of buffer. Flow-through containing unlabeled cells was collected in a tube under the column. The column was removed from the magnet and placed in a new tube. The labelled cells (attached to magnetic beads) were then flushed out with 5 ml of buffer by firmly

pushing the plunger into the column. The purified $CD19^+$ or IgM^+ cells were counted.

2.13 FACS screening for auto-antibodies

$CD19^+$ and IgM^+ B cells from healthy donor PBMCs were positively selected using anti-human CD19 or -IgM MicroBeads (Miltenyi Biotec Ltd., Surrey UK) with the method described in 2.12. Prior to beginning the experiment, patients' sera was inactivated for 30 min at 56°C. The Fc receptors of the isolated cells were blocked by incubation with 2 µl (0.5mg/ml) anti-mouse CD16/32 (eBioscience, Ltd., Hatfield UK) + 98 µl PBS on ice for 30 min, followed by washing 1 x with PBS at 1500rpm for 5 min. 100µl of 1:100 diluted inactivated patient's serum was added to either isolated IgM+ or CD19+ B cells and incubated for 1-2 hours on ice, then stained with anti-human CD19 APC, anti-human IgM PE-Cy5 and anti-human IgG PE (all from BD Biosciences, Oxford UK). As a positive control for the detection of IgG auto-antibodies, 0.5µg Rituximab (MabThera, Roche, Welwyn Garden City UK) was added to a healthy donor serum. For the detection of IgM auto-antibodies a patient with high rheumatoid factor was used as a positive control. Cells were acquired by flowcytometry LSR II (BD Biosciences, Oxford UK) and analyzed with FlowJo 7.5.

3 Results

3.1 Auto-antibodies against B cells

3.1.1 Background

20-30% of CVID patients suffer from autoimmune complications and in approximately 10% of patients low total B cell numbers are described. In a cohort of 180 CVID patients, 36 have been identified with low B cell numbers and/or autoimmune manifestations. The majority of CVID patients have autoimmune cytopenias but autoimmune thyroiditis, vitiligo, colitis and Crohn's disease are also seen in CVID patients. In our cohort, autoimmune cytopenias (25%) were most prevalent compared to the other autoimmune manifestations in CVID.

Within the identified CVID cohort of 36 patients, 22 had an absolute CD19+ count of < 0.1 $x10^9$/l, ten had autoimmunity as well as an absolute CD19+ count of < 0.1 $x10^9$/l, and four had autoimmune manifestations only (Table 6). The reason why CVID patients have decreased B cell numbers and why CVID patients develop autoimmune manifestations is still unknown.

22 samples low B cell numbers (absolute CD19+ count of < 0.1 $x10^9$/l) **but no** autoimmune manifestation	**10 samples** low B cell numbers (absolute CD19+ count of < 0.1 $x10^9$/l) and autoimmune manifestation	**4 samples** autoimmune manifestation **but** normal B cell numbers
	1 ITP and AIHA 5 ITP 1 Vitiligo 1 Crohn's disease 1 celiac disease 1 autoimmune thyroid	2 ITP 1 AIHA 1 autoimmune thyroid

Table 6: Summary of all CVID patients screened in this test

3.1.2 Hypothesis

Based on the above observation, we hypothesised that CVID patients with low B cell numbers and/or autoimmunity may have auto-antibodies against B cells, suggesting that CVID is a primary autoimmune condition, which would explain the hypogammaglobulinemia through autoimmunity.

3.1.3 Experimental set up

3.1.3.1 Detection of IgG auto-antibodies against B cells

To detect putative auto-antibodies against B cells, a FACS based assay was established. Many CVID patients have low B cell numbers and therefore the assay using CVID patients' serum was done on healthy donor B cells. IgM^+ B cells were purified from PBMCs using MACS magnetic bead separation. Serum of 36 CVID patients (Table 6) was incubated with IgM^+ purified B cells from healthy donors to detect auto-reactive IgG antibodies. The assumption was that if patients have IgG auto-antibodies against B cells they will bind to healthy donor IgM^+ sorted B cells and shift from a single positive IgM population to a double positive IgM/IgG population. (Fig. 1) This approach assumes that patients' B cells and healthy donor B cells carry the same epitopes/antigens.

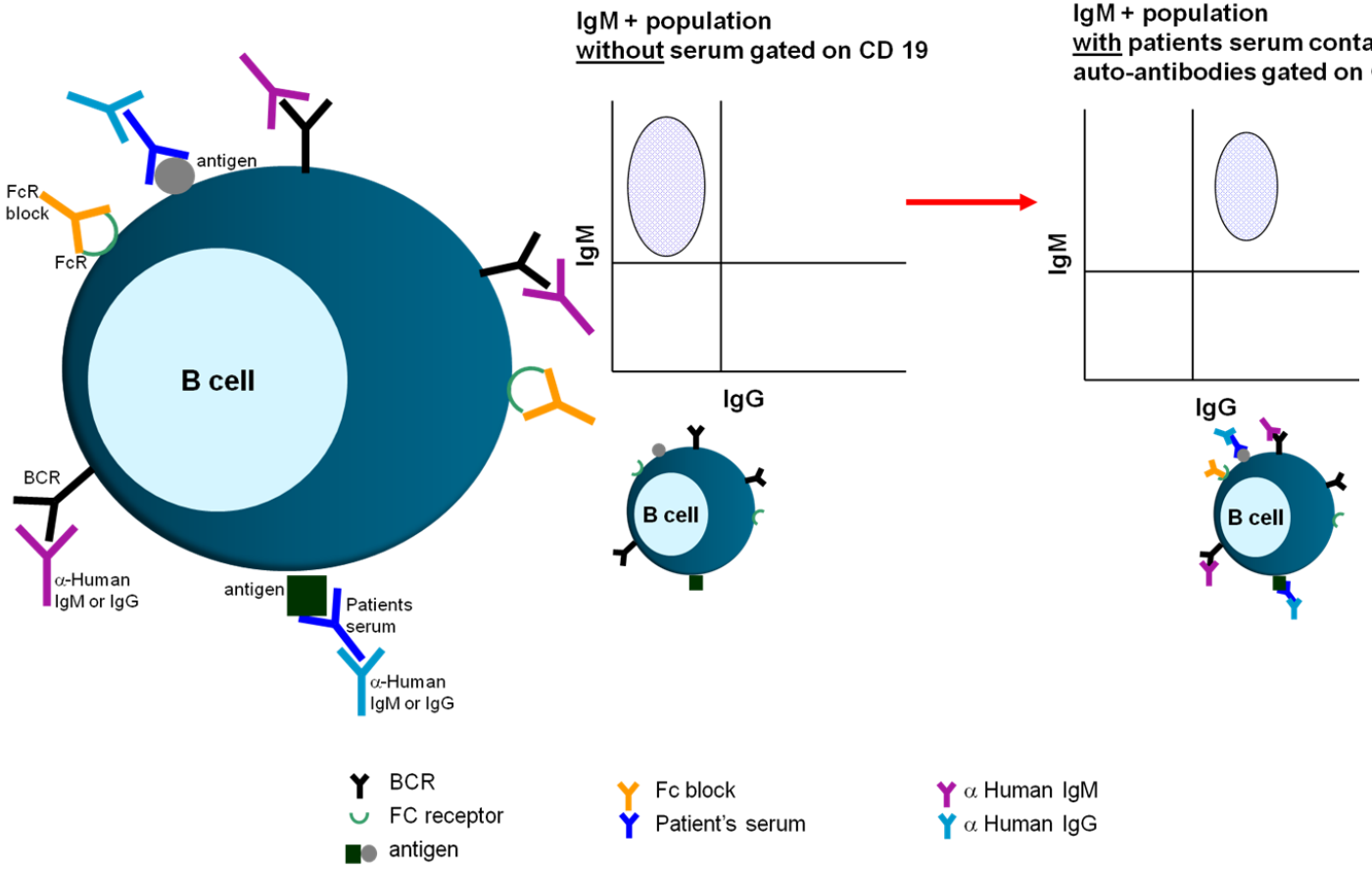

Figure 1: Schematic of experimental set up

IgM positive B cells from healthy donor were blocked with anti-mouse CD16/32 and then incubated with heat inactivated patients serum. Cells were then stained with anti-human IgM and anti-human IgG. If there were IgG auto-antibodies binding to an antigen on the surface of B cells then the single positive IgM population would shift to double positive as shown below.

To keep non-specific binding of antibodies in the serum to a minimum, a serial dilution of healthy donor serum was carried out. This was performed on IgM $^{+}$ B cells of healthy donors (Fig.2A). Whereas the dilution 1:50 showed non-specific IgG binding, the dilutions 1:100 and 1:200 did not. Thus the dilution 1:100 was used in all further experiments.

Furthermore, potential binding of patients' IgG to Fc-receptors on B cells needs to be blocked to avoid false positive staining. The binding of IgG to the Fc receptor (FcγRII-B1; CD32) on B cells has an inhibiting effect on B cell activation, antigen presentation and Ig production (Toshiyuki Takai *et al* 2002). Blocking of Fc-receptors can be achieved by adding serum of a different species. In a first approach, goat's serum was used to block Fc-receptors before patient's heat inactivated serum was added as shown in Fig.2B. This experiment was conducted on PBMCs while, all other experiments were done on IgM $^{+}$ B cells. All serum samples were heat inactivated at 56°C for 30 minutes to avoid cell lysis from complement binding. Alternatively, a commercially available anti-mouse CD16/32 (Fig.2C) was tested. No difference between blocking and no blocking was seen. However, the anti-mouse CD16/32 was used in further experiments as blocking reagent.

Anti-human CD20 IgG (Rituximab) served as a positive control. A serial dilution was done to determine the appropriate dilution (Fig.3). The concentration of 50ng/ml showed a proper shift to a double positive population and was used in all experiments. As negative control a healthy donor serum was used in every experiment.

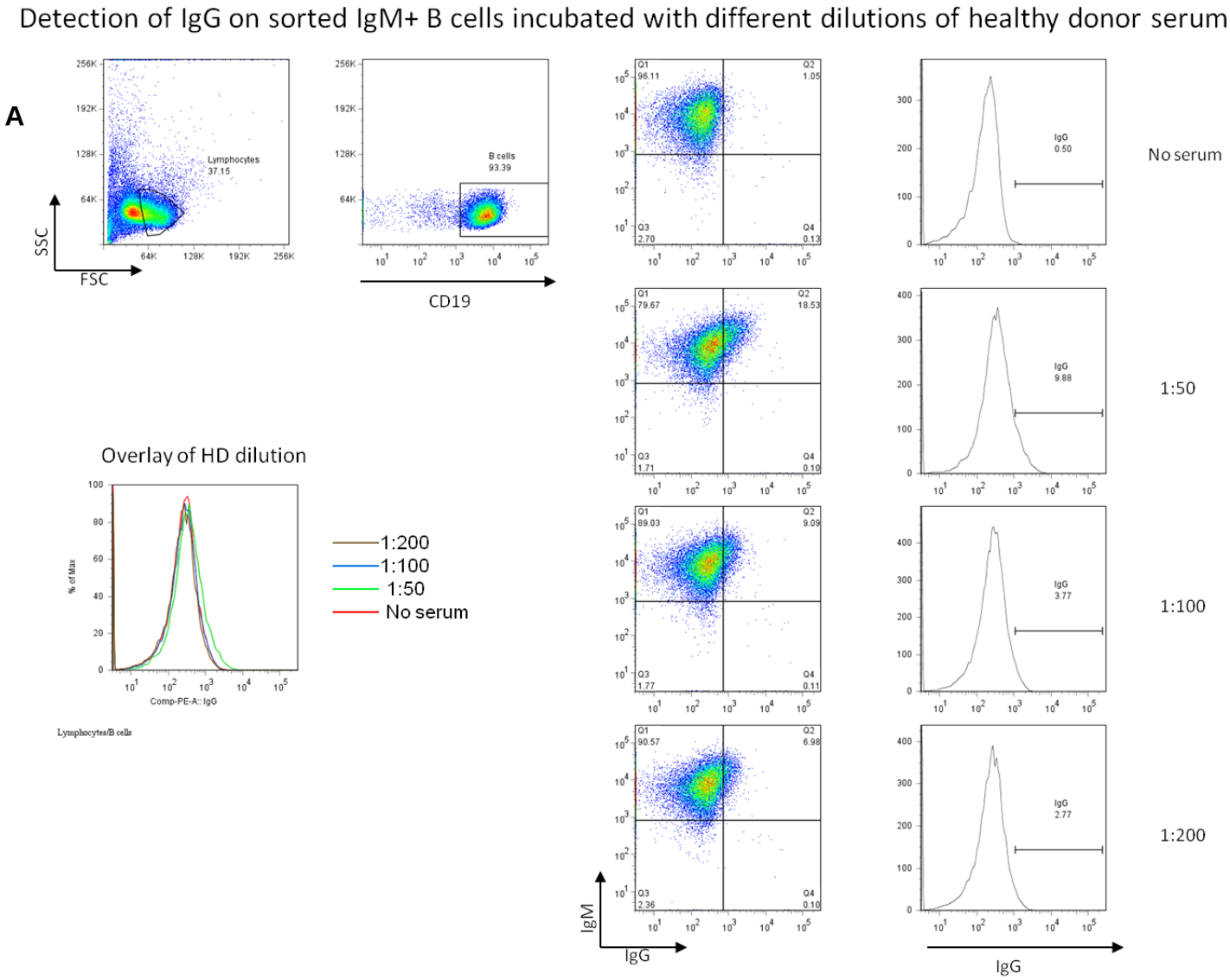

Figure 2: Serial dilution of serum and Fc blocking options

A: Detection of IgG with staining of IgM^{+} $CD19^{+}$ B cells; First gate: Lymphocytes gated according to SSC and FSC; second gate: B cells gated on CD19 expression of IgM and IgG. Dilution of healthy control samples : no serum, 1:50, 1:100 and 1:200 were tested to determine working dilution of serum samples added to healthy donor target cells.

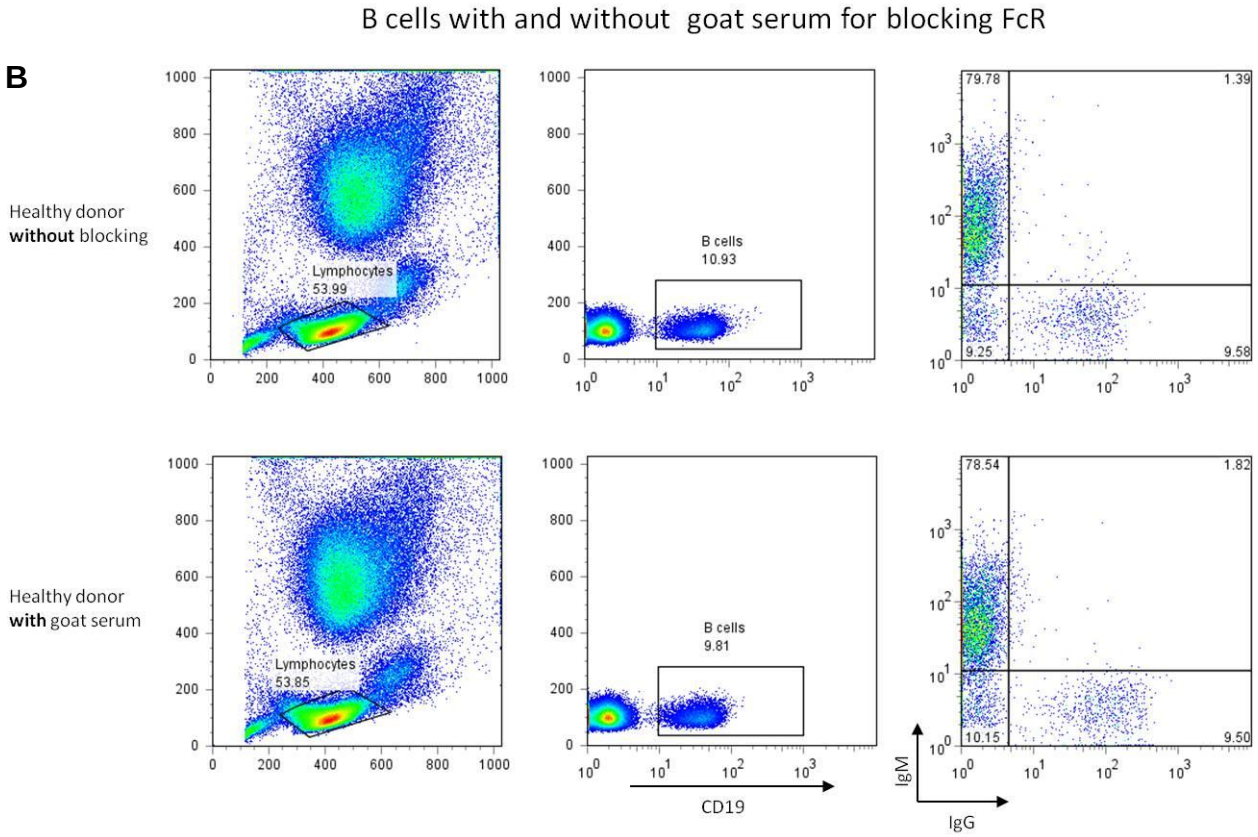

Figure 2: Fc blocking options

B: First gate: Lymphocytes gated according to SSC and FSC; second gate: B cells gated on CD19 expression of IgM and IgG. Healthy donor PBMC used as target cells, with or without blocking of FcR with goat's serum before adding 1:100 diluted healthy donor serum.

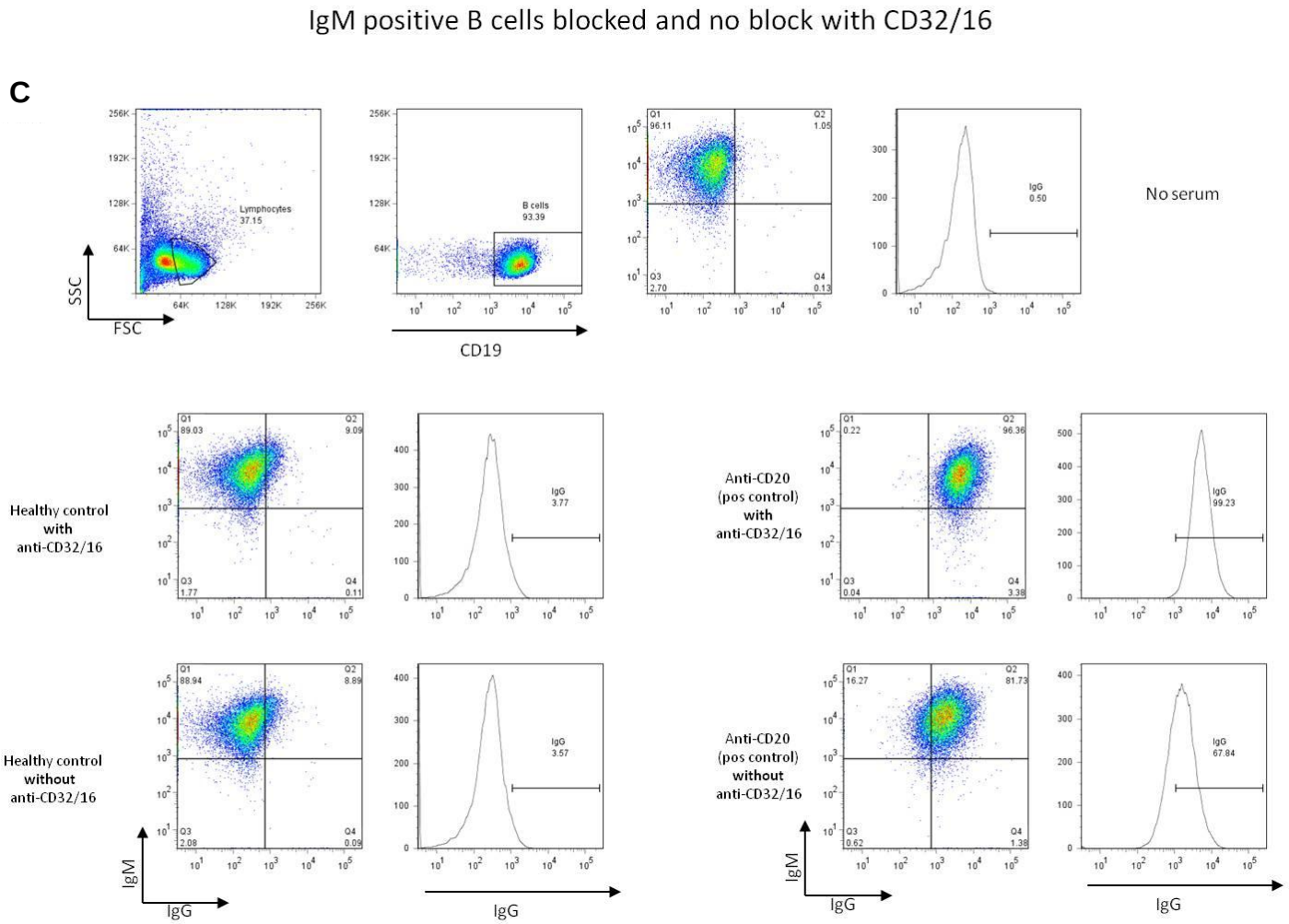

Figure 2: Fc blocking options

C: IgM^{+} B cells gated as in **A**; healthy control serum with or without added anti mouse CD32/16 for blocking of FcR. Additionally, staining with anti-CD20 human IgG with and without anti-mouse CD32/16.

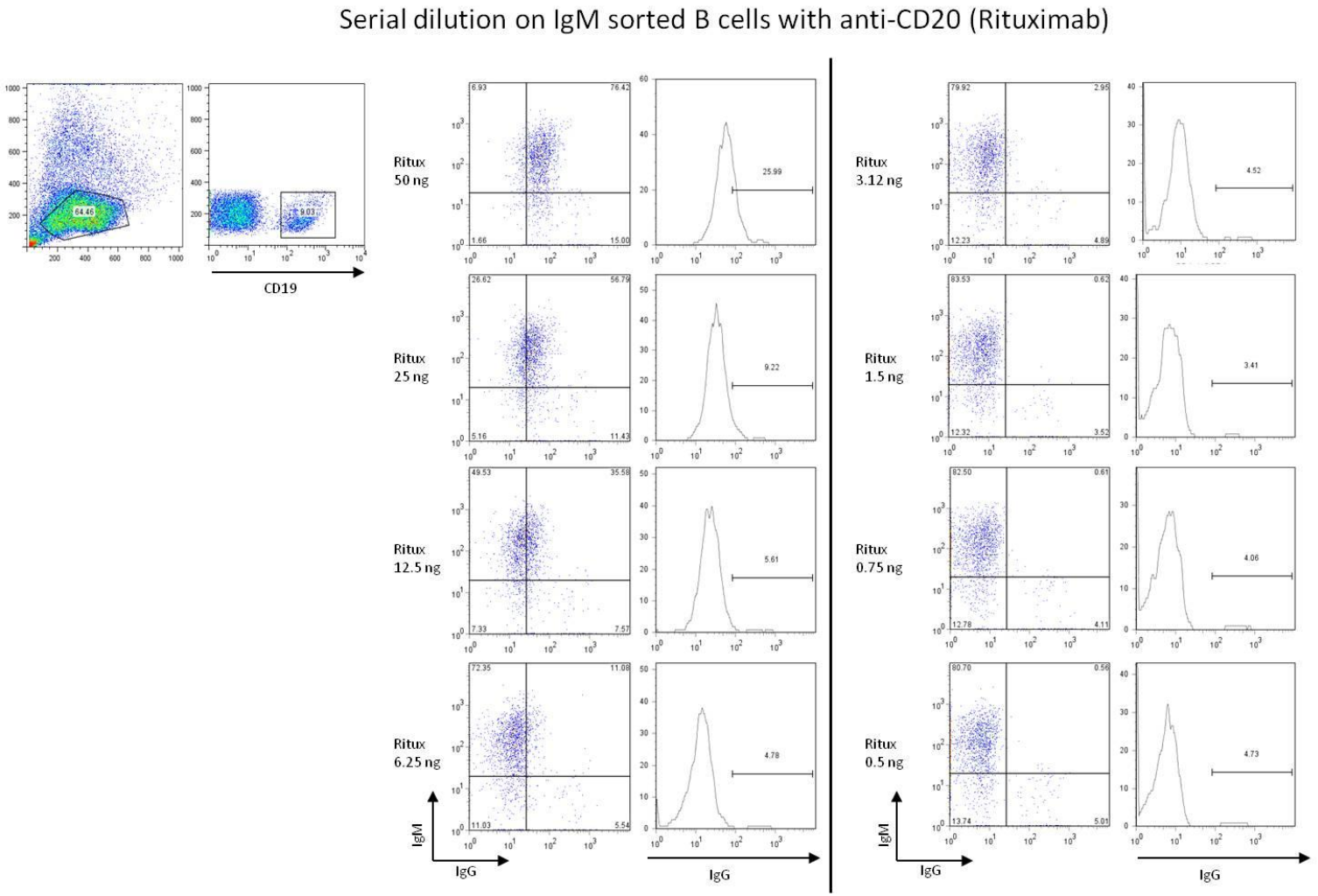

Figure 3: Anti-CD20 positive control dilution

Anti-CD20 IgG (Rituximab) served as positive control; a serial dilution was done to find the appropriate amount of anti-CD20 IgG to be used in further experiments. Staining of IgG and IgM lymphocytes gated according to SSC and FSC, then gated on CD19.

3.1.3.2 Screen for IgM auto-antibodies against B cells

To detect IgM auto-antibodies against B cells, the most straightforward approach would have been to isolate IgG $^{+}$ B cells. However this is a very small population, hence it was too difficult to isolate enough cells and a clean population for appropriate cell numbers to conduct this experiment. To circumvent this problem, CD19 $^{+}$ B cells were isolated. The same experimental approach was conducted as for the detection of IgG auto-antibodies against B cells. In search for a positive control, a patient's serum with high rheumatoid factor was thought to be appropriate, since these patients have anti-IgG IgM antibodies in their serum (Fig. 4). It was difficult to establish a titration curve of this serum. Therefore the same dilution of screened patient's sera of 1:100 dilution of the serum was added to the $CD19^{+}$ B cells. Addition of the serum of patient with high rheumatoid factor to the healthy control cells gave rise to a cell population that resulted in a slight significant shift to the double positive IgG^{+} IgM^{+} quadrant.

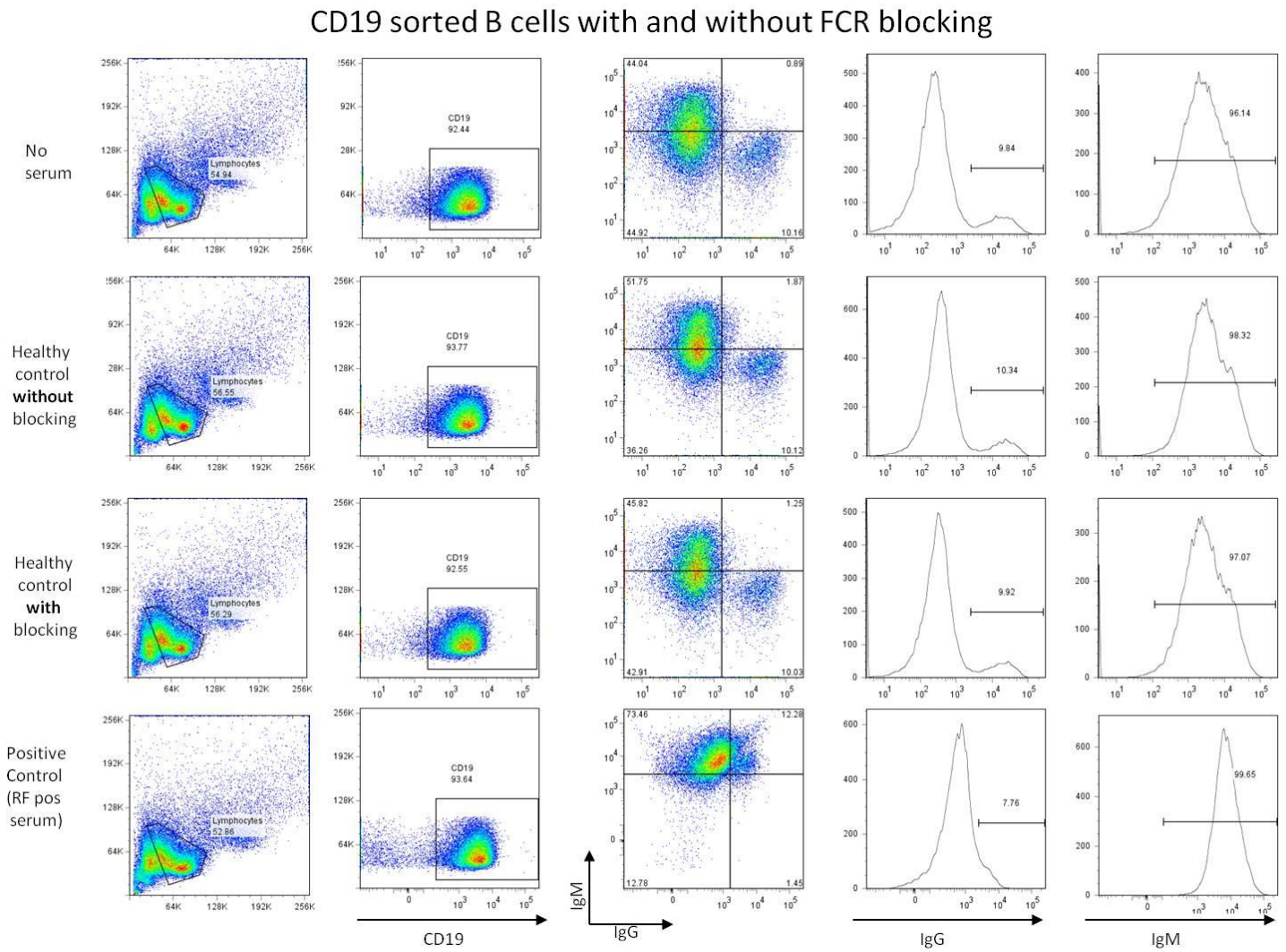

Figure 4: results of IgM screen

CD19^{+} B cells were used to detect possible IgM auto-antibodies against B cells. First gate: lymphocytes gated according to SSC and FSC; second gate: B cells gated on CD19 expression of IgM and IgG. **A:** No serum added served as negative control whereas the serum of a patient with high rheumatoid factor was used as a positive control. Healthy donor and patients' serum were added to target cells at a dilution of 1:100.

3.1.4 Results

3.1.4.1 FACS assay to detect IgG auto-antibodies against B cells

Using the FACS staining protocol described in 2.13, 36 CVID patient sera were analysed for the presence of IgG antibodies directed against purified healthy donor IgM $^{+}$ B cells.

One patient showed a shift from the IgM single positive to the IgM and IgG double positive population indicating the binding of serum IgG to the healthy donor B cells (Fig.5). Analysis of clinical data showed however that this patient had been treated with Rituximab (anti-human CD20) six months before the serum sample had been taken and therefore the staining was probably due to small amounts of Rituximab still present in the serum rather than due to IgG auto-antibodies. All other patient sera showed no detectable auto-antibodies/ IgG binding to the healthy donor B cells.

3.1.4.2 IgM auto-antibodies against B cells

After no IgG auto-antibodies against B cells were found, it was important to additionally exclude IgM auto-antibodies against B cells. (Fig.6) However, as none of the patients' sera showed a shift of the cell population we therefore concluded that there were no IgM auto-antibodies against B cells in CVID patients with autoimmunity and /or low B cells.

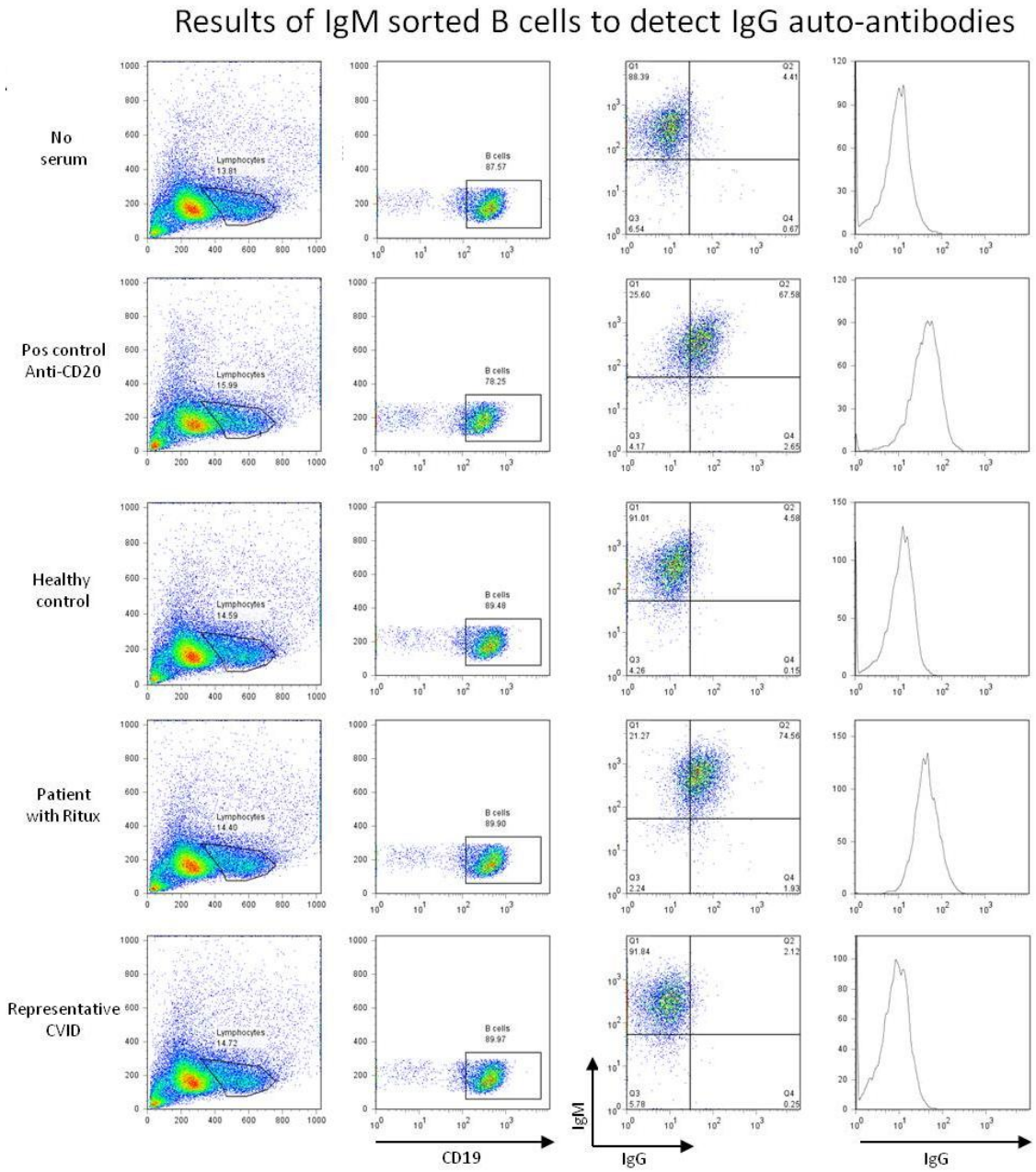

Figure 5: Results of IgG screen

IgM positive selected B cells. First gate: Lymphocytes gated according to SSC and FSC; second gate: B cells gated on CD19 expression of IgM and IgG. No serum added served as negative control whereas anti-CD20 IgG (Rituximab) 50ng/ml as positive control. Healthy donor and patients serum were added to target cells at a dilution of 1:100. Patient treated with Rituximab showed double positive shift.

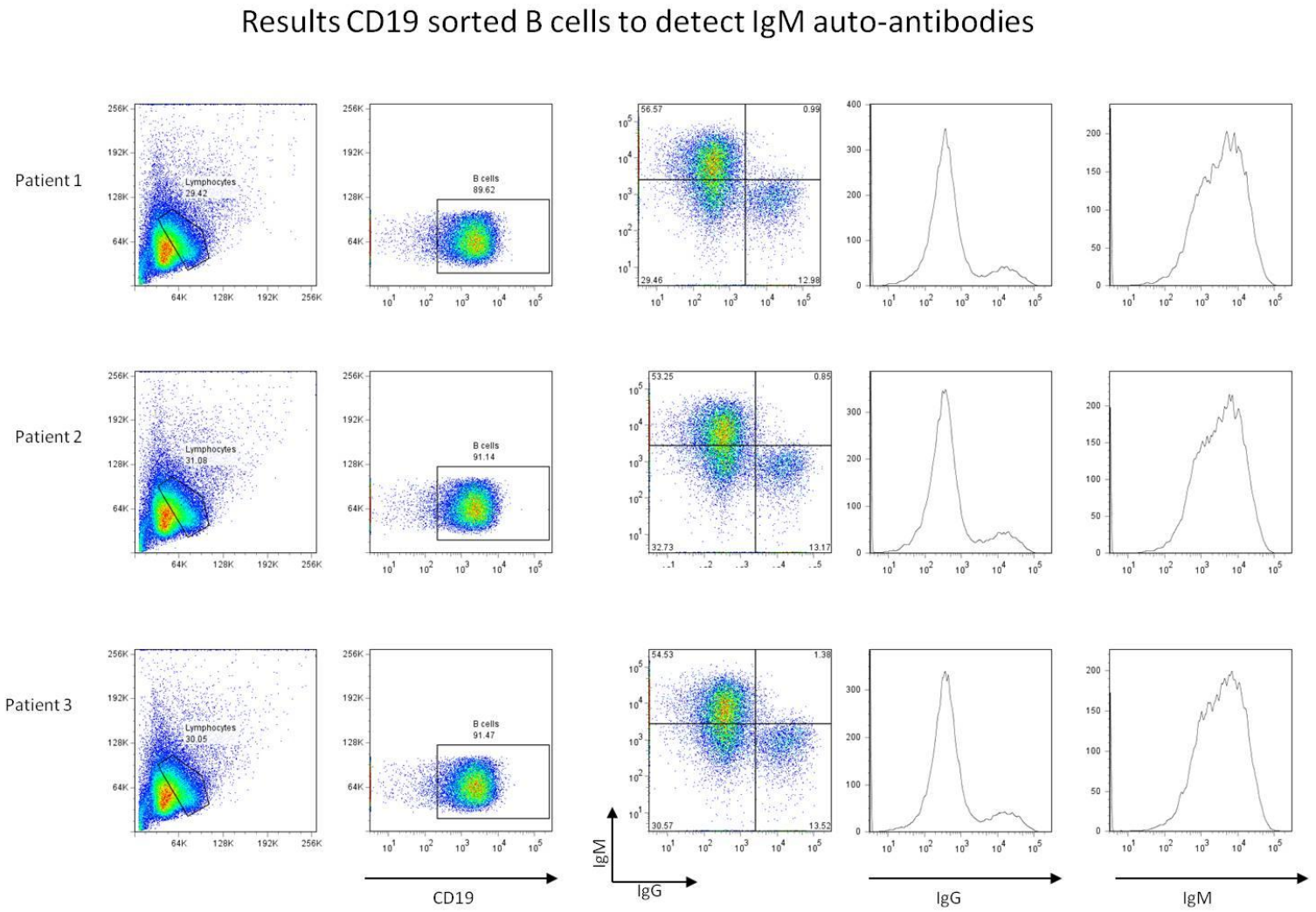

Figure 6: results of IgM screen

$CD19^{+}$ B cells were used to detect possible IgM auto-antibodies against B cells. First gate: lymphocytes gated according to SSC and FSC; second gate: B cells gated on CD19 expression of IgM and IgG. Patients serum were added to target cells at a dilution of 1:100.

3.1.5 Summary: Auto-antibodies against B cells

In 1977, a patient with Hypogammaglobulinemia was reported to have IgM antibodies against B lymphocytes (Tursz T *et al* 1977). A different publication described a screen of healthy controls showing antibodies against B cells (Park M K *et al* 1977). The approach used in these publications was a microcytotoxicity test and both could describe only cold antibodies against B lymphocytes. The literature does not reveal any other publication since 1977 investigating auto-antibodies against lymphocytes. To understand why some CVID patients have low B cell numbers and autoimmune manifestations, we aimed to determine if these CVID patients have auto-antibodies against B cells. Due to the low B cell numbers in the patients tested, it was easiest or in some cases even the only option to use healthy donor PBMCs in the FACS assay to screen patient's serum. No auto-antibodies were detected using this approach (Fig. 5 and 6). However, there remains the possibility that there are unique antigens expressed or epitopes exposed on the patients' B cells which are not present on healthy donor B cells. If this was the case, potential auto-antibodies against these antigens would not be able to bind to healthy donor PBMCs and could therefore not be detected with this assay. No auto-antibodies in CVID patients' serum have been detected in the screen.
As the results suggest that there are no auto-antibodies against B cells in CVID patients, the question why these patients develop autoimmune manifestations and have low B cell numbers still remains to be answered.

3.2 B cell survival signals in CVID patients

3.2.1 Introduction

To further investigate why CVID patients have low B cell numbers, we focused on B cell survival signals. BAFF (B cell activating factor) is one of the most important B cell survival signals and belongs to the TNF family. It is expressed on the surface of monocytes, dendritic cells, neutrophils, stromal cells, activated T cells, malignant B cells and epithelial cells and binds to three different receptors: BAFF-R, TACI and BCMA. These are expressed at various times during B cell development. Most of the expressed BAFF is cleaved from the cell surface and circulates in a soluble form binding to BAFF-R. BAFF enhances long term B cell survival and is important in the differentiation into plasma cells. Reduced B cell survival due to disturbed BAFF/BAFF-R signalling might be another reason for the low B cell numbers seen in 10% of CVID patients.

Mutational analysis has been conducted on BAFF (*TNFSF13B*) and BAFF-R (*TNFRSF13C)* (Losi C *et al* 2005 and 2006). Polymorphisms have been described in these two genes but are not known to be disease causing.

BAFF is located on chromosome 13q34 and has six exons. Exon one codes for the transmembrane domain and its flanking regions. Exon two codes the furin processing site and exons three to six code for the TNF homology domain (THD) that binds to receptors. The BAFF-R is located on chromosome 22q13 and has three exons. Exon one encodes the cystein rich domain (CRD). Exon two encodes the transmembrane domain and flanking regions and exon three encodes the intracellular domain.

In collaboration with Prof. Eibel from the University Hospital Freiburg, BAFF serum levels of 85 CVID patients' in our cohort were measured by ELISA (Kreuzaler M *et al* 2012). Interestingly, there was a cohort with highly elevated BAFF serum levels and a cohort with extremely low BAFF serum levels.

3.2.2 Hypothesis

We hypothesised that CVID patients with low B cell numbers and low BAFF serum levels have a mutation in BAFF, whereas CVID patients with low B cell numbers and high BAFF serum levels have a mutation in BAFF-R. We therefore identified those patients that have low B cells numbers (absolute CD19+ count of < 0.1 $x10^9$/l) from the cohort of 85 patients whose serum was run on the ELISA screen and subjected them to sequence analysis.

3.2.3 Results

3.2.3.1 BAFF and BAFF-R genomic DNA sequencing

To identify patients with possible BAFF or BAFF-R mutations, screened patients were selected for sequencing according to their BAFF serum levels and B cell phenotype. Healthy ranges of BAFF serum levels were determined in 250 healthy controls by collaborators of Prof. Eibel; the healthy ranges and other ranges seen in various diseases are listed in Table 7. The data interpretation for further sequencing of BAFF and BAFF-R are based on these findings.

Evaluated BAFF levels	0 ng/ml	0.5-2 ng/ml Healthy range	2-5 ng/ml After Rituximab treatment	5-10 ng/ml Autoimmune Diseases	>10 ng/ml CVID, Agammaglobulinemia, WAS and HIGM
Distribution of 85 Screened patient	1	24	28	11	20

Table 7: BAFF ELISA results of 85 CVID patients. The healthy ranges were assessed by screening with Prof. Eibels collaborators

Nineteen patients were selected for *TNFSF13B* (BAFF) sequencing; they had normal to low BAFF levels between 0.5 – 5 ng/ml and an absolute CD19+ count of < 0.1 $x10^9$/l. No mutations were found in these 19 patients. Seventeen patients had elevated BAFF levels (>5 ng/ml) with an absolute CD19+ count of < 0.1 $x10^9$/l and in addition showed >10% transitional B cells, which is a hallmark for CVID. These patients were selected for the sequencing of *TNFRSF13C* (BAFF-R). Out of the 17 patients screened, four had a previously described heterozygous P21R (CCG>CGG proline to arginin) SNP in exon 1 of BAFF-R (Losi C *et al* 2005) (Fig.7A*)*. One patient showed a heterozygous polymorphism G64V (GGC->GTT/ G>T and C>T) in exon 2 of BAFF-R (Fig.7B), which has also been described previously (Losi C *et al* 2005). A novel polymorphism, heterozygous R106Q (CGG->CAG/ G>A), was found in exon 2 of BAFF-R in one patient (Fig.7C). All other patients had a wild-type sequence in either BAFF or the BAFF-R, respectively.

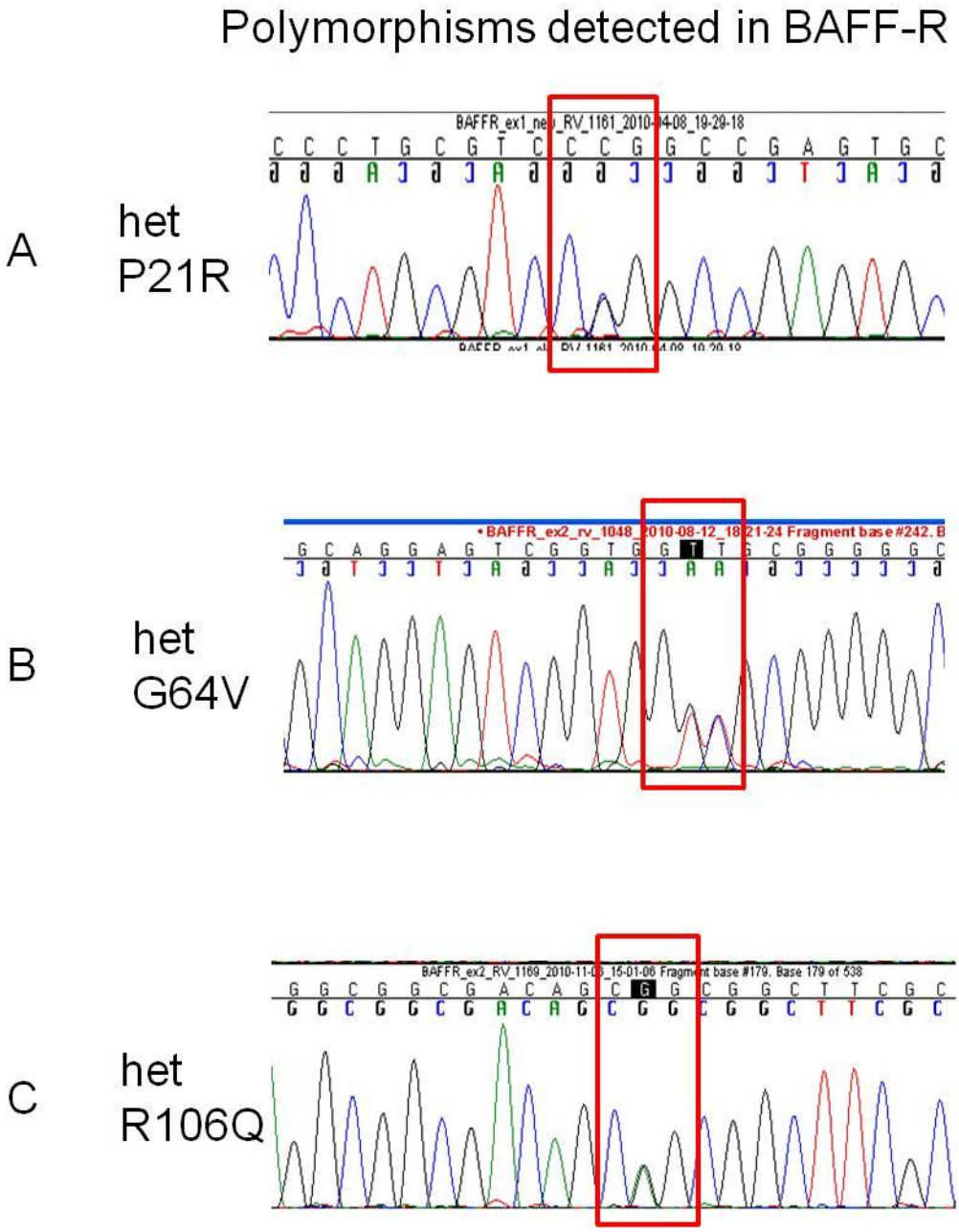

Figure 7: Sequencing results of 2 rare polymorphisms and one novel polymorphism in *TNFRSF13C,* the gene encoding BAFF-R. **A:** a rare heterozygous P21R polymorphism in exon 1 of BAFF-R showing a SNP from CCG → CGC in four patients; **B:** rare heterozygous G64V polymorphism in exon 2 of BAFF-R showing two SNPs from GGC → GTT in one patient and **C:** showing a novel SNP R106Q CGG → CAG in exon 2 of BAFF-R in one patient.

3.2.3.2 BAFF mRNA detection

The 19 patients with low BAFF serum levels that were shown to be BAFF wild-type by genomic DNA (gDNA) sequencing were further analyzed on the cDNA level to exclude promoter mutations or others that might have been missed by gDNA sequencing but would affect gene expression (Fig.8). Total RNA including mRNA was extracted from PBMCs of twelve patients and reverse transcribed into cDNA. Amplification of a PCR product with BAFF-specific primers confirmed the expression of BAFF mRNA in all twelve patients. Further sequencing was attempted to exclude splice site mutations but was not feasible due to high background in the sequencing reactions.

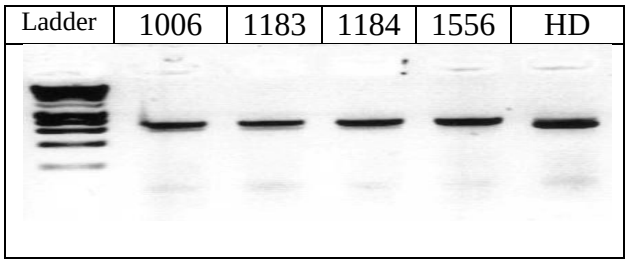

Figure 8: cDNA amplification of BAFF
cDNA amplification of patients representatively showing no mutations on genomic level.

3.2.3.3 Surface expression of BAFF-R on B cells of three patients with rare polymorphisms in *TNFRSF13C* encoding BAFF-R

Cell surface expression of BAFF-R was conducted on the three CVID patients who had polymorphisms in BAFF-R to find out if the polymorphisms have any impact on the expression on B cells. Patient's PBMCs were used for the staining. BAFF-R expression as well as transitional ($CD19^{+}$ $CD24^{hi}$ $CD38^{hi}$), memory ($CD19^{+}$ $CD24^{+}$ $CD38^{-/dim}$) and mature ($CD19^{+}$ $CD24^{dim}$ $CD38^{dim}$) B cell numbers were determined in this experiment. Two experiments were conducted on different days due to the availability of patient samples (Fig. 9A

and B). The expression level of BAFF-R was compared to that of a healthy donor stained for BAFF-R of total $CD19^+$ cells. Using an overlay of total $CD19^+$ population, surface expression of BAFF-R on B cells shows a slight reduction of BAFF-R expression in two patients with a het P21R and one patient with het R106Q, compared to healthy controls. The overlay of the B cell subpopulations also showed a slight reduction of BAFF-R expression. However, the percentage of cells was variable between subpopulations.

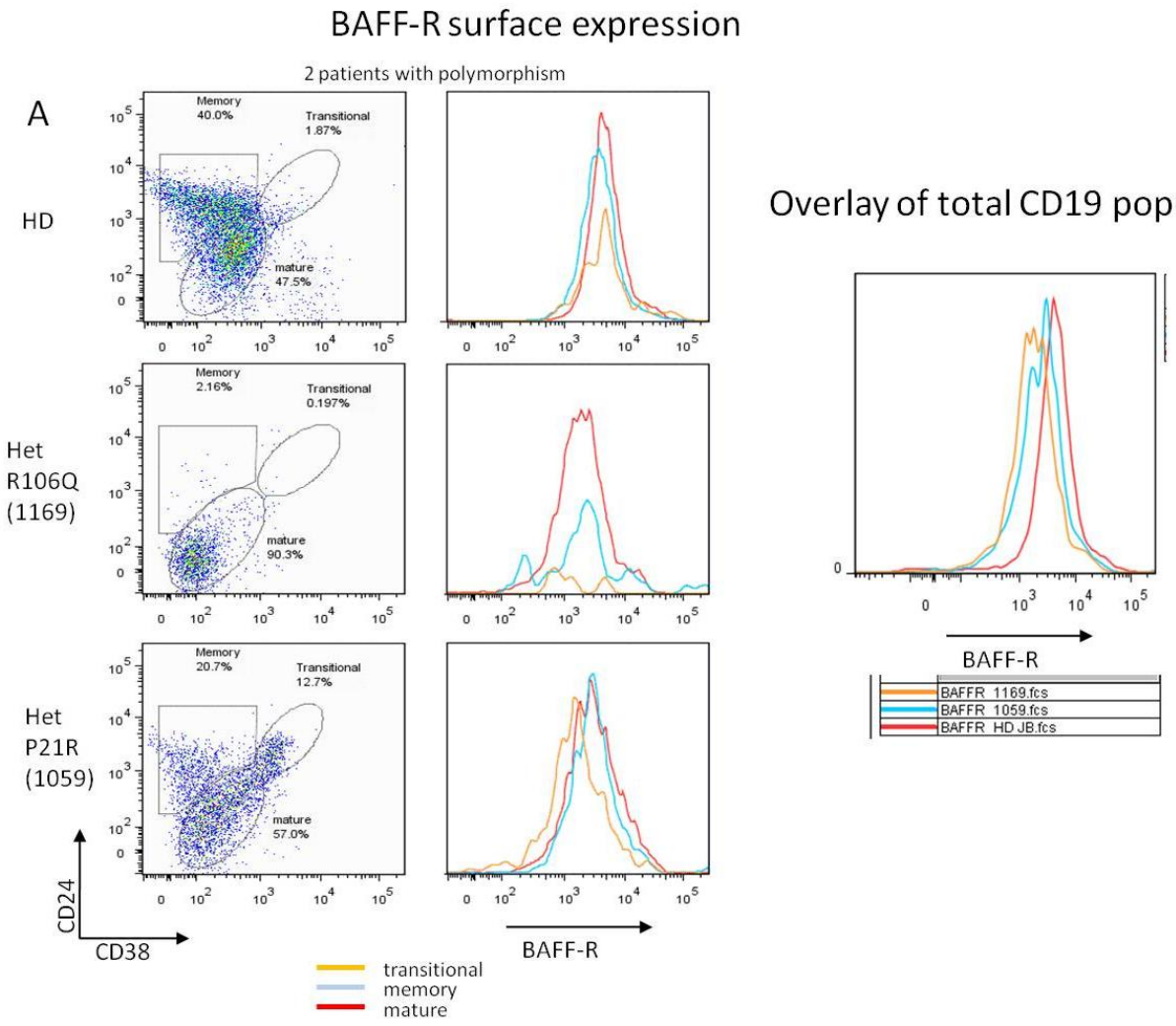

Figure 9A: BAFF-R expression

Frozen PBMCs were used in these experiments. Lymphocytes gated according to SSC and FSC (gate not shown); first gate: B cells gated on CD19, expression of CD24 and CD38. Memory, transitional and mature B cell subpopulations are shown.
A: patient with het R106Q and one patient with het P21R are compared to healthy control. Overlay of subpopulations and total CD19 population

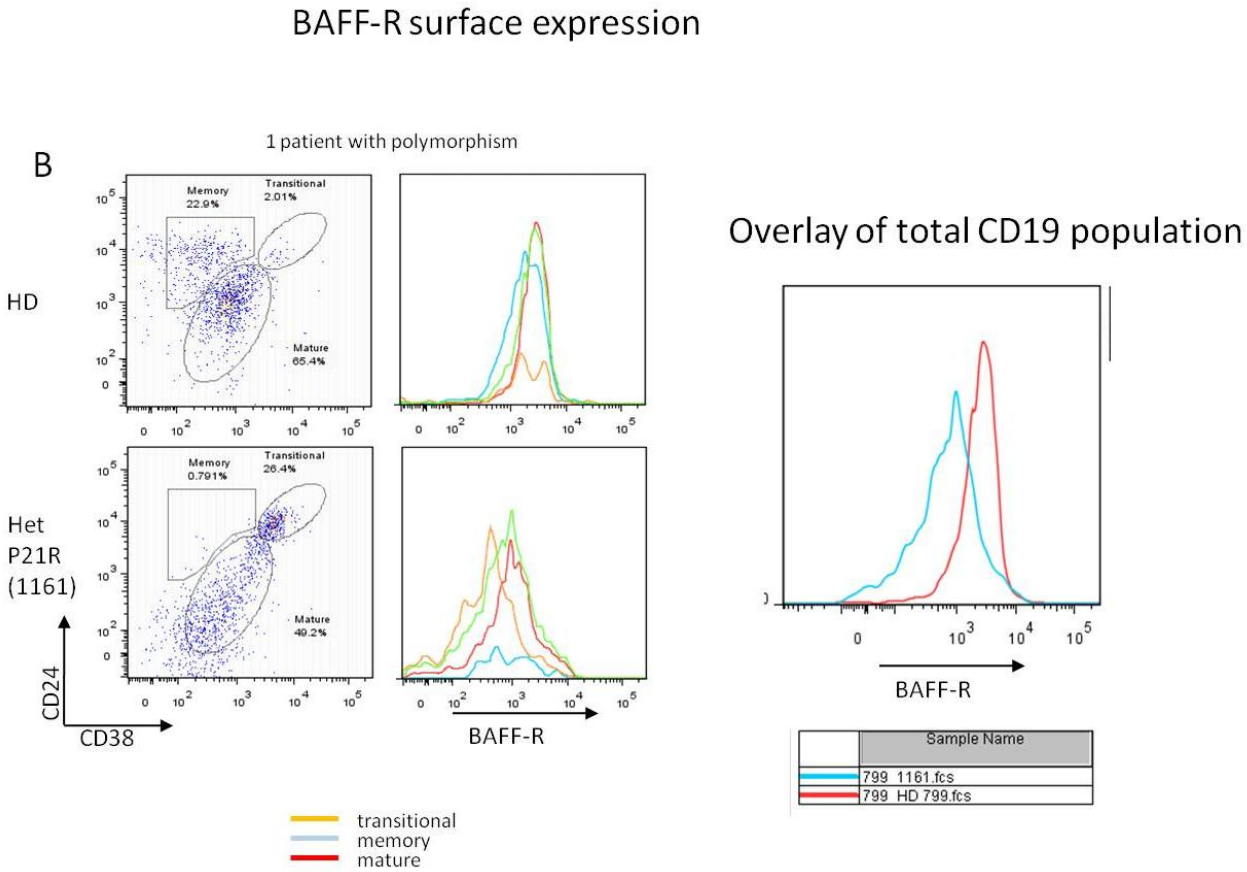

Figure 9B: BAFF-R expression

Frozen PBMCs were used in these experiments. Lymphocytes gated according to SSC and FSC (gate not shown); first gate: B cells gated on CD19, expression of CD24 and CD38. Memory, transitional and mature B cell subpopulations are shown. **B**: Patient with het P21R is compared with healthy control. Overlay of subpopulations and total CD19 population

3.2.4 Summary: B cell survival signals

High serum levels of BAFF have been detected in a cohort of CVID patients. One possibility is that this can be due to mutations in BAFF-R so that there might not be enough BAFF-R expression to capture circulating BAFF. We found six patients out of 17 with rare polymorphisms. Four showed a heterozygous P21R polymorphism in exon 1, one had a heterozygous G64V polymorphism in exon 2 and one had a het R106Q polymorphism also in exon 2. These polymorphisms are located in the extracellular domain of the receptor and might be involved in BAFF binding (Losi *et al* 2005). The BAFF-R expression on B cells of the three patients with BAFF-R polymorphisms showed no major impairment.

BAFF-R expression was not analyzed for the other three patients, because patients either had very low to no B cells (1x het P21R and 1x het G64V) or because we had no access to the patients' PBMCs (1x het P21R). Therefore, our hypothesis that CVID patients with low B cell numbers and low BAFF serum levels have a mutation in BAFF, and CVID patients with low B cell numbers and high BAFF serum levels have a mutation in BAFF-R could not be confirmed. An alternative hypothesis is that CVID patients with low BAFF serum levels have antibodies against BAFF, and this is under investigation.

4 Discussion

Extensive research on CVID has been carried out in the last decade. Genetic analysis performed on CVID patients showed mutations in various genes including *ICOS* (Grimbacher B *et al* 2003, Salzer U *et al* 2004), *CD19* (Van Zelm MC *et al* 2006, Kanegane H *et al* 2007), BAFF-R (Warnatz K *et al* 2005), TACI (Salzer U *et al* 2005, Castigili E *et al* 2005), *CD20* (Kuipers TW *et al* 2010), *CD21* (Thiel J *et al* 2011), *CD81* (Van Zelm MC *et al* 2010) and *MSH5* (Sekine H *et al* 2007). The cellular compartment has also been investigated to a very high degree with emphasis on understanding B cell function and signalling pathways (Warnatz K *et al* 2002, Wehr C *et al* 2008). The outcome of past research has led to a comprehensive tool used in the clinical setting: the classification of CVID patients using flow cytometric B cell phenotyping which can aid in the diagnosis of patients.

The aims of this project were to enhance the understanding of possible causes of CVID in patients with CVID and autoimmunity. In order to test the hypothesis whether CVID patients with low B cells and/or autoimmunity have auto-antibodies against B cells, 36 CVID patients with low B cells and autoimmunity were screened using a FACS based assay to detect possible auto-antibodies against B cells. Similar approaches have been established to detect antibodies against T cells (Liao JJ *et al* 2009) and neutrophils (Stella R *et al* 2010).

B cells from healthy controls were incubated with serum of these patients to see if auto-antibodies against B cells can be detected. No auto-antibodies

could be found using this approach (Fig. 5 and 6). To get a definite answer, however, patients B cells would need to be incubated with their own serum. This experiment could not be conducted because the patient cohort had very low (absolute CD19+ count of < 0.1 $x10^9$/l) to absent B cells. The experimental design we used is therefore limited as it will fail to detect unique antigens expressed or epitopes exposed on the patients' B cells which are not present on healthy donor PBMCs.

In this study, IgG or IgM auto-antibodies against B cells were not detected. As a positive control to detect possible IgG auto-antibodies, Rituximab was used and we identified one patient with auto-antibodies against B cells who had a Rituximab infusion approximately six months prior to drawing the serum sample. As the half-life of normal IgG in serum is said to be 21 days, we take this result as proof of a high sensitivity of our test.

In order to detect IgM auto-antibodies, $CD19^+$ B cells were isolated. The easier experiment would have been to isolate IgG^+ B cells, but since healthy donors only have about 10% of IgG^+ B cells this lead to the limitation of not having enough cells to conduct this experiment. The $CD19^+$ isolated population included both IgM^+ and IgG^+ B cells in the screening, with predominantly IgM^+ B cells. Since there was no shift in the single IgM population and none in the IgG population these results again suggest that there are no IgG and IgM auto-antibodies against B cells in CVID patients. As a positive control a patient's serum with high rheumatoid factor was used. The question why these patients develop autoimmune manifestations and have low B cell numbers still remains to be answered.

In addition to BCR signalling BAFF is the most important survival signal for B cells. Therefore, in order to find a cause for low B cell numbers in CVID, a screen for BAFF serum levels in collaboration with Prof. H. Eibel at the University Hospital in Freiburg was carried out (Kreuzaler M *et al* 2012).

The BAFF serum levels detected in our cohort of CVID patients were bimodal and at extreme ends of the normal range. Patients with high BAFF serum levels could possibly have mutations in BAFF-R resulting in insufficient BAFF-R expression, unable to capture circulating BAFF. Hence we performed BAFF-R sequencing. Six out of 17 patients showed rare polymorphisms in BAFF-R: four showed a heterozygous (het) P21R polymorphism and one a het G64V polymorphism. A novel polymorphism, het R106Q, was found in the sixth patient (Fig.7C). These polymorphisms are located in the extracellular domain of the BAFF-R and therefore can either lead to impaired BAFF binding or to impaired expression of the receptor. BAFF-R expression on B cells was analyzed on two patients with the het P21R allele and one patient with the het R106Q polymorphism but no major impairment (Fig.9A and B) in expression could be detected. B cells of the remaining three patients with rare polymorphisms could not be stained, either due to very low B cell count (1x het P21R and 1x het G64V) or simply because we did not have access to the patients' PBMC's (1x het P21R).

Conversely, DNA of 19 patients with very low to normal BAFF serum levels and low B cell numbers, with an absolute CD19+ count of < 0.1 $x10^9$/l, were screened for BAFF mutations. None of these 19 patients showed any

mutations on genomic DNA in their exonic BAFF sequences. To further exclude mutations in the promoter region and splice sight mutations, mRNA expression of twelve out of the 19 patients was evaluated. PCR amplification showed mRNA expression of BAFF with specific mRNA primers, making mutations in BAFF unlikely.

The results obtained showed that CVID patients with low B cell numbers and high BAFF serum levels had no mutations in the BAFF-R leading to impaired BAFF-R expression, and that CVID patients' with low B cell numbers and low BAFF serum levels had no mutation in BAFF.

Even though all experiments conducted did not support the hypothesis that CVID is a primary autoimmune condition, which would explain the hypogammaglobulinemia through autoimmunity and that CVID patients might have impaired B cell survival signals due to possible mutations in the BAFF-R and BAFF, our results contribute to ruling out disease causing factors in CVID (Janda A *et al* 2011; Kreuzaler M *et al* 2012; Losi C *et al* 2005 and 2006). Further investigation of whether CVID patients have auto-antibodies against other important factors involved in B cell survival should be pursued, experimental design permitting. Unfortunately, in this work it was not possible to find a reason why certain patients with CVID have low or absent B cells and/or develop autoimmunity.

CVID is a very heterogeneous disease and a better understanding of underlying pathogenesis can have important implications for patient care and management in the clinical setting.

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7 Abbreviations

AD-CVID	autosomal dominant common variable immunodeficiency
APC	allophycocyanin
APC	antigen presenting cells
APRIL	a proliferation-inducing ligand
AR-CVID	autosomal recessive common variable immunodeficiency
BAFF	B cell activating factor
BAFF-R	B cell activating factor receptor
BCMA	B cell maturation factor
BCR	B cell receptor
cDNA	complementary deoxyribonucleic acid
CVID	common variable immunodeficiency
DC	dendritic cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid disodium salt dehydrate
FACS	Fluorescence activated cell sorting
FcR	Fc receptor
FCS	fetal calve serum
FITC	fluorescein isothiocyanate
gDNA	genomic deoxyribonucleic acid
ITP	Idiopathic Thrombocytopenia
LN	lymph node
LN2	liquid nitrogen
NFκB	nuclear factor kappa B

PALS	periarteriolar lymphoid sheath
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	R-phycoerythrin
PE-Cy5	phycoerythrin and cyanine dye
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RT	reverse transcriptase
TACI	transmembrane activator and calcium-modulator and cyclophilin ligand interactor
TNF	Tumor necrosis factor