# Antigen-dependent induction of a CD40 signal in peripheral B cells

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# Summary

Monoclonal antibodies have emerged as one of the most promising therapeutics in oncology over the last decades. The generation of fully human tumorantigen-specific antibodies suitable for anti-tumor therapy is laborious and difficult to achieve. Autoreactive B cells expressing those antibodies are detectable in cancer patients and represent a suitable source for human antibodies. However, the isolation and cultivation of this cell type is challenging.

A novel method was established to identify antigen-specific B cells. The method is based on the conversion of the antigen independent CD40 signal into an antigen-specific one. For that, the artificial fusion proteins ABCos1 and ABCos2 (**A**ntigen-specific **B** cell **co-s**timulator) were generated, which consist of an extracellular association-domain derived from the constant region of the human immunoglobulin (Ig) G1, a transmembrane fragment and an intracellular signal transducer domain derived of the cytoplasmic domain of the human CD40 receptor. By the association with endogenous Ig molecules the heterodimeric complex allows the antigen-specific stimulation of both the BCR and CD40.

In this work the ability of the ABCos constructs to associate with endogenous IgG molecules was shown. Moreover, crosslinking of ABCos stimulates the activation of NF-κB in HEK293-lucNifty and induces proliferation in B cells. The stimulation of ABCos in transfected B cells results in an activation pattern different from that induced by the conventional CD40 signal. ABCos activated B cells show a mainly IgG isotype specific activation of memory B cells and are characterized by high proliferation and the differentiation into plasma cells. To validate the approach a model system was conducted: B cells were transfected with IVT-RNA encoding for anti-Plac1 B cell receptor (antigen-specific BCR), ABCos or both. The stimulation with the BCR specific Plac1 peptide induces proliferation only in the cotransfected B cell population. Moreover, we tested the method in human IgG$^+$ memory B cells from CMV infected blood donors, in which the stimulation of ABCos transfected B cells with a CMV peptide induces antigen-specific expansion.

These findings show that challenging ABCos transfected B cells with a specific antigen results in the activation and expansion of antigen-specific B cells and not only allows the identification but also cultivation of these B cells. The described method will help to identify antigen-specific B cells and can be used to characterize (tumor) autoantigen-specific B cells and allows the generation of fully human antibodies that can be used as diagnostic tool as well as in cancer therapy.

# Zusammenfassung

Monoklonale Antikörper stellen heute einen beachtlichen Teil des tumortherapeutischen Arsenals dar und haben sich fest in der klinischen Anwendung etabliert. Die Herstellung von humanen Antikörpern für die Behandlung von Tumoren ist äußerst schwierig. B Zellen, welche gegen tumorassoziierte Antigene Antikörper produzieren, stellen eine mögliche Quelle für solche humanen Antikörper dar. Allerdings ist bis heute die Identifizierung und Kultivierung dieser speziellen autoreaktiven B Zellen herausfordernd.

In dieser Arbeit wurde eine Methode entwickelt, die es ermöglicht, antigenspezifische B Zellen in einem Gesamtrepertoire an B Zellen zu identifizieren. Die Methode basiert auf der Umwandlung des Antigen-unabhängigen CD40 Signals in ein Antigen-abhängiges. Hierfür wurden die artifiziellen Fusionsproteine ABCos1 und ABCos2 (**A**ntigen-specific **B** cell **co-s**timulator) entwickelt, welche eine extrazelluläre Assoziierungsdomäne abgeleitet vom humanen IgG1, eine Transmembrandomäne und eine intrazelluläre CD40 Signalinduktions Domäne besitzen. Durch die Heterodimerisierung mit dem endogenen Immunglobulin entsteht ein artifizieller Rezeptor, welcher die gleichzeitige antigenspezifische Induktion vom B-Zell-Rezeptor (BZR) und CD40 Signal in B Zellen ermöglicht. Die ABCos Konstrukte sind in der Lage, durch Quervernetzung ein NF-κB Signal in HEK293-lucNifty und Proliferation in B Zellen zu induzieren. Die Antikörper-vermittelte Quervernetzung von ABCos führt zu einem phenotypischen Aktivierungsmuster in B Zellen, welches sich vom konventionellen CD40 Signal unterscheidet. Durch die ABCos spezifische Aktivierung werden hauptsächlich IgG$^+$ Gedächtnis B Zellen aktiviert, welche stark proliferieren und zu Plasmazellen differenzieren. Um die Methode zu validieren, wurden Modelsysteme verwendet: Zuerst wurden B Zellen entweder mit einen Plac1 Peptid spezifischen BZR, mit ABCos oder gleichzeitig mit BZR und ABCos transfiziert. Die Stimulierung mit dem BZR spezifischen Plac1 Peptid führt nur in ABCos und BZR doppelt-transfizierten B Zellen zu einer Aktivierung und der Induktion von Proliferation. Darüber hinaus wurde die Methode an humanen IgG$^+$ Gedächtnis B Zellen von CMV infizierten Patienten getestet. Nur IgG$^+$ Gedächtnis B Zellen, die mit ABCos transfiziert wurden, konnten mittels eines CMV spezifischen Peptids zur Proliferation gebracht werden und somit identifiziert werden.

Diese Daten zeigen, dass ABCos transfizierte B Zellen durch antigenspezifische Stimulierung aktiviert und expandiert werden können. Diese Methode ermöglicht die Identifizierung und Kultivierung von antigenspezifischen B Zellen und erlaubt die Charakterisierung von (tumor) autoantigenspezifischen B Zellen. Weiterhin ermöglicht

sie die Generierung von voll-humanen Antikörpern, welche für die Diagnostik oder Therapeutik von Interesse sind.

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# Abbreviations

| | |
|---|---|
| °C | **Degree C**elsius |
| µ | **Mikro** |
| 7-AAD | **7-A**mino**a**ctinomycin **D** |
| A | **A**mpere |
| aa | **A**mino **a**cid |
| ADCC | **A**ntibody-**d**ependent **c**ellular **c**ytotoxicity |
| ABCos | **A**ntigen-specific B cell co-stimulator |
| APC | **A**ntigen-**p**resenting **c**ell |
| ATP | **A**denosine **tri**phophate |
| BAFF-R | **B cell a**ctivating **f**actor of the TNF **f**amily |
| BCMA | **B cell m**aturation |
| BCR | **B cell r**eceptor |
| bp | **B**ase **p**air |
| CDC | **C**omplement **d**ependent **c**ytotoxicity |
| CDR | **C**omplementarity **d**etermining **r**egion |
| CFSE | **C**arboxyfluorescein **s**uccinimidyl **e**ster |
| CH / VH | **C**onstant **h**eavy / **v**ariable **h**eavy |
| CHO | **Ch**inese **h**amster **o**vary |
| CL / VL | **C**onstant **l**ight / **v**ariable **l**ight |
| CLP | **C**ommon **l**ymphoid progenitors |
| CMV | **C**yto**m**egalo**v**irus |
| CO₂ | **C**arbon **dio**xide |
| CPD | **C**ell **p**roliferation **d**ye |
| CpG-ODN | **C**ytosine **p**hosphodiester **g**uanine-**O**ligo**d**esoxy**n**ukleotide |
| DC | **D**endritic **c**ell |
| DMSO | **Di**methyl **s**ulf**o**xide |
| DNA | **D**eoxyribo**n**ucleic **a**cid |
| EBV | **E**pstein-**B**arr-**V**irus |
| EDTA | **E**thylene**d**iamine**t**etraacetic **a**cid |
| EGFR | **E**pidermal **g**rowth **f**actor **r**eceptor |
| ELP | **E**arliest **l**ymphocyte **p**rogenitors |
| ER | **E**ndoplasmic **r**eticulum |
| ERK | **E**xtracellular signal-**r**egulated **k**inases |
| EtBr | **Et**hidium**br**omid |
| F | **F**araday |
| FACS | **F**luorescence **a**ctivated **c**ell **s**orting |
| Fc | **F**ragment **c**rystallizable region |
| FCShi | **F**etal **c**alf **s**erum (**h**eat **i**nactivated) |
| FcγR | **F**ragment **c**rystallizable **r**egion **g**amma **r**eceptor |
| FDC | **F**ollicular **d**endritic **c**ell |
| FRC | **F**ollicular **r**eticular **c**ell |
| FSC | **F**orward **sc**atter |
| GC | **G**erminal **c**enter |
| GFP | **G**reen **f**luorescent **p**rotein |
| HA | **He**m**a**gglutinin |
| HACA | **H**uman **a**nti-**c**himeric **a**ntibody |
| HAHA | **H**uman **a**nti-**h**uman **a**ntibody |
| HAMA | **H**uman **a**nti-**m**ouse **a**ntibody |
| HEK | **H**uman **e**mbryonic **k**idney |
| HEV | **H**igh **e**ndothelial **v**enules |
| HIGS | **H**yper **Ig**M **s**yndrom |
| HSC | **H**ematopoietic **s**tem **c**ells |

| | |
|---|---|
| IC | **I**mmune **c**omplex |
| Ig | **I**mmuno**g**lobulin |
| IgH / IgL | **I**mmuno**g**lobulin **h**eavy / **l**ight chain |
| IL | **I**nter**l**eukin |
| IMDM | **I**scove's **M**odified **D**ulbecco's **M**edia |
| IVT | *In vitro* **t**ranscribed |
| JAK | **Ja**nus-**K**inase |
| JNK | **J**un **amino**-terminal **k**inases |
| kDa | **K**ilo **D**alton |
| L | **L**iter |
| LC / HC | **L**ight **c**hain / **h**eavy **c**hain |
| LMPP | **L**ymphoid-primed **m**ulti**p**otent **p**rogenitors |
| M | **M**olar |
| mAb | **M**onoclonal **a**nti**b**ody |
| MACS | **Ma**gnetic **c**ell **s**eparation |
| MAPK | **M**itogen-**a**ctivated **p**rotein **k**inase |
| MFI | **M**ean **f**luorescence **i**ntensity |
| min | **Min**ute |
| MOPS | 3 *(*n **mo**rpholino*)***p**ropane **s**ulfonic acid |
| MPP | **M**ulti**p**otential **p**rogenitors |
| mRNA | **M**essenger **r**ibo**n**ucleic **a**cid |
| MZ | **M**arginal **z**one |
| n | **N**ano |
| NE-AA | **N**on-**e**ssential **a**mino **a**cid |
| NF-κB | **N**uclear **f**actor **kappa**-light-chain-enhancer of activated **B cell**s |
| NK cell | **N**atural **k**iller cell |
| PAGE | **P**oly**a**crylamide **g**el **e**lectrophoresis |
| PBMC | **P**eripheral **b**lood **m**ononuclear **c**ell |
| PBS | **P**hosphate **b**uffered **s**aline |
| PMA | **P**horbol **m**yristate **a**cetate |
| pMHC | **P**eptide **m**ajor **h**istocompatibility **c**omplex |
| ppm | **P**arts **p**er **m**illion |
| PVDF | **P**oly**v**inyli**d**ene **f**luoride |
| RAG | **R**ecombination **a**ctivating **g**enes |
| RNA | **R**ibo**n**ucleic **a**cid |
| rpm | **R**ounds **p**er **m**inute |
| RPMI | **R**oswell **P**ark **M**emorial **I**nstitute |
| SAPK | **S**tress-**a**ctivated **p**rotein **k**inases |
| SCS | **S**ub**c**apsular **s**inus |
| SDS | **S**odium **d**odecyl **s**ulfate |
| SLC | **S**urrogate **l**ight **c**hain |
| SSC | **S**ideward **sc**atter |
| STAT | **S**ignal **t**ransducer and **a**ctivator of **t**ranscription |
| TACI | **T**ransmembrane **a**ctivator and **c**alcium-modulating and cyclophilin ligand **i**nteractor |
| TCR | **T c**ell **r**eceptor |
| T$_{FH}$ cell | **T f**ollicular **h**elper **c**ell |
| T$_H$ cell | **T h**elper **c**ell |
| TM | **T**rans**m**embrane |
| TNF | **T**umor **n**ecrosis **f**actor |
| TRAF | **T**NF receptor **a**ssociated **f**actor |
| TRIS | **Tris**(hydroxymethyl)aminomethane |
| U | **U**nit |
| UTR | **U**n**t**ranslated **r**egion |
| VDJ | **V**ariable, **d**iversifying, **j**oining |

# 1 Introduction

## 1.1 The adaptive immune system in health and diseases

All organisms are continuously in contact with substances and organisms of the environment and many of these influences are potentially dangerous for the organism. To protect the body against pathogens higher organisms developed a great arsenal of defense strategies, namely the immune system. A complex system of multiple factors and cell types controls the normal physiological functions and eliminates pathogens. The defense mechanisms are based on the recognition of structural components of invading pathogens (pathogen-associated molecular pattern) or misled physiological functions (Chaplin, 2003). The immune system can be divided into two arms based on origination and specificity of the components: the innate and the adaptive immune system. Both arms have effector cells (cellular immunity) and soluble effector molecules (humoral immunity).
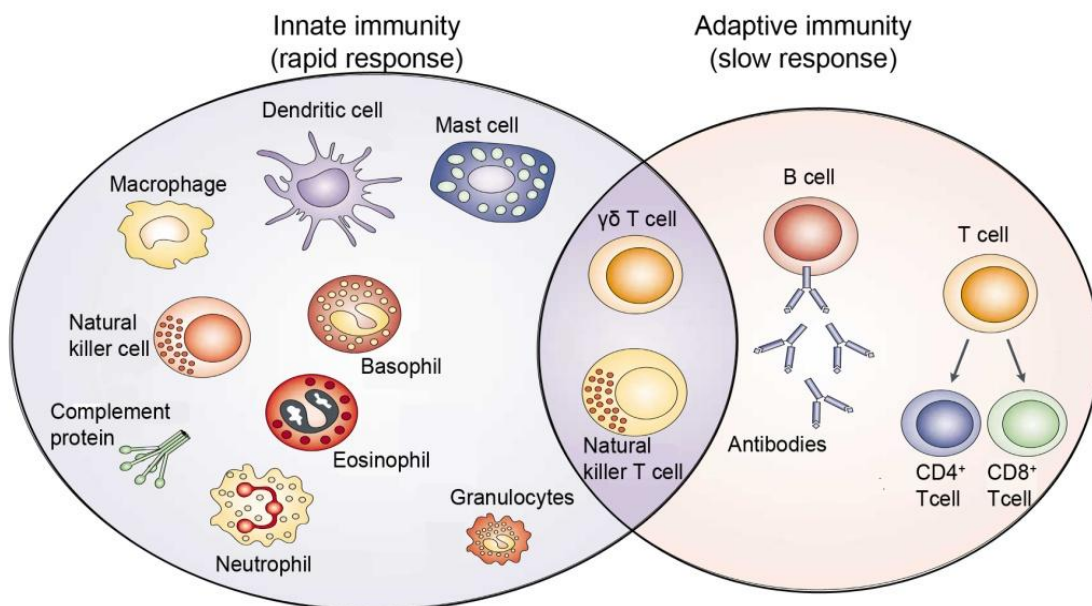


**Figure 1: *Components of the immune system.***
**Schematic draft of the two arms of the immune system: Innate and adaptive immunity with different components of the humoral and cellular immunity (Dranoff, 2004).**

The adaptive immune system is characterized by antigen-specificity and the formation of memory. It is specific for molecular structures (antigens) from individual pathogens.

These antigens are recognized by antigen-specific receptors expressed on the surface of two different cell types: the T cells responsible for the cellular arm of adaptive immunity and the B cells responsible for the humoral arm of adaptive immunity. The extremely high variability of antigenic structures requires a similarly high variability of the antigen-specific receptors. Antigen-specific receptors expressed on B cells and T cells are generated by random DNA gene segments recombination during the cell development (chapter 1.3.2) (Chaplin, 2003).

The adaptive immunity is orchestrated by professional antigen presenting cells (APC), like dendritic cells, B cells or macrophages. For that, the APCs take up antigens, which they process intracellularly and present via major histocompatibility complex (MHC) molecules on their surface. T cells recognize the peptide-MHC (pMHC) complex on APCs and become activated, which can lead to proliferation and cytokine secretion.

For full B cell activation this cognate interaction with T cells is required. This interaction results in B cell proliferation and clonal expansion and additionally determines the cell fate to become an effector cell or memory cell. Further details of cognate B cell-T cell interaction are described in chapter 1.4. Some of the activated B cells develop into antibody secreting plasma cells. Antibodies specific for a certain antigen are able to mediate cell killing by complement dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC). Additionally, the antibody may affect the physiological function of the recognized antigen. Some of the activated T cells and B cells develop into memory cells which allow the immune system to react to a second infection of the same pathogen faster and more efficiently.

The hallmark of the immune system is to discriminate between self and non-self structures. Disturbance of this capability can lead to cellular and humoral immune responses acting against the body own cells and tissues, which may result in the development of autoimmune diseases. This phenomenon was first hypothesized by Paul Ehrlich more than 100 years ago and named *horror autotoxicus (Silverstein, 2001).*

## 1.1.1 Autoreactive B lymphocytes in health and diseases

A major hallmark of humoral immunity is the diversity and specificity of the antibody repertoire as stated above. The antibody diversity is produced by the random processes of V(D)J recombination and somatic hypermutation, resulting in a variety of polyreactive specificities, including self-reactive B cells. By the cloning of antibodies

from single B cells at different developmental stages the frequencies and checkpoints of central and peripheral tolerance were determined. Approximately 60 % of all early immature B cells are self-reactive, whereas less than 5 % of the peripheral mature B cells show self-reactive. Two developmental checkpoints regulate the reduction of self-reactivity: at the immature B cell stage in the bone marrow and during the transition from new emigrant to mature naïve B cell in the periphery (Wardemann et al., 2003).

Data from CD40L deficient mice and observations in MHC-II deficient patients suggest that neither CD40/CD40L interactions nor antigen presentation are important for the central tolerance checkpoint (chapter 1.3.1). However, these interactions are essential for the second peripheral tolerance checkpoint, which remove self-reactive B cells from the repertoire by mechanisms involving apoptosis of B cell with strong self-reactive BCR signals, receptor editing or anergy by intermediate self-reactive BCR signals (Herve et al., 2007).

Another process during which autoreactive B cells could develop is the T cell dependent antigen mediated activation of naïve IgM$^+$ B cells. This activation induces the formation of germinal centers and drives Ig gene somatic hypermutation and class switching to other isotypes. These B cells differentiate into antibody secreting plasma cells or memory B cells (MacLennan, 1994). B cells that leave the germinal center reaction are selected for high reactivity and specificity to the activating antigens. Recent studies show that peripheral IgG$^+$ memory B cells of healthy donors are notably enriched in autoreactive clones compared to IgM$^+$ naïve B cells. The increased autoreactivity in IgG$^+$ memory B cells compared to IgM$^+$ naïve B cells from which they arose is the result of somatic hypermutations during the affinity maturation. IgG$^+$ memory B cells do not secret antibodies but are able to differentiate into antibody secreting plasma cell after antigen recognition (Tiller et al., 2007a). However, self-reactive antibodies are not per se mediators of autoimmunity and could play an essential role in normal physiology, like autoantigen clearance to prevent autoimmunity (Kim et al., 2002). Some experiments recently showed that endogenous antibodies reactive against myelin are required for rapid and robust myelin clearance and axon generation of the peripheral nerve system (Vargas et al., 2010). Furthermore, the strategy to treat systemic lupus erythematosus (Leandro et al., 2005) or rheumatoid arthritis (Edwards et al., 2004) patients with anti-CD20 antibodies that deplete naïve and memory B cells but not long-lived plasma cells suggests that IgG$^+$ memory B cells and their corresponding autoantibodies play a role in autoimmunity.

## 1.2 Immunotherapy with antibodies

Monoclonal antibodies have emerged as one of the most promising therapeutic biologicals in immunotherapy over the last decade, especially monoclonal antibodies against tumor associated or tumor specific antigens. The development of monoclonal antibody (mAb) technology represented a considerable achievement and resulted in numerous applications. Recent production technologies generate rodent antibodies through the immunization of mice. In the field of immunotherapy, rodent mAbs have proved to be of limited use because of their strong immunogenicity in man. Due to their low immunogenicity in patients, fully human mAbs are becoming increasingly important for the treatment of cancer and other diseases. The generation of fully human antibodies suitable as therapeutic is laborious and difficult to achieve. Autoreactive B cells expressing those antibodies are detectable in cancer patient and represent a suitable source for human antibodies (Pavlou and Reichert, 2004; Reichert et al., 2005).

### 1.2.1 Modern production methods for therapeutic antibodies

The procedure for the production of monoclonal antibodies by hybridoma technology was invented by C. Milstein, G.J.F. Köhler and N.K. Jerne in 1975 for which they were awarded the Nobel Price for physiology or medicine in 1984.

The first step to produce hybridomas is the immunization of a donor organism to obtain adequate B cell clones. B cells are taken from the spleen of an immunized mouse and then fused with myeloma tumor cells. After two weeks the hybridoma cells can easily be selected by subcloning through limiting dilution (Kohler and Milstein, 1975).

In principle, every immunogenic structure can be used for the generation of monoclonal antibodies. Targets for immunotherapy, especially tumor therapy, should be membrane associated and expressed in a highly selective manner on targets cells, like tumor cells or other disease inducing cells.

The hybridoma technology generates murine antibodies, which induce immunogenicity in patients. The humoral immune response against therapeutic antibodies can lead to severe side effects (Kuus-Reichel et al., 1994; Dillman, 2011). Anti-isotypic antibodies are specific for the constant region of the therapeutic antibody and may decrease the antibody concentration below the effective levels. Additionally, anti-idiotypic antibodies which are specific for the variable region of antibodies were observed. These antibodies can block the antigen-binding of the therapeutic antibody to the target structure and accelerate the plasma clearance. Furthermore, post-translational

modifications and polymorphisms may represent an antigenic structure and lead to side effects or decreased pharmaco-efficiency. These effects are lower in antibodies with higher human proportion. In addition, the different Fc-domains of the murine and human antibodies have different potential to activate the complement system or to recruit effector cells (Dillman, 2011; Weiner, 1999). These disadvantages of murine antibodies provoked the development of new strategies, which allow the substitution of the murine Fc-part by a human Fc-part. These chimeric antibodies have a sequence identity of 86 - 93 % to the fully human counterpart. In the humanized form additional substitutions were introduced into the variable region and these antibodies show 92 – 95 % sequence homology to the human counterpart. The chimerization and humanization of the antibodies result in an enhanced effector-function and a higher half-life in blood. Whether fully human antibodies will further reduce or completely eliminate immunogenicity remains unclear (Hsu et al., 1993; Chen et al., 1994).

Two major approaches are currently used to produce human-like antibodies, namely antibody display libraries and transgenic mice. The libraries are obtained from human immune cells and contain antigen binding fragments (scFvs or Fabs) displayed on the surface of bacteriophages (Huse et al., 1989; McCafferty et al., 1990) or ribosomes (Hanes and Pluckthun, 1997). Several approaches are established to select antibodies that specifically bind to a particular antigen. A disadvantage of this technology is that no affinity maturation process occurs and the antibodies derived from display libraries are of relatively low affinity. The transgenic mice were generated by introduction of human immunoglobulin loci transgenes into embryonic stem cells to engineer B cell-affected mice that are unable of expressing murine immunoglobulin heavy and kappa light chain genes (Lonberg et al., 1994; Mendez et al., 1997; Kuroiwa et al., 2000). These mice can be immunized with a certain antigen and thereby produce fully human antibodies. By repetitive immunization the mice will generate high affinity monoclonal antibodies through the normal affinity maturation process.

As mentioned above a major problem of the different antibody formats are their immunogenicity. Establishment of an immune response can negatively impact the pharmacokinetic, safety and efficacy profile. According to the therapeutic antibody the response are called human anti-mouse antibodies (HAMA) (Hooks et al., 1991), human anti-chimeric antibodies (HACA) (Tcheng et al., 2001; Baert et al., 2003) or human anti-human antibodies (HAHA) (Weinblatt et al., 1995). Additionally, the immune response can be directed against a conjugated effector molecule like a toxin or a drug. Theoretically, antibodies are immunogenic per se, whether or not they are human, due to the individuality of the idiotype. Nonetheless, human or humanized antibodies are poorly immunogenic in humans (Stein, 2002). Clinical trials with human antibodies

obtained from genetically modified mice technology are relatively non-immunogenic. In comparison, human therapeutic antibodies generated through phage display are selected *in vitro* and have not undergone the *in vivo* immunological process that would abolish antibodies with atypical conformations or amino acid sequences. Furthermore, the random pairing process and mutations subsequently introduced for affinity maturation enhance immunogenicity. Consistent with hypothesis, 12 % of rheumatoid arthritis patients treated with phage display derived human monoclonal anti-TNFα antibodies developed human anti-human antibody response (van de Putte et al., 2004; Bartelds et al., 2007). In contrast, genetically modified mice generated human antibodies, like anti-IL8 and anti-EGFR, show no human anti-human antibody response and no altered pharmacokinetic profile in clinical studies (Figlin et al., 2002; Krueger, 2002; Schwartz et al., 2002).

Fully human antibodies are potentially of low immunogenicity and permit a safer and more effective therapy. Therefore novel strategies to achieve fully human antibodies are needed. These antibodies with validated targets offer a huge area for potential clinical development of antibody therapeutics.

## 1.3 B lymphocytes

Leukocytes, or white blood cells, are cells of the immune system and involved in adaptive and innate immune responses. All leukocytes originate from pluripotent hematopoietic stem cells (HSC) in the bone marrow. These stem cells are able to proliferate for maintenance or differentiate into the myeloid cell lineage or lymphoid cell lineage. The myeloid cell lineage encompasses granulocytes, macrophages, dendritic cells (DC) and mast cells, whereas T cells, B cells and natural killer cells belong to the lymphoid cell lineage. While T cells develop in the thymus, B cells develop in the fetal liver and the adult bone marrow

### 1.3.1 Development of B lymphocytes

B cells arise from HSC in the bone marrow. There HSC are juxtaposed to osteoblasts, endothelial cells or reticular cells which provide important cytokines like CXCL12. These cells build the inner bone marrow anatomy with the microenviromental niches which are required for hematopoiesis. Through different stimuli HSC are able to differentiate into hematopoietic multipotential progenitors (MPP) and subsequently to

lymphoid-primed multipotent progenitors (LMPPs). LMPP cells lack erythro-megakaryocytic differentiation potential but retain complete lymphoid cell spectrum, which includes the development to T cells, DC, NK cells, and B cells (Adolfsson et al., 2005). With the emergence of the lymphoid recombination activating gene (RAG) 1 and RAG2 expression the LMPP cells develop into the earliest lymphocyte progenitors (ELPs), which subsequently differentiate into common lymphoid progenitors (CLP) (Izon et al., 2001; Kondo et al., 1997). The RAG enzymes are responsible for the gene rearrangement which is necessary for immunoglobulin production (Oettinger et al., 1990; Schatz et al., 1989). Nevertheless, these kinds of progenitors are still negative for membrane associated immunoglobulin (Busslinger, 2004).

For the entry into the B cell pathway the lymphoid progenitors have to express different lineage specific transcription factors. These transcription factors regulate B cell development by repressing lineage-inappropriate genes and by activating B cell specific genes. Especially PAX5 positively regulates genes which are important for differentiation to pre B cell, like BLNK, Igα, CD19, and others (Nutt et al., 1998). These precursor B cells are divided into four subsets based on the expression pattern of cell surface marker. These four subsets are named fraction A (or pre-pro B cell), fraction B (or pro B cell), fraction C (or pro B cell), and fraction D (or pre B cells) (Busslinger, 2004; Nutt et al., 1997; Nutt et al., 1999).

The diversification of the BCR is generated by complex mechanisms of gene rearrangements (chapter 1.3.2). This random process generates a BCR of random specificity and to avoid autoimmunity the self-reactive B cells have to be deleted from the repertoire by the induction of apoptosis (Hartley et al., 1991; Nemazee et al., 1991; Sandel and Monroe, 1999), anergy (Goodnow et al., 1988) or by secondary recombination (Radic et al., 1993; Tiegs et al., 1993) (chapter 1.3.1). During the B cell development the pre B cells express the pre-BCR, which consist of a fully rearranged heavy chain (IgH), associated with the germline-encoded surrogate light chain (SLC). In contrast to the light chain (IgL) the SLC is a heterodimeric complex of two invariant proteins, VpreB and λ5 (Nishimoto et al., 1991). Signaling from the pre-BCR gives rapid feedback about the functionality of the IgH and induces clonal proliferation. Besides, this signal results in positive selection which leads to complex genetic changes, like down regulation of pre-BCR components and recombination of the IgL genes (Keenan et al., 2008) and is associated with progression of B cell development (Karasuyama et al., 1996).
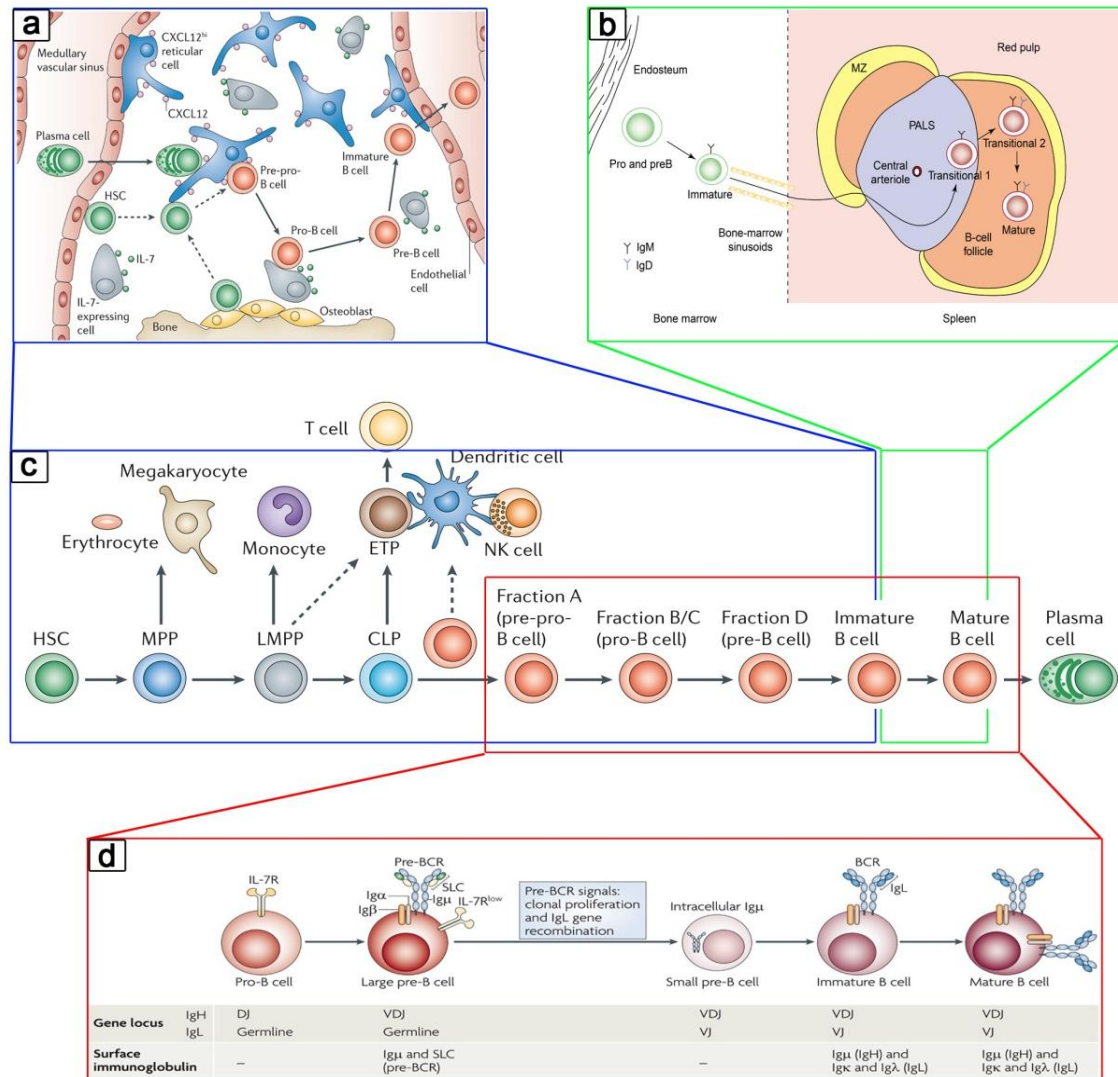
**Figure 2:** *B cell development.*
**The different B cells stages are located in different tissues. Early B cell differentiation requires different environmental factors in the bone marrow (a, c). Haematopoetic stem cells are located in the bone marrow and can differentiate to pre B cells (a, b, c, d). The B cells test the recombined pre-BCR for functionality in bone marrow. Subsequently the immature B cells leave the bone marrow and migrate to the spleen (b, d). (modified and adapted from Chung et al., 2003; Herzog et al., 2009; Nagasawa, 2006)**

Successful IgL gene rearrangement in pre B cells results in the expression of a complete BCR of the subclass µ. This BCR consists of two light chains covalently linked by disulfide bridges to the membrane spanning heavy chain (Reth et al., 1987). The membrane domain of the IgM is obligatory associated with Ig-α and Ig-β (CD79-a and CD79-b) (Brummer et al., 2004). Ig-α and Ig-β not only work as chaperones to facilitate correct protein folding, they also join the IgM as membrane signaling elements, which are crucial for further development and survival of B cells (Kraus et al., 2004). The immature IgM+ B cell leave the bone marrow, enter the blood and migrate to the spleen. B cells enter the spleen as transitional type 1 cells (T1 B cells) and are still only positive for IgM. At this stage, these B cells are sensitive to negative selection,

which is responsible for the establishment of the tolerance against self-proteins (Loder et al., 1999; Wardemann et al., 2003). At the end of the T1 stage B cells begin to express IgD and lose their sensitivity to negative selection. At this time point the process of negative selection is completed and the B cell repertoire is mostly depleted of autoreactive specificities. The T1 B cells are able to differentiate in the spleen to transitional type 2 B cells as indicated by surface IgD expression and subsequently to marginal zone (MZ) B cells or follicular B cells (Allman et al., 2001; Chung et al., 2002; Su and Rawlings, 2002).

Peripheral B cells are mainly located in secondary lymphoid tissues or can be found on their way from one lymphoid tissue to another. Parts of the secondary lymphoid tissues are the lymph nodes, the spleen, Peyer´s patches and other mucosal associated lymphoid tissues. B cells migrate through the blood vessels into the secondary lymphoid organs where they remain approximately one day. Afterwards the B cells return to the circulation and patrol to another tissue (Drayton et al., 2006). In the lymphoid tissue the B cells are located in follicles on the opposite site of antigen entry: lymph node follicles are placed beneath the subcapsular sinus (SCS), splenic follicles are located beneath the blood-filled marginal sinus, and in the mucosa the follicles are situated adjacent to antigen-transporting M cells. The presentation of certain antigens is tightly regulated in the follicular zone of lymphoid tissues and involves several cell types. The full activation of B cells requires recognition of the specific antigen by binding to the BCR and the support of T cells. Subsequently, these interactions regulate the cell fate as antibody secreting plasma cell or memory B cells and are described in detail in cognate B cell – T cell interaction (chapter 1.4).

### 1.3.2 Generation of immunoglobulin diversity by V(D)J recombination

Immunoglobulins are one class of effector molecules of B cells, expressed on the surface of B cells as BCR or secreted as antibody. It consists of two heavy chains (HC) and two light chains (LC), covalently linked by disulfide bridges and formes a Y shaped molecule. Different gene segments are available for the C-terminal part of the heavy chain, which defines the isotype subclass of the immunoglobulin based on the gene segment used, namely α (IgA), δ (IgD), ε (IgE), γ (IgG), or μ (IgM) (Woof and Burton, 2004). This constant part is responsible for the effector functions of the antibody, like complement dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC). In addition further attributes are also defined by the constant domain, such as serum half-life and glycosylation pattern at the $C_H2$ domain. The antigen-binding sites

of immunoglobulins are located at the tip of the variable domain and are composed of the heavy and light chain domains (Weiner, 2007).
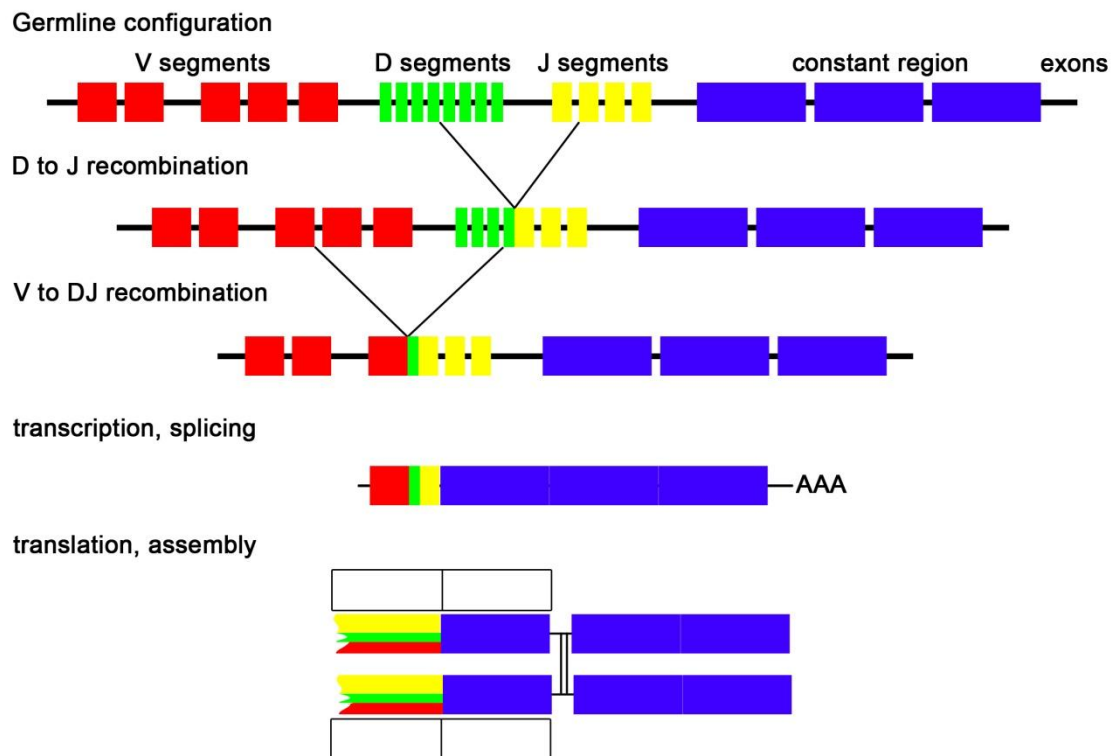


**Figure 3: *Heavy chain gene rearrangement.***
**Germline gene segments are randomly arranged to produce a virtually unlimited diversity of antibody specificity. Variable (V), diversity (D) and joining (J) segments are combined in two steps: D to J and V to DJ recombination. The white boxes simulate the light chain which is similarly rearranged as the heavy chain.**

B cells can respond to specific antigens by generating a practically unlimited diversity of B cell receptors and thereby antibodies. This remarkable attribute is achieved by a process called V(D)J recombination in which certain immunoglobulin gene segments are randomly rearranged (Oettinger et al., 1990; Schatz et al., 1989). Furthermore, the receptor diversity and functionality is increased by somatic hypermutation induced affinity maturation and isotype class switching. IgH genes are located on the human chromosome 14 and contain 65 variable segments ($V_H$), 27 diversity segments ($D_H$), and 6 joining ($J_H$) segments. By DNA gene rearrangement of these segments 10530 different heavy chains are theoretically possible (Cook et al., 1994; Tomlinson et al., 1995). Furthermore, the variability is increased by the association of the light chain. The variable domain of the light chain is rearranged in a similar way to the heavy chain. The light chain originates from the rearrangement of different V and J gene segments, which are located on chromosome 2 as kappa locus (Malcolm et al., 1982) or on chromosome 22 as lambda gene locus (Anderson et al., 1984). In contrast to the heavy

chain the light chain contains no D segments. The DNA gene rearrangement starts in pro B cells with the recombination of one D and one J segment of the heavy chain. The DNA between both segments is deleted from the chromosome as circular DNA. Subsequently, the rearranged DJ segment is connected to one V segment forming a completely rearranged VDJ heavy chain. Besides the VDJ genes unspliced RNA contains the constant μ and δ chains (V-D-J-$C_μ$-$C_δ$). By the addition of a polyadenylated tail after the $C_μ$ chain and the removal of DNA parts between VDJ segments and $C_μ$ chain the primary RNA sequence is processed to the final mRNA (Jung et al., 2006).

The developmental stages are temporally and spatially separated. The heavy chain is presented on the surface in association with the SLC. A pre-BCR signal induces clonal proliferation and IgL gene rearrangement, resulting in the gene rearrangement of the V segment and J segment of the IgL gene locus. The immature B cell stage is the first developmental stage with surface IgM molecules composed of the complete Igμ heavy chain and Igκ or Igλ light chain. The mature B cell is the last developmental stage of an antigen inexperienced B cells and expresses IgM and IgD molecules with the same specificity.

After antigen recognition B cells enter germinal center reaction in the lymph node. In this process the antibody diversification is further increased by somatic hypermutation.

The B cells start to proliferate and the BCR gene locus undergoes a tremendously high rate of mutation in this somatic gene. In contrast to modifications in germ line genes somatic alterations are limited to the individual. The mutation frequency is up to $10^5$ to $10^6$ fold higher as other mutation rates across the genome. However, the mutations are mainly occurring into hotspots of the variable gene segments, known as hypervariable regions or also named complementary determining regions (CDR). The CDRs are more variable as the rest of the variable domain and are separated by framework regions. The framework regions form the β-sheets, whereas the CDRs form β-bends or loop structures which are in the folded domain located on the tip of the variable domain and are responsible for antigen recognition (Figure 4).
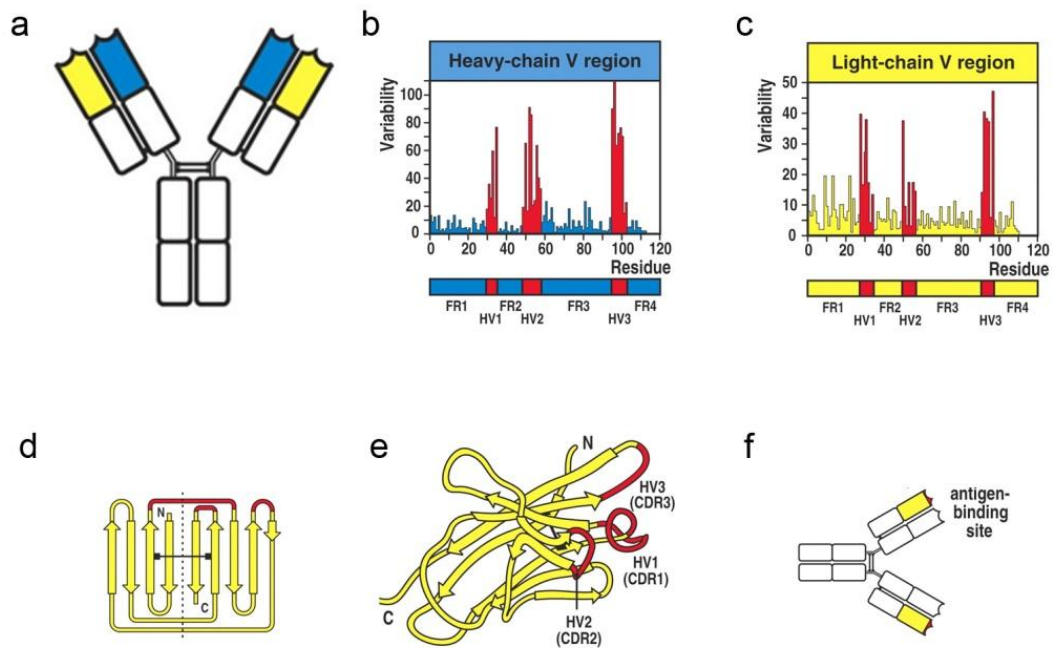
**Figure 4: *Hypervariable domains of human antibodies*.**
**Antibody diversity is produced by rearrangement of variable gene segments and somatic hypermutations. a) Structure of an antibody with highlighted variable light chain (yellow) and variable heavy chain (blue). Variability of the amino acid sequence of the (b) heavy chain and (c) light chain. The hypervariable domains (or complementary determining region, red) are separated by framework region (blue or yellow). d) Secondary structure of the variable light chain domain (framework region in yellow, hypervariable regions in red). e) Tertiary structure of the variable light chain domain (framework region in yellow, hypervariable regions in red). f) The hypervariable domain (red) is located on the tip of the variable light chain domain (yellow) (Janeway et al., 2005).**

## 1.3.3 Biosynthesis of BCR and antibodies

As mentioned above, immunoglobulin proteins are molecules which are composed of two heavy chains and two light chains, linked by disulfide bridges. Heavy chain and light chain are composed of multiple domains, four to five domains for the heavy chain ($V_H$-$C_H$1-$C_H$2-$C_H$3) depending on the isotype and 2 domains for the light chain ($V_L$-$C_L$). Each domain consists of approximately 110 amino acids which are folded in a highly conserved twisted barrel-like β-sheet structure. These structures are in most cases stabilized by buried intrachain disulfide bridges. Two heavy chains are linked by two disulfide bonds in the hinge region, which is located between the $C_H$1 and $C_H$2 domain. Each light chain is coupled to the heavy chain by one disulfide bound between the $C_L$ and $C_H$1 (Janeway et al., 2005).

Ig molecules can exist as either surface associated protein in the membrane of mature B cells or as secreted protein produced by plasma cells. Ig synthesis in mature B cells is very low and constitutes only 1 % of the total cell protein synthesis (Miller and

Philips, 1969). Mature B cells do not secrete Ig molecules, however occasional shedding of Ig membrane associated molecules lead to a slow turnover of the BCR. Each mature B cell carries up to $10^5$ membrane associated Ig molecules with a half-life of 24 to 48 hours (Andersson et al., 1974; Marchalonis and Cone, 1973; Melchers and Andersson, 2011; Vitetta and Uhr, 1973). Plasma cells are highly efficient in Ig production and secretion. The Ig production is 50 to 100 fold increased compared to mature B cells. Plasma cells produce very large amounts of Ig molecules and invest up to 50 % of the total cell protein synthesis for Ig production. Plasma cells are extremely efficient to secrete Ig molecules and synthesize up to $2 \times 10^3$ antibodies per second, but retain incorrectly assembled subunits (Andersson et al., 1974; Hendershot and Sitia, 2004).

The heavy and light chains are independently cotranslationally translocated into the endoplamatic reticulum (ER) and the folding process starts before the complete mRNA is translated. The molecular chaperone BIP transiently binds to several Ig domains before the folding is completed. When BIP binds transiently to the $V_L$ domain the $C_L$ domain can fold autonomously to a monomeric state (Goto and Hamaguchi, 1982; Haas and Wabl, 1983). All constant domains have seven strands (abcdefg). The topology is stabilized by a buried disulfide bridge, which links the strand b and f (Bork et al., 1994; Huber et al., 1976) facilitating the building of the tertiary structure. The conserved strands a and b and strands e and f are linked by a small helices, which are important elements for correct folding of the Ig domain (Feige et al., 2008).
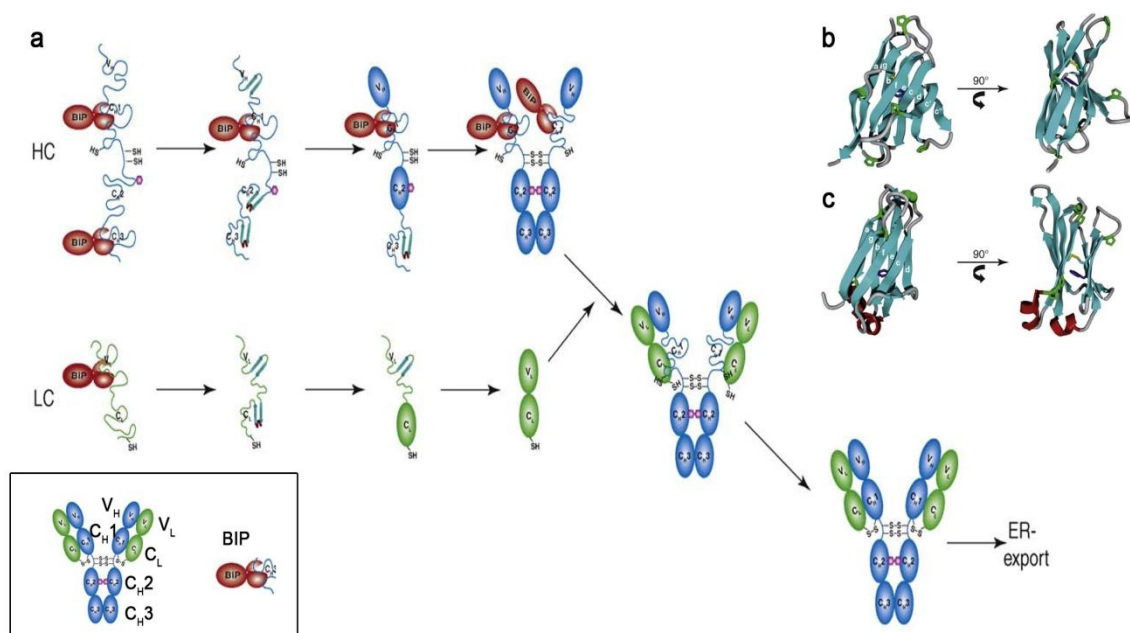


**Figure 5: *Schematic draft of antibody folding.***
**a) Light and heavy chain fold independently with the aid of BIP (red) before assembling and transport to the endoplasmatic reticulum. Architecture of the variable (b) and constant light chain**

**(c). Shown are the small helices (red) which connect the strand (turquoise). The strand nomenclature is indicated. Disulfide bridge (yellow) and proximal tryptophan residue (blue) (modified and adapted from Feige et al., 2010).**

The folding mechanism of the $C_L$ domain is readily transferable to the $C_H2$ domain, whereas the effect of the sugar moieties on the domain folding reaction are not completely understood (Feige et al., 2004). Nonetheless, the $C_H2$ domains of two HC interact only via N-linked glycans, which were added covalently to the domain cotranslationally. These glycans determine the orientation and spacing of the two $C_H2$ domains (Bergman and Kuehl, 1978; Feige et al., 2009b; Huber et al., 1976; Krapp et al., 2003). After successful folding of the $V_H$ and $C_H2$ domain the $C_H3$ domain folds autonomously to an obligate homodimer followed by dimerization of two heavy chains (Isenman et al., 1979; Thies et al., 2002). The heavy chain dimer is completely folded, except the $C_H1$ domain which is still coupled to BIP. The $C_H1$ BIP interaction is stable without light chain and prevents that unassembled heavy chains are transported to the cell surface. When the light chain displaces BIP from the $C_H1$ domain, the $C_L1$ domain induces folding of the $C_H1$ domain. The interchain disulfide bridge between $C_L1$ and $C_H1$ is essential for the correct folding (Feige et al., 2009a).

## 1.4    Cognate interaction of B cells and T cells

The antigen-specific interaction of B cells and cognate T cells is required for the full activation of B cells. This interaction takes place in secondary lymphoid organs like lymph nodes, tonsils or spleen. All human beings have approximately 500 to 600 lymph nodes distributed in the body. While lymph nodes filter extracellular fluid called lymph, Tonsils filter ingested or inhaled substances and the spleen filters the blood (Janeway et al., 2005; von Andrian and Mempel, 2003).

The lymph node is encased by a fibrous capsule. Inside the lymph node the fibrous capsule extends into trabeculae, which end in the medullary sinus. The filtered lymph leaves the lymph node through the efferent lymphatic vessel. The lymphocytes enter the lymph node from the blood stream through the capillary venules (HEV = high endothelial venules) (Janeway et al., 2005; von Andrian and Mempel, 2003). Inside the lymph node B cells are located in primary lymphoid follicles in close contact to the paracortex where T cells are mainly located. The distribution of B cells and T cells into special compartments in the lymph node is achieved by a special chemokine micromilieu (Bajenoff et al., 2006; Mempel et al., 2006). The fibroblast reticular cells

(FRC) secrete the fibers that form the network in both areas. While follicular FRCs secrete CXCL13 and attract B cells (Anderson and Anderson, 1975), paracorticl FRCs secrete CCL19 and CCL21 for T cell attraction.
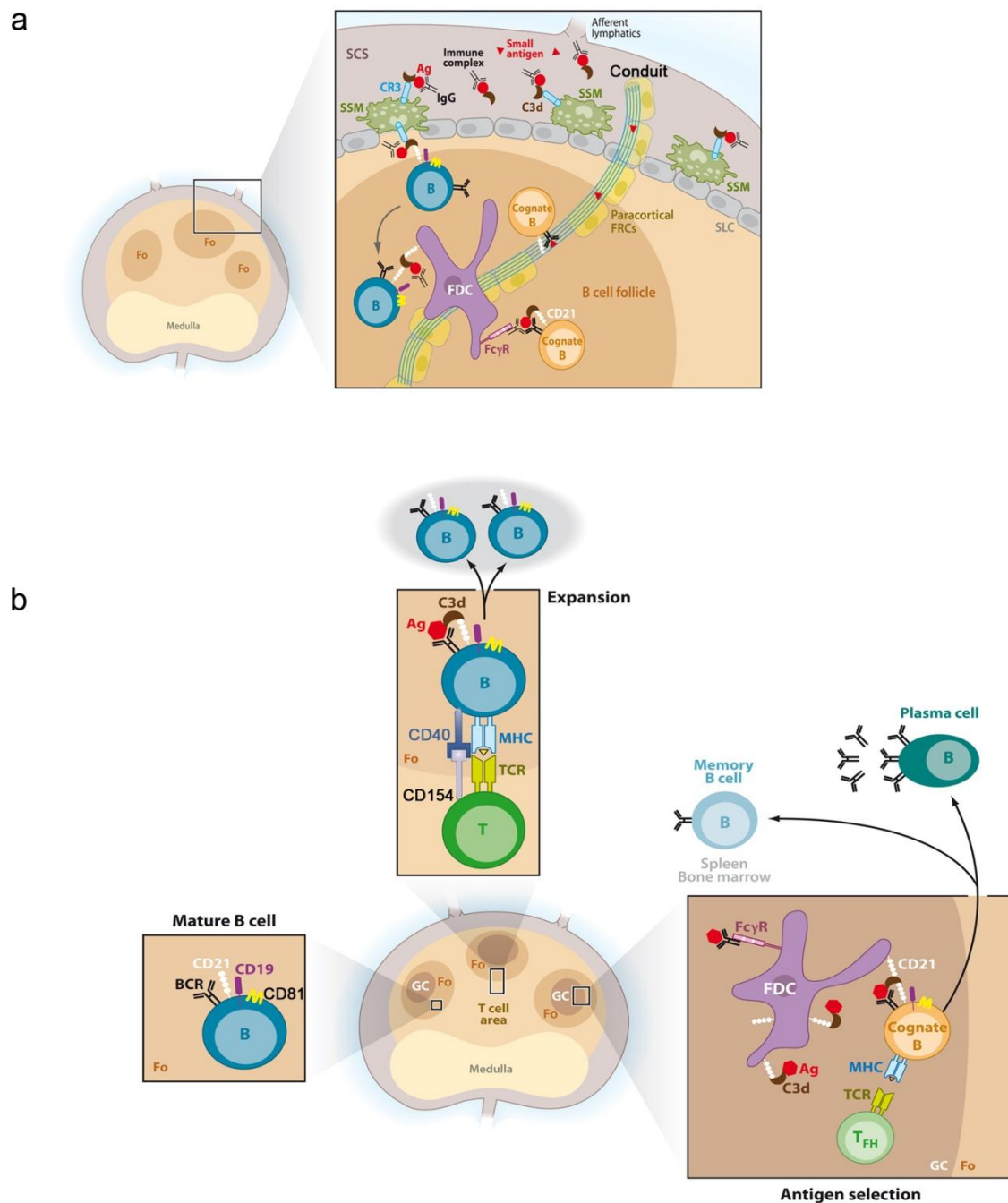


**Figure 6: *Trafficking of antigens in lymph nodes and cognate B cell –T cell interactions.***
**a) Antigen transport into the lymph node and interactions with B cells. Antigens enter the lymph node via the afferent vessel. While antigen immune complexes (indicated as IC) are transported through subcapsular sinus to FDCs, small antigens directly migrate to the FDCs through follicular conduits. The FDCs are the main depot for B cell antigens. b) Mature B cells (as indicated) are located in follicles in the lymph node. Full B cell activation and expansion (as indicated) after cognate interaction with T cells. Complex interaction of different cell types and and B cell antigen selection (as indicated) determines the B cell fate (modified and adapted from Gonzalez et al., 2011).**

The lymph continuously drains to the lymphatic vessels and thereby transports soluble antigens or antigens presented by APCs to the lymph nodes. The lymph enters the lymph node through the afferent lymph vessels and distributes into the subcapsular sinus. In the subcapsular sinus the antigens are quickly taken up by macrophages that line the subcapsular sinus. These macrophages transport the antigens to the subjacent tissue in the lymphoid follicles, making the antigens available for B cells (Junt et al., 2007; Phan et al., 2007; Carrasco and Batista, 2007; Martinez-Pomares and Gordon, 2007). The antigen recognition by the B cell receptor will lead to activation, up-regulation of costimulatory molecules and movement of the B cells towards the paracortical region (Batista and Harwood, 2009). The BCR together with the bound antigen is then internalized and transferred to an intracellular compartment called MIIC, where newly synthesized major histocompatibility complex class II (MHC-II) molecules and peptides derived from antigens are formed into peptide-MHC-II (pMHC-II) complexes. These pMHC-II complexes are subsequently transported to the cell surface, where they are recognizable for T cells.

Another route of antigen entry into the lymph node is via mature dendritic cells that have taken up antigens in the periphery and migrate to the lymph node. These mature antigen experienced DCs express pMHC-II complexes and recruit antigen-specific T cells, inter alia T helper cells ($T_H$ cells). Antigen-specific interaction of DC and $T_H$ cell induces clonal $T_H$ cell expansion and differentiation into effector $T_H$ cell (Malherbe et al., 2008). Recent studies indicate that it is not the antigen amount that decides about naïve $T_H$ cell fate, but rather the binding strength between TCR and pMHC-II (Fazilleau et al., 2009).

B cells which are activated by a specific antigen need the second stimulatory signal provided by T cells. For that antigen activated B cells move towards the T cell zone to receive help during cognate interaction. Without this help, most of the antigen primed B cells will die without expansion or differentiation. Simultaneously, antigen-specific activated follicular T helper cells ($T_{FH}$) migrate toward the B cell zones. At the border of both cell specific zones the pMHC-II specific effector $T_{FH}$ cells and antigen primed B cells interact through TCR-pMHC-II and CD40-CD154 binding (McHeyzer-Williams and McHeyzer-Williams, 2005; Garside et al., 1998; MacLennan, 1994). These interactions are needed for entry of effector $T_{FH}$ cells and B cells into the germinal center reaction. The germinal center is the place in the follicles where the antigen dependent clonal expansion, immunoglobulin diversification, and affinity maturation takes place (MacLennan, 1994; Allen et al., 2007; Klein and Dalla-Favera, 2008; Rajewsky et al., 1969). All these attributes are needed for the production of high affinity

antibodies, which increase the potency of a humoral immune response against a certain pathogen. The gradual increase of antibody affinity is induced by somatic hypermutations and subsequent selection of high affinity B cell clones (Goidl et al., 1968; McKean et al., 1984; Nussenzweig and Benacerraf, 1967). For this purpose the germinal centers are divided into two parts, the light zone and the dark zone. The B cells in the dark zone are large fast dividing B cells (centroblasts) and undergo clonal expansion and random somatic hypermutations. Subsequently, the B cells enter the light zone and remain as non dividing B cells (centrocytes) (MacLennan, 1994; Allen et al., 2007; Klein and Dalla-Favera, 2008; Rajewsky, 1996; Nieuwenhuis and Opstelten, 1984; Tarlinton, 2008). These B cells are interacting with follicular dendritic cells and $T_{FH}$ cells for selection of improved affinity. The selected B cells either return to the dark zone for further proliferation and mutation or remain in the light zone for immunoglobulin class switch and differentiation into plasma cells or memory B cells (Meyer-Hermann et al., 2001; Oprea and Perelson, 1997).

## 1.5 CD40 receptor and secondary signal for B cells

In addition to the BCR signal for the full activation B cells need a secondary co-stimulatory CD40 signal provided by T cells expressing the CD40 ligand (CD154) (described in detail in chapter 1.4). The 30 kDa CD40 receptor is a type I transmembrane glycoprotein cell surface receptor of the tumor necrosis factor (TNF) receptor superfamily (Braesch-Andersen et al., 1989; Chatzigeorgiou et al., 2009; Lam and Sugden, 2003) and was initially identified as both a B cell costimulatory receptor and a tumor antigen expressed in bladder carcinoma (Paulie et al., 1985). Human CD40 is a 277 amino acid protein that consists of a 173 amino acid extracellular domain, a 22 amino acid helical transmembrane domain, and a 62 amino acid cytoplasmic domain responsible for the induction of signaling (van Kooten and Banchereau, 2000).

The CD40 receptor gene is located on chromosome 20 and constitutively expressed by professional antigen presenting cells, like dendritic cells, B cells and macrophages. However, besides being expressed by antigen presenting cells, CD40 expression has been shown for monocytes, basophils, eosinophils, epithelial cells, endothelial cells, smooth muscle cells, microglia hematopoietic progenitors and activated T cells. Fibroblasts and keratinocytes also express low levels of CD40 constitutively and up-regulate CD40 expression in response to interferon-γ (Banchereau et al., 1995; Bourgeois et al., 2002; van Kooten and Banchereau, 1997).

Its ligand CD40L (CD154) is a 29 kDa type II transmembrane glycoprotein belonging to the TNF gene superfamily and was initially isolated from membranes of activated T cells. Human CD154 is a 261 amino acid protein that consists of a 215 amino acid extracellular domain, a 24 amino acid helical transmembrane domain, and a 22 amino acid cytoplasmic domain (van Kooten and Banchereau, 2000). CD154 is transiently expressed on activated CD4^{+} T cells and can also be induced upon activation on CD8^{+} T cells, mast cells, basophils, eosinophils, ephitelial cells, NK cells, and platelets. Further studies showed that CD154 is constitutively expressed in low levels on endothelial cells, smooth muscle cells, and macrophages and is up-regulated after cell activation (Carbone et al., 1997). The diverse expression pattern of the costimmulatory pair indicates the pivotal role in different cellular immune processes.

Engagement of CD40 by its ligand leads to trimeric clustering of CD40 and the recruitment of adaptor proteins known as TNF receptor-associated factors (TRAFs) to the cytoplasmic tail of CD40 (Karpusas et al., 1995). Clustered trimeric CD40 directly interacts with TRAF2, TRAF3, TRAF5, and TRAF6, and indirectly with TRAF1 (Bishop, 2004). Binding of TRAFs results in the formation of a signaling complex that leads to the activation of mitogen and stress activated protein kinase (MAPK/SAPK) cascades, phospholipase Cγ (PLCγ) pathway and canonical and non canonical nuclear factor κB (NFκB) signaling pathway (Bishop et al., 2007). Besides TRAF signaling, recent evidence shows that CD40 can directly recruit Janus family kinase 3 (Jak3) which leads to the phosporylation of signal transducer and activator of transcription 5 (STAT5) (Bishop, 2004; Bishop et al., 2007).
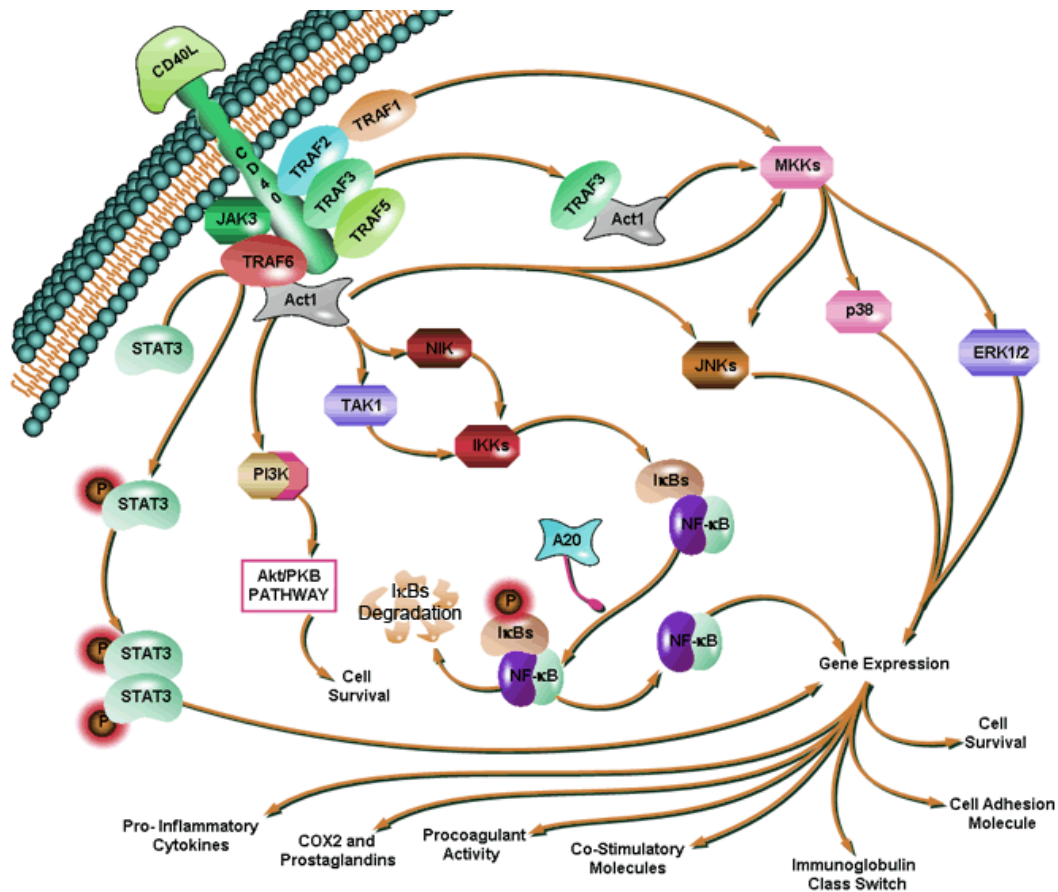
**Figure 7:** *CD40 signaling pathway.*
**Crosslinking of CD40 leads to intracellular signal transduction. Different molecules, like TRAFs, JAK3 and Act1 can initiate the signal transduction. Resulting in the activation of STAT3, ERK1/2, JNK and NF-κB finally modifying gene expression. CD40 activation induces variety of cellular responses, for example in B cells it augments cell survival, expression of adhesion and co-stimulatory molecules and others (adapted from SA Biosciences, Qiagen).**

These cascades eventually result in survival, maturation, proliferation, production of inflammatory cytokines, expression of additional costimmunlatory molecules, development of germinal center B cells (GC B cells), immunoglobulin isotype switching, somatic hypermutation of immunoglobulin genes and the formation of long-lived plasma cells and memory B cells (Danese et al., 2004).

As a powerful immune activator misregulation of CD40 can contribute to the development of a variety of chronic inflammatory diseases, like rheumatoid arthritis (Brennan and McInnes, 2008; Noss and Brenner, 2008), allergic diseases, autoimmune diabetes (Balasa et al., 1997), Graves disease and autoimmune thyroiditis (Faure et al., 1997). Additionally, CD40 may play a role in psoriasis (Boehncke and Schon, 2007) and inflammatory bowel diseases (Liu et al., 2000; Stuber et al., 1996). However, functional CD40 expression and signaling is extremely important for adequate thymus-dependent humoral immune responses. CD40-CD154 interactions contribute to B cell proliferation and isotype switch. Patients with X-linked hyper-IgM- syndrome (HIGS)

frequently carry a mutation in the CD154 gene (Allen et al., 1993; Aruffo et al., 1993; DiSanto et al., 1993). Thereby, CD4$^+$ T cells are not able to cluster CD40 on B cells and induce CD40 signaling. Yet, these patients have normal B cell numbers, but a reduced number of IgG antibodies and an increased number of IgM antibodies (Kawabe et al., 1994; Xu et al., 1994). Additionally, several experiments showed the importance of CD40 signal for the formation of germinal centers (Foy et al., 1994; Kawabe et al., 1994; Xu et al., 1994). Finally the impaired CD40 signaling leading to the ablation of isotype switched high affinity IgG$^+$ memory B cells is responsible for susceptibility to infections and impairs the effective pathogen clearance (Foy et al., 1994; Lam and Sugden, 2003; Xu et al., 1994). Recent studies demonstrate that CD40-CD154 interaction is more like an adjustable signal inducer than an on/off switch. Variation of the signal intensity can play a role in the regulation of different B cell subset activation, resulting in proliferation, differentiation or proliferation and differentiation of IgM$^+$/IgD$^+$ naive B cells and IgG$^+$ memory B cells (Neron et al., 2005).

## 1.6  *Ex vivo* CD40 system for B cell expansion

20 years ago Jacques Banchereau and Françoise Rousset published their idea to grow human B cells *ex vivo* in the presence of anti-CD40 antibodies to expand and cultivate them (Banchereau and Rousset, 1991). The strategy published by Banchereau is a two step procedure. One step is the isolation of the antigen-specific B cells requiring the antigen which binds to the BCR for detection. The second step is the expansion of the antigen-specific B cell clones. Therefore, they generated a continuously CD32 expressing fibroblastic cell line which was able to bind the stimulating anti-CD40 antibodies. The CD40 stimulation and added cytokines expand B cells and induce phenotypic changes, like isotype swichting and plasma cell differentiation.
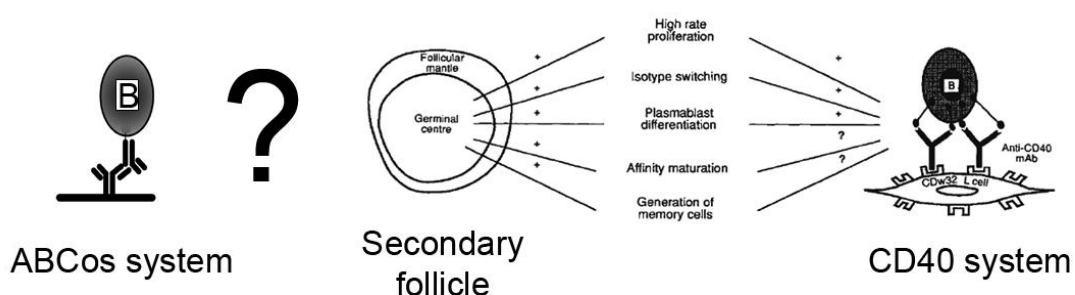


**Figure 8:** *CD40 mediated expansion in vivo in germinal centers and artificia ex vivo systems.*
**Comparison of biological properties of the both CD40 systems with those of germinal center from secondary follicles. Left is shown the proposed project which used the ABCos construct for CD40 signaling. Right is shown the Banchereau method based on the antibody mediated CD40 signal. (adapted and modified from Banchereau and Rousset, 1991).**

This system mimics the *in vivo* germinal center reaction which is highly effective to expand antigen-specific B cells and increase the affinity of the antibody. Five characteristics are defining the germinal center reaction: strong proliferation, somatic hypermutations, isotype switch and B cell differentiation into memory B cells or plasma cells. To perform the germinal center reaction in the lymph node B cells require the help of T helper cells and follicular dendritic cells (chapter 1.4). The high proliferation rate is needed to expand antigen-specific B cells. These B cells are able to introduce mutations into the variable domains by somatic hypermutations of their antibodies to modify the affinity which will finally increase antigen-specificity. Also in this process the context-dependent isotype switch to IgG, IgA or IgE takes place. The successful amplification of high-affinity B cells results in the differentiation into plasma cells and long-lived memory B cells. In the *ex vivo* simulation of germinal center reactions described by Banchereau the CD32 expressing fibroblastic cell line represents the follicular dendritic cells and the supplemented cytokines represent the follicular T helper cells.

An approach to become B cells independent of other factors which grow autonomously is the infection with EBV. The infection of peripheral B cells alone leads to a mainly IgM isotype, whereas the combination of the CD40 expansion approach and EBV transduction results in an IgA and IgG phenotype. These cells could be an important source for Ig mRNA which could be used for the large scale production of monoclonal antibodies.

## 1.7   Aim of the thesis

Antibody-based cancer therapies have been successfully introduced into the clinic and have emerged as one of the most promising therapeutics in oncology over the last decade. The approved tumor therapeutic antibodies do not cover the complete medical demand. The generation of fully human tumorantigen-specific antibodies suitable for anti-tumor therapy is laborious and difficult to achieve. Autoreactive B cells expressing those antibodies are present in cancer patient and represent a suitable source for human therapeutic antibodies. However, the isolation and cultivation of this cell type is challenging. To make these autoreactive B cells accessible this study designed a novel signaling platform.

Based on the described theorem from Jacques Banchereau this study was aiming to modify this technology from a two step technology to a T cell or anti-CD40 independent one step technology. This novel technology platform was developed to identify and

isolate antigen-specific B cells in the complete B cell repertoire of peripheral blood samples in one step. For the activation of B cells two signals are required, the antigen-specific B cell receptor signal and the antigen-independent CD40 signal provided by T helper cells. The aim was to establish a novel method mimicking the *in vivo* process. This method is based on the conversion of the antigen-independent CD40 signal into an antigen-specific one. To achieve this, a synthetic fusion protein was designed, which is able to induce antigen-specific CD40 signal, called **ABCos** (**A**ntigen-specific B cell co-stimulator). The strategy is pictured in the figure 9 and is based on the intracellular association of the synthetic fusion protein and the endogenous immunoglobulin mediated by the association domains $C_H2$-$C_H3$ derived from IgG1. This fusion protein should be anchored in cell membrane and after clustering by antigen recognition induces an antigen-specific CD40 signal, which results in germinal center like reactions.
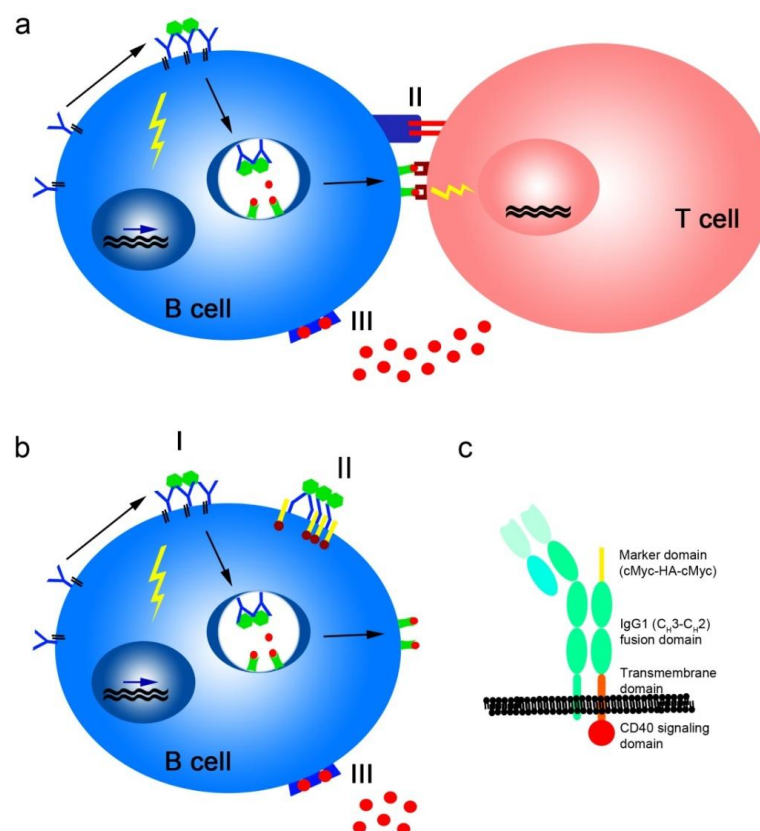


**Figure 9:** *Schematic draft of the antigen-specific activation via ABCos constructs.*
**a) For full B cell activation different signals are required, (I) antigen-specific signal resulting from BCR clustering through antigen recognition, (II) antigen independent CD40 signal and (III) stimulating cytokines provided by cognate T helper cells. b) To mimic the *in vivo* system of B cell activation B cells were transfected with ABCos construct and transform the antigen-independent signal into an antigen-specific one (II). Both signals (I) BCR and (II) CD40 are induced by certain antigen and could supplement with different (III) cytokine cocktails. c) Detailed description of the heterodimerized ABCos construct (right) with the endogenous immunoglobulin (left).**

The aim of this study was to test the functionality of the constructs and the strategy. For that the ability of the ABCos constructs to heterodimerize with the endogenous immunoglobulin and to induce a CD40-like signal should be analyzed. A primary objective of this work was to test the capacity of the ABCos constructs to induce antigen-specific expansion, as well as to analyze the effects triggered by constructs on B cells and B cell subsets.

# 2    Material and Methods

## 2.1    Material

### 2.1.1    Hardware

| Name | Manufacturer |
|------|--------------|
| Bath circulator Julabo F10 | Julabo Labortechnik, Seelbach, Germany |
| BioAnalyzer 2100 | Agilent Technologies, Palo Alto, USA |
| Biofuge pico | Heraeus Instruments, Hanau, Germany |
| Cell incubator | Heraeus Instruments, Hanau, Germany |
| Centrifuge 5810 | Heraeus Iinstruments, Hanau, Germany |
| Centrifuge Mikro 22R | Hettich, Tuttlingen, Germany |
| Clean bench Herasafe | Heraeus Instruments, Hanau, Germany |
| $CO_2$ Incubator | Heraeus Instruments, Hanau, Germany |
| Cuvette centrifuge IST | Th. Betzel, Hattersheim, Germany |
| Delfia platewash | PerkinElmer, Waltham, USA |
| Electrophoresis power supply ST 606 | Gibco BRL/Invitrogen, Karlsruhe, Germany |
| FACS Calibur | Becton Dickinson, Heidelberg, Germany |
| FACS Canto2 | Becton Dickinson, Heidelberg, Germany |
| Fluorescence microscope DM RXA | Leica Microsystems, Wetzlar, Germany |
| Gel documentation system Gel Jet Imager | Intas, Göttingen, Germany |
| Gene Pulser II | BioRad, München, Germany |
| Incubation hube IH 50 | Noctua, Wien, Austria |
| Inverted microscope Wilovert | Hund, Wetzlar, Germany |
| Luminescence reader infinite M200 | Tecan, Crailsheim, Germany |
| Microbiological incubator | Heraeus Instruments, Hanau, Germany |
| Microscope Wilovert S | Hund, Wetzlar, Germany |
| Midi gel electrophoresis system MGU-602T | C.B.S. Scientific, Del Mar, USA |
| Minishaker MS2 | IKA Labortechnik, Staufen, Germany |
| Table centrifuge mikro 22R | Hettich, Tuttlingen, Germany |
| Thermocycler T3 | Biometra, Göttingen, Germany |
| Thermomixer compact | Eppendorf, Hamburg, Germany |
| Ultrospec 2100 pro | Amersham Pharmacia, Uppsala, Sweden |
| Vortexer VF2 | IKA Labortechnik, Staufen, Germany |

### 2.1.2 Consumables

| Designation | Manufacturer |
|---|---|
| 24 well plates | Costar, New York, USA |
| 6 well plates | Costar, New York, USA |
| 8-Strip tubes, 0.2 mL | Molecular BioProducts, San Diego, USA |
| 96 well plate (white) | Nunc, Wiesbaden, Germany |
| 96 well plates round bottom | Costar, New York, USA |
| 96 well plates V bottom | Greiner Bio one, Essen, Germany |
| Cell culture petri dishes | Becton Dickinson, Heidelberg, Germany |
| Cell scrapers | Becton Dickinson, Heidelberg, Germany |
| Cell strainer, 40 µm, Nylon | Becton Dickinson, Heidelberg, Germany |
| Cryo tube 1.8 mL | Nunc, Wiesbaden, Germany |
| Electroporation cuvettes, 4 mm gap | BioRad, Hercules, USA |
| Eppendorf tubes, 1.5 and 2 mL | Eppendorf, Hamburg, Germany |
| Round bottom 5 mL FACS tubes | Becton Dickinson, Heidelberg, Germany |
| Falcon tubes, 15 and 50 mL | Greiner BioChemica, Flacht, Germany |
| Filter tips, AvantGuard 100-1250 µL | Light Labs, Dallas, USA |
| Filter tips, Biosphere 10-100 µL | Sarstedt, Nümbrecht, Germany |
| Filter tips, Neptune 1-10 µL | Continental Lab Products, San Diego, USA |
| Gene pulser cuvette | BioRad, München, Germany |
| MACS separation columns | Miltenyi Biotec, Bergisch-Gladbach, Germany |
| PCR tubes, 0.5 mL | Molecular BioProducts, San Diego, USA |
| RNase-free tubes, 1.5 and 2 mL | Eppendorf, Hamburg, Germany |
| Serological Pipette, 5 mL, 10 mL, 15 mL | Greiner Bio One, Frickenhausen, Germany |
| Surgical disposable scalpels | B. Braun, Melsungen, Germany |
| Tissue culture flasks | Becton Dickinson, Heidelberg, Germany |

### 2.1.3 Kits

| Name | Manufacturer |
|---|---|
| Magnetic acitivated cell sorting (MACS) Kit CD19$^+$ B cells, IgG$^+$ memory B cells | Miltenyi Biotec, Bergisch Gladbach, Germany |
| MEGAclear $^{TM}$ | Ambion, Austin, USA |
| mMESSAGE mMACHINE SP6 Kit | Ambion, Austin, USA |
| mMESSAGE mMACHINE T7 Ultra Kit | Ambion, Austin, USA |
| NucleoSpin Kit | Macherey and Nagel, Düren, Germany |
| NucleoSpin Plasmid Mini Kit | Macherey-Nagel, Düren, Germany |
| Qiafilter Plasmid Maxi (Midi) Kit | Qiagen, Hilden, Germany |
| QiaQuick Gel Extraction Kit | Qiagen, Hilden, Germany |
| Qiaquick MinElute Gel Extraction Kit | Qiagen, Hilden, Germany |
| Qiaquick MinElute PCR Purification Kit | Qiagen, Hilden, Germany |
| Vybrant CFDA/SE Cell Labeling Kit | Invitrogen, San Diego, USA |

## 2.1.4 Chemicals and enzymes

| Designation | Manufacturer |
|---|---|
| AB serum | Cambrex, Rutherford, USA |
| Acetic acid | AppliChem, Darmstadt, Germany |
| Adenosine 5`-triphosphatase (ATPase) | Sigma, St. Louis, USA |
| Agar | Difco, Detroit, USA |
| Agarose | Sigma, St. Louis, USA |
| Ammoniumpersulfate (APS) | Sigma, St. Louis, USA |
| Ampicillin | Sigma, St. Louis, USA |
| BamH1 | Fermentas, St. Leon-Rot, Germany |
| Benzonase | Merck, Darmstadt, Germany |
| Bovine serum albumin (BSA) fraction V pH 7.0 | Serva, Heidelberg, Germany |
| Chloroform | Merck, Darmstadt, Germany |
| CpG-ODN $_{2006}$ | InvivoGen, San Diego, USA |
| Diethylenpyrocarbonate (DEPC) | Sigma, St. Louis, USA |
| Dimethyl sulfoxide (DMSO) | Appli-Chem, Darmstadt, Germany |
| D-Luciferin | Becton Dickinson, Heidelberg, Germany |
| DNase I | Roche, Mannheim, Germany |
| Dulbeccos modified eagle medium (DMEM) | Invitrogen, San Diego, USA |
| EciI | New England Biolabs, Frankfurt, Germany |
| Ethanol absolute p.A. | Appli-Chem, Darmstadt, Germany |
| Ethidiumbromide | Sigma, St. Louis, USA |
| Ethylendiamintetraacetate (EDTA) | Merck, Darmstadt, Germany |
| Ethylendiamintetraacetate (EDTA) 0.5 M pH 8.0 | Sigma, St. Louis, USA |
| FACS Clean/Flow/Rinse | Becton Dickinson, Heidelberg, Germany |
| Fetal calf serum (FCS) | PAA laboratories, Cölbe, Germany |
| Ficoll-Hypaque | Amersham Biosciences, Uppsala, Sweden |
| Formaldehyde | Merck, Darmstadt, Germany |
| GeneRuler 1kB DNA Ladder | MBI Fermentas, Burlington, Canada |
| GeneRuler 50bp DNA Ladder | MBI Fermentas, Burlington, Canada |
| Geneticin | Invitrogen, San Diego, USA |
| Glycerin (waterless) | Merck, Darmstadt, Germany |
| Human serum albumin 20 % (HSA) | CSL Behring, Marburg, Germany |
| IL2 | Miltenyi Biotec, Bergisch Gladbach, Germany |
| IL4 | Miltenyi Biotec, Bergisch Gladbach, Germany |
| IL6 | Miltenyi Biotec, Bergisch Gladbach, Germany |
| IL7 | PeproTech, London, UK |
| IL10 | PromoKine, Heidelberg, Germany |
| IL12 | PeproTech, London, UK |
| IL13 | PromoKine, Heidelberg, Germany |
| IL15 | R&D System, Minneapolis, USA |
| IL21 | PromoKine, Heidelberg, Germany |
| Isopropanol p.a. | Merck, Darmstadt, Germany |
| Kanamycin | Sigma, St. Louis, USA |

| Lumi-Light western blotting substrate | Roche, Mannheim, Germany |
|---|---|
| Methanol | Merck, Darmstadt, Germany |
| Milk powder | Gabler Saliter, Obergünzburg, Germany |
| MOPS | Sigma, St. Louis, USA |
| Non-essential amino acids (NEAA) | Invitrogen, San Diego, USA |
| PBS | Bio Whittaker Europe, Verviers, Belgien |
| PBS pH 7.2 | Invitrogen, San Diego, USA |
| Penicillin (10.000 U/ml)/ streptomycin (10 mg/ml) | Invitrogen, San Diego, USA |
| Phenol | Roth, Karlsruhe, Germany |
| Ponceau S | Sigma, St. Louis, USA |
| Propidium iodide | Sigma, St. Louis, USA |
| RNA size marker | Ambion, Huntingdon, UK |
| Roti Load 1 | Roth, Karlsruhe, Germany |
| RPMI 1640 + GlutaMAX | Invitrogen, San Diego, USA |
| Sac1 | Fermentas, St. Leon-Rot, Germany |
| Sodium acetate | Roth, Karlsruhe, Germany |
| Sodium acetate (3M; pH 4.5) | Ambion, Austin, USA |
| Sodium chloride | Roth, Karlsruhe, Germany |
| Sodium dihydrogenphosphate monohydrate | Merck, Darmstadt, Germany |
| Sodium dodecylsulfate (SDS) | Sigma, St. Louis, USA |
| Sodium hydroxide | Merck, Darmstadt, Germany |
| Sodium pyruvate 100 mM | Invitrogen, San Diego, USA |
| Trishydroxymethylaminomethan (TRIS) | AppliChem, Darmstadt, Germany |
| Triton-X 100 | Merck, Darmstadt, Germany |
| TrypLE Express | Invitrogen, San Diego, USA |
| Trypton | Difco, Detroit, USA |
| Tween 20 | Merck, Darmstadt, Germany |
| Water Molecular Biology Grade | Cambrex, Rutherford, USA |
| Whatman paper | Whatman International, Waitstone, UK |
| X-Vivo 15 | Lonza, Verviers, Belgium |
| Xho1 | Fermentas, St. Leon-Rot, Germany |

## 2.1.5 Antibodies

| Reactivity (human) | Donor | Clone | Isotype | Conjugation | Manufacturer |
|---|---|---|---|---|---|
| CD19 | mouse | J3-119 | IgG1 | PE, FITC, APC | Beckman Coulter |
| CD20 | mouse | B9E9 | IgG2a | PE, FITC, APC | Beckman Coulter |
| CD27 | mouse | L128 | IgG1 | PE, APC | BD |
| CD32 | mouse | AT10 | IgG1 | FITC | Caltag |
| CD38 | mouse | HIT2 | IgG1 | PE, APC | BD |
| CD40 | mouse | MAB89 | IgG1 | pure | Abcam |
| CD138 | mouse | MI15 | IgG1 | PE, APC | BD |
| cMyc | mouse | 9E10 | IgG1 | pure, FITC | Sigma |
| kappa | mouse | MEM-09 | IgG1 | APC | Abcam |
| IgD | goat | polyclonal | IgG | PE | Abd Serotec |

## 2.1.6 Vector

The pST1-A120 vector backbones were previously described by Holtkamp (Holtkamp et al., 2006). The Plasmid vector constructs were used as templates for *in vitro* transcription of ABCos1 and ABCos2 RNA The pSTI-A120 vector, derived from pCMV-Script-Plasmid, features a T7-promoter, two serial human β-globin 3´ UTRs, a 120bp-long poly A-tail and a Neomycin resistance gene.

## 2.1.7 Peptide and proteins

| Peptide | Source | Sequence | Producer |
|---------|--------|----------|----------|
| Plac1-SED | Human Placenta antigen 1 | SEDWSLHTDDMIGSM | Jerini, Berlin, Germany |
| Plac1-EAQ | Human Placenta antigen 1 | EAQPLQPSHFLDISE | Jerini, Berlin, Germany |
| pp150 | CMV | Immunodominat regions | Abcam, Cambridge, UK |

## 2.1.8 Amino acid sequences of ABCos1 and ABCos2

ABCos1 = 1 – 386 amino acid

```
1          10         20         30         40         50
MYRMQLLSCI ALSLALVTNS APTGSEQKLI SEEDLYPYDV PDYAEQKLIS
EEDLRSDKKV EPKSSDKTHT CPPCPAPELL GGPSVFLFPP KPKDTLMISR
TPEVTCVVVD VSHEDPEVKF NWYVDGVEVH NAKTKPREEQ YNSTYRVVSV
LTVLHQDWLN GKEYKCKVSN KALPAPIEKT ISKAKGQPRE PQVYTLPPSR
DELTKNQVSL TCLVKGFYPS DIAVEWESNG QPENNYKTTP PVLDSDGSFF
LYSKLTVDKS RWQQGNVFSC SVMHEALHNH YTQKSLSLSP ELQLEGPQDR
LRALVVIPII FGILFAILLV LVFIKKVAKK PTNKAPHPKQ EPQEINFPDD
LPGSNTAAPV QETLHGCQPV TQEDGKESRI SVQERQ
```

aa1-23 =    IL-2 secretion signal
aa26-54 =   Myc-HA-Myc tag
aa55-294 =  IgG1 $C_H2$-$C_H3$
aa295-324 = CD40 TM domain
aa325-386 = CD40 intracellular domain

ABCos2 = 1 – 395 amino acids

```
1          10         20         30         40         50
MYRMQLLSCI ALSLALVTNS APTGSEQKLI SEEDLYPYDV PDYAEQKLIS
EEDLRSDKKV EPKSSDKTHT CPPCPAPELL GGPSVFLFPP KPKDTLMISR
TPEVTCVVVD VSHEDPEVKF NWYVDGVEVH NAKTKPREEQ YNSTYRVVSV
LTVLHQDWLN GKEYKCKVSN KALPAPIEKT ISKAKGQPRE PQVYTLPPSR
DELTKNQVSL TCLVKGFYPS DIAVEWESNG QPENNYKTTP PVLDSDGSFF
LYSKLTVDKS RWQQGNVFSC SVMHEALHNH YTQKSLSLSP ELQLEESCAE
AQDGELDGLW TTITIFITLF LLSVCYSATV TFFKKVAKKP TNKAPHPKQE
PQEINFPDDL PGSNTAAPVQ ETLHGCQPVT QEDGKESRIS VQERQ
```

aa1-23 =    IL-2 secretion signal
aa26-54 =   Myc-HA-Myc tag
aa55-294 =  IgG1 $C_H2$-$C_H3$

aa295-333 = IgG TM domain
aa334-395 = CD40 intracellular domain

## 2.1.9 Cell lines

| Cell line | Origin of cells | reference | culture |
|---|---|---|---|
| CHO-K1 WT | Chinese hamster ovary cells | DSMZ No: ACC100 | DMEM:F12 + 10 % FBS$_{hi}$, 1% Pen/Strep |
| CHO-K1-CD32 | Chinese hamster ovary cells stably transfected with CD32 | In house | DMEM:F12 + 10 % FBS$_{hi}$, 500 µg/mL Hygromycin-B; 1% Pen/Strep |
| CHO-K1-pC15 | Chinese hamster ovary cells stably transfected with secreted IgG1 | Ganymed Pharmaceuticals AG | DMEM:F12 + 10 % FBS$_{hi}$, 1% HT, 1% Pen/Strep |
| HEK293-lucNifty-miRNA81 clone 23 | Human embryonal Kidney epithelial cells transfected with miRNA81 and pNifty-Luc | DSMZ No: ACC305 | D-MEM; 10% FCS, 1% NEAA; 1% Na-Pyruvat; 1% Na-Pyruvat, 1% Pen/Strep, for cultivation with and for assay without 250 µg/mL Geneticin; 100 µg/mL Zeocin |

## 2.1.10 Buffer and solutions

**Buffer and solutions for molecular biology methods**

**MOPS gel (1 %)**
1.5 g Agarose
120 mL DEPC water
dissolve agarose by boiling
• cool down to 55 °C
• add 15 mL 10 x MOPS stock solution
• add 15 mL 37 % formaldehyde

**MOPS 10 x stock solution**
42.8 g MOPS
6.8 g Sodium acetate
3.7 g EDTA
• pH 7.0

**Loading buffer**
0.25 % Bromphenol blue
0.25 % Xylene cyanole FF
0.25 % Orange G
1 mM EDTA
40 % Sucrose
• in $H_2O_{dest}$

**RNA sample buffer**
120 µL 10 x MOPS
500 µL Formamide
350 µL Formaldehyde 37 %
150 µL Blue marker
100 µL Ethidium bromide (0.05 M)

**TAE 50 x stock solution**
242 g TrisBase
57.1 mL Glacial acetic acid
100 mL 0,5 M EDTA pH 8.0
• add 1 L $H_2O_{dest}$
• autoclave

**TAE gel (1 %)**
1.5 g Agarose
150 mL 1 x TAE
• dissolve agarose by boiling
• cool down to 55 °C
• add 75 µL ethidium bromide (0.05 M)

**DEPC water**
1 mL DEPC
• ad 1 L $H_2O_{dest}$
• shake over night at room temperature
• autoclave

**LB medium**
5 g Yeast extract
10 g Tryptone
10 g sodium chloride
• ad 1 L $H_2O$dest
• autoclave

**SOC medium**
5 g Yeast extract
20 g Tryptone
0.5 g sodium chloride
2.5 ml potassium chloride (1 M)
• add 1 L $H_2O_{dest}$
• autoclave
• add 20 ml Glucose (1 M, sterile)

**LB agar**
1 L LB medium
15 g Agar
• autoclave


**Buffers and solutions for cell biological and immunological methods**


**FACS buffer**
| 500 mL | PBS |
| 5 % | $FCS_{hi}$ |
| 5 mM | EDTA |

**FACS buffer incl. 7-AAD**
| 500 mL | PBS |
| 5 % | $FCS_{hi}$ |
| 5 mM | EDTA |
| 10 µg/mL | 7-AAD |

**MACS buffer**
500 mL          PBS
5 %             HSA
5 mM            EDTA

**Refreezing medium**
90 % human AB serum
10 % DMSO
• add 50 µL Heparin

**Buffers and solutions for proteinbiochemistry**

**Running buffer Laemmli 10 x**
144 g Glycin
30.3 g TRIS
10 g SDS
• add 1 L $H_2O_{dest}$

**Anode buffer 1**
300 mM TRIS pH 9.5

**Anode buffer 2**
30 mM TRIS pH 9.5

**Cathode buffer 10 x**
250 mM TRIS
400 mM ε-Aminocaproic acid

**Working solution**
1 volume Cathode buffer (10 x)
7 volumes $H_2O$ dest.
2 volumes Methanol

**Wash buffer 10 x**
0.5 M TRIS
3 M NaCl
2 % Tween 20
• adjust to pH 7.5

**Blocking buffer**
5 % Milk powder (w/v)
0.1 % Tween20 (v/v)
• dissolve in PBS

## 2.2   Methods

### 2.2.1  Molecular biological methods

The molecular biological methods were conducted based on the published standard protocols "Molecular cloning" from Sambrook and colleagues (Sambrook et al., 1989).

### 2.2.1.1       Transformation

Chemically competent XL-1 blue cells (Stratagene) were thawed on ice and 50 µL cell suspension was supplemented with 0.85 µL β-mercapthoethanol. The cell suspension was mixed gently by tapping every 2 min while incubated on ice for 10 min. 0.1 – 50 ng of the plasmid DNA was added to the cell solution and the mixture was incubated for further 30 min on ice. Subsequently the heat shock at 42 °C for 45 seconds caused a short formation of pores in the cell membrane allowing the DNA uptake. During the following incubation in 450 µL SOC medium without antibiotics at 37 °C for 1 h the cells were able to regenerate and express specific antibiotic resistance enzymes provided by the introduced plasmid.

To propagate transformed cells 250 µL of the cell suspension were spread on LB-agar plates containing the antibiotic, either ampicillin (100 µg/mL) or kanamycin (25 µg/mL).

### 2.2.1.2       Preparation of plasmid DNA from bacteria culture

Antibiotic resistant clones were picked from an LB-agar plate with a sterile pipette tip and transferred in 3 mL selective LB-medium. The inoculated culture was then incubated over night at 37 °C on continual shaking. The NucleoSpin Plasmid DNA Purification Kit (Macherey-Nagel) was used for the purification of plasmid DNA. This method is based on the publication from Birnboim and Doly (Birnboim and Doly, 1979). The buffer N3 neutralized the lysate and adjusted the sample to high-salt binding conditions in one step. This high-salt condition leaded to protein denaturation and precipitation of chromosomal DNA, cellular debris and SDS. The small plasmid DNA renatures correctly and resides in solution after centrifugation. The supernatant was applied to the silica membranes of spin columns provided in the kit. The DNA was washed and eluted in 30 – 50 µL $H_2O$. To control the correctness of the purified DNA,

the eluate was digested with appropriate restriction enzymes and analyzed by agarose gel electrophoresis. Plasmids displaying the expected band pattern were sequenced. Correct constructs were either stored as DNA at -20 °C or as glycerol stock by mixing the cell suspension and glycerol to a final concentration of 50 % glycerol.

For large scale plasmid DNA purification, 50 – 200 mL LB medium containing appropriate antibiotic was inoculated with 50 – 200 µL of the respective glycerol stock. Plasmids were then isolated and purified using Qiafilter Plasmid Midi or Maxi Kits (Qiagen) according to the manufacturer's instructions.

### 2.2.1.3     Determination of nucleic acid concentration

In order to determine the concentration of nucleic acids (DNA and RNA) the common spectrophotometry was used. Molecules absorb light at a distinct wavelength. In the case of nucleic acids the absorption maximum is by 260 nm whereas proteins absorb light at 280 nm due to their amino acid aromatic site chains.

The concentration of RNA or DNA was calculated by the spectrometer directly according to the Lambert-Beer law. The purity was analyzed by the quotient between the absorbance values at 260 nm and 280 nm. Only nucleic acids with a purity value between 1.8 to 2 were used.

### 2.2.1.4     Agarose gel electrophoresis for analysis of nucleic acids

A common technique to analyze, quantify or purify DNA or RNA molecules is the agarose gel electrophoresis. The agarose gel electrophoresis separates DNA and RNA molecules according to their size. The DNA or RNA molecules migrate in the electric field due to their negatively charged phosphate backbone. To visualize the DNA or RNA ethidum bromide (EtBr) was added.

To separate DNA fragments after restriction digests or PCR amplifications gels consisting of 1 x TAE buffer and 5 ppm EtBr dye were used. The agarose percentage of the gel differed between 1 - 2 % w/v depending on the size of the DNA fragments. The samples were mixed with 1/6 v/v of 6x DNA loading dye (Fermentas) and then added in the gel chambers.

In order to control the quality of the isolated RNA or *in vitro* transcribed mRNA 1 – 2 % w/v agarose gels containing 1x MOPS were used. Before the gel was casted 37 % formaldehyde was added. The formaldehyde prevents the formation of secondary structures of the RNA molecules which would alter the migration velocity. RNA probes were mixed with sample buffer (including EtBr) and denatured for 10 min at 70 °C before loading to the gel.

To determine the size of the loaded nucleic acids, a suitable DNA or RNA size marker was loaded. Electrophoresis buffer contained 1x TAE or 1x MOPS, respectively. Electrophoresis was performed at 100 - 170 V depending on size of the gel and the gel chamber.

### 2.2.1.5        DNA sequencing

To analyze the presence of mutations or the length of the poly-A tail the purified DNA were sequenced with the appropriate sequencing primers by a commercial provider (MWG Eurofins, Eberberg, Germany).

### 2.2.1.6        Generation of IVT-RNA

All constructs for the production of IVT-RNA were inserted into pST1 plasmids. For the generation of RNA the pST1 plasmids were linearized by Eci1 or Sap1 digestion (2.2.1.7) and purified by phenol/chloroform extraction and sodium acetate precipitation. For that the DNA was mixed with equal volumes of phenol/chloroform. After centrifugation (2 min, 10 000 g) the mixture builds two phases separated by an interphase. The lower phase is the organic phase and includes proteins, while precipitated proteins reside in the interphase. The upper aqueous phase contains the DNA and was transferred into a new tube. For further purification and removal of phenol contamination the DNA was mixed a second time with chloroform and centrifuged (2 min, 10 000 x g). The aqueous phase was separated and mixed with 0.1 volume of  3 M sodium acetate as well as 2.5 volumes ethanol for DNA precipitation. After vigorous vortexing the probes were incubated over night at -20 °C for precipitation. Subsequently the DNA was pelleted by centrifugation (45 min, 10 000 x g, 4 °C). After removing the supernatant the pellet was washed with 80 % ethanol to remove salt, dried and resolved in an appropriate volume of DEPC-$H_2O$.

The DNA concentration was analyzed by spectrophotometry (2.2.1.3). According to the manufacture´s instruction the linearized vector DNA was *in vitro* transcribed into mRNA with the Ambion mMessage mMachine T7 ultra kit. This kit uses a modified cap analogue, called ARCA cap (anti reversed cap analogue). Due to –$OCH_3$ substituted – OH groups the generated RNA only contains caps in the correct orientation (Stepinski et al., 2001).

The reaction batch for the *in vitro* transcription was composed of:

| Reagent | Volume in µL |
|---|---|
| T7 2 x NTP/ARCA | 10 |
| 10 x T7 buffer | 2 |

| linearised DNA (1 µg/µL) | 1 |
|---|---|
| T7 polymerase | 2 |
| RNAse free aqua dest. $H_2O$ | 5 |
| **Final volume** | **20** |

The reaction batch was incubated for approx. 2 – 3 h at 37 °C. Subsequently template DNA was digested by adding 1 µL Turbo DNase I for 30 min at 37 °C. To remove reaction batch components and DNA contaminations the RNA was purified using Ambion MegaClear Kit. Quantification and quality control were done by spectrophotometry and Bioanalyzer.

## 2.2.1.7    Restriction digest of nucleic acids

For cloning of vectors and inserts (e.g. PCR products) or to control ligation products nucleic acids were digested with restriction enzymes.

**Analytic digest**                                    **Preparative digest**

0,5 – 1 µg    Plasmid                          5 – 10 µg    Plasmid

1 U/µg restriction enzyme                  5 – 10 U/µg    restriction enzyme

➔ 1 – 2 h                                        ➔ 2 – 4  h
➔ enzyme specific                          ➔ enzyme specific
  temperature                                     temperature

The samples were mixed with 1x enzyme specific buffer and diluted with $H_2O$ to a volume that decreases the glycerol concentration to 5 % v/v. For that the total volume is 10 times higher than the volume of the restriction enzyme. Subsequently the samples were analyzed with agarose gel electrophoresis (2.2.1.4) or purified by anionic exchange columns.

## 2.2.1.8    Ligation of nucleic acids

For ligation the vectors were dephosphorylated by addition of CIAP (Calf Intestine Alkaline Phophatase, Fermentas) to prevent self-ligation of the vectors. To ligate the insert and vector the T4-DNA Ligase (Fermentas) was used.

For the ligation digested and purified insert and vector were mixed in a molar ratio of 3 : 1 or 5 : 1. The reaction batch includes 1 µL enzyme (10 U/µL) and 1 µL 10x buffer in a final volume of 10 µL. The ligation batch was incubated at 22 °C for 1 – 2 h or over night at 14 °C.

## 2.2.1.9 Purification of nucleic acids after gel electrophoresis

The desired fragments were excised from an agarose gel with a clean scalpel. The DNA was isolated from the gel by using QIAquick Gel Extraction Kit (Qiagen) according to manufacturer´s instruction. 1 volume gel and 3 volumes QG buffer were mixed and incubated for 10 min at 50 °C. Subsequently the completely dissolved gel was applied to a spin column and incubated for 1 min. The column was washed with buffer QG and buffer PE. The PE buffer includes ethanol and removes agarose contaminations, ethidum bromide and salts. After centrifugation the clean dry DNA fragments were eluted with 30 – 50 µL $H_2O$.

In order to purify enzymatically modified or amplified DNA fragments the QIAquick PCR Purification Kit (Qiagen) was used. The samples were diluted with 5 volumes PB buffer rinsed onto a spin column. The columns were washed with PB and PE buffer to remove enzymes, unused nucleotides and salts. After centrifugation the clean dry DNA fragments were eluted with 30 – 50 µL $H_2O$. To concentrate DNA MiniElute columns were loaded with DNA and eluted with only 10 µL $H_2O$. All DNA samples were stored at -20 °C.

## 2.2.2  Cell biological and immunological methods

The cell culture techniques are based on the published standard protocols "Kultur tierischer Zellen" from SJ Morgen (Morgen and Darling, 1994).

### 2.2.2.1      Maintenance of cell lines

All cells were cultured under aseptic conditions in specified medium including 1 % penicillin/streptomycin. All cells were cultured in an incubator at 37 °C with 95 % humidity and 5 % $CO_2$. The adherent cells were cultured subconfluent. To maintain subconfluent cells the medium was removed, cells were washed with PBS and detached by adding trypsin. The enzymatic reaction was stopped by dilution with complete medium and subsequently disseminated in desired cell density into new culture dishes. Cells growing in suspension were diluted in fresh medium following transfer into a new flask.

### 2.2.2.2      Determination of cell numbers

The cell numbers were determined with the "Neubauer cell-count chamber" via microscopy. An aliquot of the cell suspension was diluted with 0.1 % trypan blue solution. Trypan blue is a polyanionic azo dye that is not able to penetrate cells with an intact membrane potential. For this reason vital cells remain unstained (refractive), while due to the leaky cell membrane dead cells are stained with trypan blue. According to requirements the cell suspension was diluted 1 : 2, 1 : 5, or 1 : 10 with trypan blue in a total volume of 100 µL. The product of cell number per quadrants, dilution and chamber factor and volume is the total cell number.

### 2.2.2.3      Freezing and thawing of cells

Cryopreservation is useful to preserve important cells. To alleviate the negative effects by freezing we added cryoprotectants (DMSO) and used special container for slow cooling with a defined cooling rate from 1 °C/min. In contrast, thawing should be rapid to diminish damaging effects.

For cryopreservation, the cells were harvested, counted and centrifuged (300 x g, 4 °C, 8 min) before adjusted in precooled freezing medium to $1 - 2 \times 10^7$ cells/mL. 1 mL cell suspension was transferred in special cryo tubes and stored over night at -80 °C in special cryo-boxes before long term storage at -196 °C.

The cells were defrosted in 37 °C warm water and washed in 20 mL 0.2 M EDTA/PBS. Subsequently, the cells were centrifuged, and the cell pellet was resuspended in fresh medium.

### 2.2.2.4 Isolation of peripheral blood mononuclear cells (PBMC) from human blood samples

PBMC were isolated from buffy coats by Ficoll-Hypaque density centrifugation. During the processing of donate blood the anti-coagulated blood is split in plasma and erythrocytes separated by the so called buffy coat. The buffy coat mainly consists of leucocytes and platelets and represents less than 1 % v/v of the donated blood.

Buffy coats were obtained from the Transfusion Center Mainz. To isolate the PBMCs the buffy coats were diluted with PBS to 140 mL and 35 mL of the diluted blood were added to a 15 mL ficoll layer. After centrifugation (678 x g, 21 °C, 25 min) the mononuclear cells separate from polynuclear cells and erythrocytes. The sedimentation constant of cells depends on their volume, density and weight. Only the erythrocytes and granulocytes pass the ficoll and build a red pellet on the bottom. Direct on top of the ficoll is a "white" ring or interphase consisting of lymphocytes and monocytes which is overlaid by the plasma and platelets phase. The PBMCs were recovered by pipetting and washed twice with 2 mM EDTA/PBS before further usage.

### 2.2.2.5 Isolation of different cell populations from human PBMC

PBMC are composed of B cells, T cells, NK cells and monocytes. Magnetic activated cell sorting (MACS) is a useful method to isolate specific subsets. The method is based on the labeling of cells by antibodies conjugated to magnetic beads (diameter app. 50 nm). To separate the magnetically labeled cells from unlabeled cells a MACS column was placed into a magnetic field. This leads to the retention of the magnetically labeled cells, while the unlabeled cells are able to pass the column. The labeled cells are eluted after the column was removed from the magnetic field.

- Isolation of $CD19^+$ B cells:

The isolation of $CD19^+$ B cells was performed according to the manufacture´s instruction and is based on the labeling of CD19 which is exclusively expressed on B cells (Miltenyi Biotec).

- Isolation of $IgG^+$ memory B cells:

The isolation of $IgG^+$ memory B cells was performed according to the manufacture´s instruction and is based on the depletion of non B cells and subsequent positive selection with anti-IgG beads (Miltenyi Biotec).

MATERIAL and Methods

## 2.2.2.6 Electroporation of primary cells or cell lines with IVT-RNA

Electropermeabilization or short electroporation is a useful technique to introduce molecules (DNA, RNA) into cells by an electrical pulse, which forms pores into the cell membrane. In order to increase the transfection and survival rate it is necessary to optimize the strength of pulse for each cell line. Because RNA is susceptible to degradation by RNases it is important to work with RNAse-free consumables (e.g. gloves, pipette tips, cuvettes) and work at 4 °C to decrease the activity of RNases.

For the electroporation the cells were harvested and washed twice in serum-free X-Vivo 15 medium to minimize the amount of RNases present. Cells were adjusted to the desired cell number ($1 - 20 \times 10^6$ cells/electroporation) and transferred into a precooled 4 mm gap sterile electroporation cuvette. After addition of IVT-RNA the RNA-cell suspension was mixed by pipetting and electroporated using Gene Pulser-II apparatus (Bio-Rad). Subsequently the cells were transferred into fresh culture medium for regeneration. The optimal electroporation parameters for each cell line were determined in advance.

## 2.2.2.7 Luciferase-Assay for NF-κB analysis

To monitor the induction of ABCos signaling in response to ABCos clustering the in-house generated HEK293 cell line which is stably transfected with lucNifty vector and miRNA81 was used. The lucNifty vector is a reporter plasmid composed of the luciferase gene under the control of an NF-κB-inducible ELAM1 composite promoter. The Firefly luciferase from Photinus pyralis is a 61 kDa monomer, which is functional after translation and catalyzes the oxidative enzymatic reaction from luciferin to oxyluciferin. This reaction needs ATP and $O_2$ as a substrate and produces as a by-product light and AMP. The produced light ($550 - 570$ nm) can be measured with a luminometer and corresponds to the activation level of NF-κB (Alam and Cook, 1990; de Wet et al., 1987).

To perform NF-κB luciferase assay, HEK 293 reporter cells transiently transfected with ABCos constructs were incubated over night. Subsequently $1 \times 10^6$ ABCos positive HEK 293 reporter cells were added in an anti-cMyc or control antibody coated 96-well plate in 100 µL over night. 6 hours before analysis PMA and Ionomycin were added as positive control. A mixture of D-Luciferin (1 µg/µl) and 5 mM ATP- 10 mM Tris base solution was added to each well and bioluminescence flux was measured using a microplate luminescence reader with 1sec integration time/well.

## 2.2.2.8     Proliferation analysis with CFSE or CPD-eF670 staining

CFDA-SE (carboxyfluorescein diacetate succinimidyl ester) is an amine-reactive molecule which easily enters cell membranes. Only after intracellular cleavage of the acetate groups by esterases the CFSE start to fluoresce. The succinimidyl ester groups covalently bind to amine-groups of intracellular proteins and when a cell divides the fluorescence is equally distributed to the daughter cells leading to a reduction of fluorescence which can be detected by FACS analysis (Lyons, 2000).

The Vybrant CFDA/SE cell Labeling Kit was used to label cells with CFSE according to the manufacture´s instruction. CFDA/SE powder was dissolved in 90 µL DMSO for reconstitution to obtain a 10 mM solution. This solution was prediluted 1 : 40 in required volume. Approximately $1x10^7$ cells/ in 1 mL PBS were stained with 20 µL prediluted CFDA/SE solution (5 µM final concentration) incubated for 5 – 10 min at room temperature. To stop the labeling reaction the cells were washed with medium or $FCS_{hi}$ and subsequently adjusted to the required concentration.

To label cells with proliferation detection dye the Cell Proliferation Dye eF670 Labeling Kit (eBioscience, San Diego, USA) was used according to the manufactures instruction. Shortly, $1x10^6$ cells in 500 µL PBS were stained with 50 µL prediluted CPD-eF670 solution (final 5 µM). After adding CPD-eF670 solution the cells were mixed and incubated for 5 – 10 min at room temperature. To remove unbound CPD-eF670 the cells were washed with medium or $FCS_{hi}$ and subsequently adjusted to the required concentration.

## 2.2.2.9     Activation and expansion of peripheral B cells

For the activation and expansion of peripheral B cells we used different stimulations. Monoclonal antibodies against CD40, cMyc or irrelevant targets or antigens coated on the cell culture plate were used for the expansion. In a 96-well format approx. 1 – 10 µg protein in 100 µL PBS per well were incubated for 1 – 2 hours at 37 °C. After coating each well was washed with 200 µL medium to remove unbound proteins. The B cells were added in a cell concentration from $1 – 20 \times 10^6$ cells/well and incubated for 5 – 7 days in complete culture medium supplemented with 5 % human AB serum, IL4, and IL21.

### 2.2.3  Protein biochemical methods

### 2.2.3.1    Flow cytometry

Flow cytometry is a useful technique to analyze large numbers of cells in cell size, cell granulation, and protein expression. For that, cells pass a laser beam in a laminar sample flow as single cell suspension. The light is either scattered or absorbed and emitted as fluorescence. The scattered light is collected by detectors in forward scatter channel which analyze cell sizes, or the side scatter channel allowing the analysis of granularity.

Protein expression is analyzed by fluorescence labeled antibodies binding to their respective antigen. Different fluorescent dyes can emit light which correlates to the amount of antibody which binds to their specific target. To discern between viable and dead cells the DNA intercalating dyes PI or 7-AAD were used.

### 2.2.3.2    Staining of cell surface markers

Cells were harvested, adjusted to the desired cell concentration and washed in 2 mM EDTA/PBS, centrifuged (8 min; 300 x g; 4 °C) and resuspended in 100 µL FACS buffer.

The FACS buffer was mixed before with 1 – 3 µL of fluorochrome labeled anti-human antibodies and the complete staining batch was incubated for 20 min at 4 °C in the dark. The cells were washed twice with FACS buffer and resuspended in 150 – 300 µL FACS buffer including 7-AAD. Flow cytometry studies were performed on a FACSCalibur or FACS Canto II machine and analyzed using Flow-Jo software.

### 2.2.3.3    Preparation of reduced or non-reduced cell lysates

Cell lysates were used for SDS-PAGE and Westernblot experiments. For that, cell pellets were resuspended with the required amount of 4x RotiLoad buffer (1 for reduced, 2 for non-reduced) and shaked vigorously. The solution was heated for 10 min at 60 °C or 5 min at 95 °C for non-reduced conditions or reduced conditions, respectively. Subsequently, the protein solution was cooled down and centrifuged for 20 sec at 12.000 rpm to regain condensed water.

## 2.2.3.4    SDS-PAGE

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, was used for separation of proteins according to their electrophoretic mobility. By the addition of the anionic tenside SDS the inherent charge of the proteins due to charged aminoacids gets covered and the protein acquires a distinct negative charge distribution, which allows a separation by size. Depending on the molecular weight of the protein of interest, different acrylamide percentages of the resolving gel were selected. After preparing the gel and fixing into the electrophoresis chamber the diluted samples were loaded to the gel slots. The proteins were separated by approximately 200 V, 25 mA for 70 min. The run was finished when the bromphenol blue dye of the sample buffer reached lower part of the gel. Subsequently the gel was used for Coomassie staining or western blot analysis.

## 2.2.3.5    Westernblot

Whole cell lysates were prepared as described above, subjected to SDS-PAGE, and blotted onto polyvinylidene difluoride (PVDF) membrane. Subsequently the membrane was blocked by the incubation with 10 % milk powder in PBST buffer over night at 4 °C. Immunostaining was performed with antibodies reactive to cMyc, kappa, or CD40, followed by detection of the primary antibodies with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies. Unbound antibodies were removed by washing steps between the different incubation steps.

## 2.2.3.6    Coomassie staining

Coomassie Briliant blue was used to visualize proteins on gels. Protein samples were separated electrophoretically by SDS-PAGE and transferred onto a PVDF membrane. The membrane was incubated in staining solution under constant shaking for 10 minutes. The membrane was washed four times with $H_2O$ to remove unbound staining solution.

## 2.2.3.7    Deglycosylation of proteins

For deglycosylation the cell lysates were treated with reducing buffer and incubated for 5 min at 95 °C. Subsequently the samples were treated with G7 buffer (50 mM Na-Phosphat, 1% Nonidet P-40) and PNGase F enzyme according to manufacturer's

instructions. The reaction batch was incubated for 2 hours at 37 °C and analyzed by SDS-PAGE and western blot analysis.

### 2.2.3.8 Fluorescence microscopy

For colocalization studies, CHO-K1 or CHO-K1-pC15 cells were transiently transfected with 10 µg/mL IVT-RNA encoding for ABCos1 or ABCos2, cultured over night on cover slides, washed, and fixed in 4% PFA/PBS at 4 °C. Subsequently, the cells were washed and incubated with antibody specific for kappa. Unbound antibody was removed by washing steps before incubation with a secondary antibody coupled to Cy3-fluorescent dyes. Finally, the cells were washed, stained with anti-cMyc coupled to FITC fluorescent dyes. Slides were washed, mounted in fluorescence mounting medium including Hoechst 33342 to stain nucleus

For analysis, a fluorescence microscope (Leica DM RXA) equipped with a 40x/0.4 NA objective lens and the QFISH software was used.

### 2.2.4 Statistics

Statistical analysis was performed in GraphPad Prism software employing paired non-parametric t test. Values of $p < 0.05$ were considered statistically significant.

# 3    Results

## 3.1    Establishment of a protocol to cultivate and expand antigen-specific B cells

### 3.1.1    Isolation of B cell subpopulations from human peripheral blood

To isolate or analyze antigen-specific B cells it is very important to work with defined and pure cell populations to avoid undesired activation or interactions with contaminating cell population. Undesired activation would increase the background proliferation of non-antigen-specific B cells which diminishes the specificity and sensitivity of the antigen-specific isolation procedure. Therefore we optimized the isolation, cultivation and manipulation steps which allow the long term cultivation of highly-pure B cell populations without side effects.
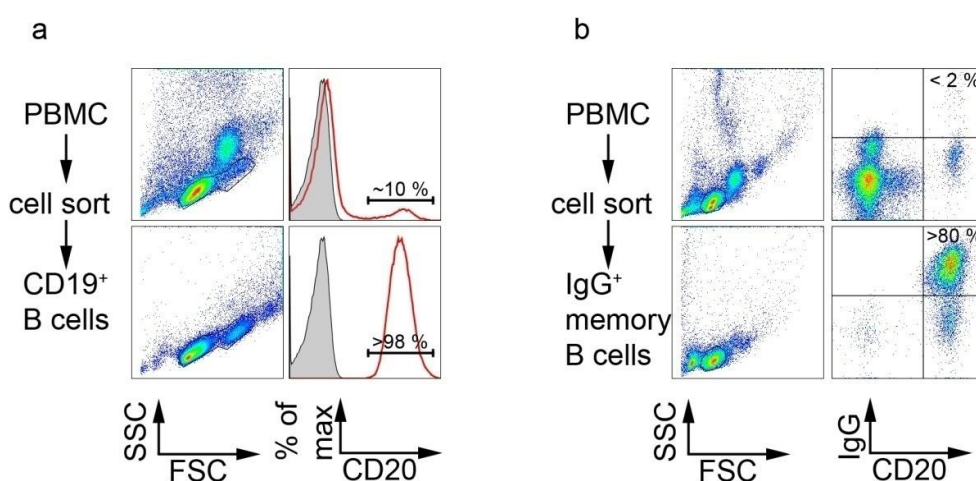


**Figure 10: *Magnetic cell separation of CD19$^+$ B cells and IgG$^+$ memory B cells from PBMC.***
**a) CD19$^+$ B cells represent approximately 10 - 15 % of the PBMC. B cell separation by anti-CD19 beads increases the purity to more than 98 %. b) IgG$^+$ memory B cells represent 10 % of the peripheral CD19$^+$ B cells. IgG$^+$ memory B cells separation by negative depletion of non B cells and subsequent isolation with anti-IgG beads increase the purity to more than 80 %.**

Peripheral blood mononuclear cells (PBMC) were obtained from buffy coats of healthy donors by Ficoll Hypaque separation and B cells were isolated by magnetic cell sorting using anti-CD19-coupled magnetic beads (Figure 10 a). For the isolation of IgG$^+$ memory B cells complete B cells were first separated from PBMC by negative selection

followed by anti-IgG-coupled magnetic bead separation (Figure 10 b). B cells represent approximately 10 % – 15 % of all peripheral mononuclear cells. After MACS enrichment the total B cell population was more than 98 % positive for the pan B cell marker CD20 and the IgG$^+$ memory B cell population was enriched to more than 80 % as determined by the surface expression of the pan B cell marker CD20 and IgG.

### 3.1.2 Optimization of culture conditions for cultivation of human B cells

The cultivation of peripheral mature B cells over a long period of time is difficult and different media compositions are described in the literature (Bernasconi et al., 2003; Dubois et al., 1998). To obtain a media composition which allows the long term cultivation together with high survival rate of the isolated B cells different combinations of described ingredients were tested. As additions Penicillin and Streptomycin were used for the protection against bacterial contamination. The human AB serum provides a source for different trace elements, lipids and variety of growth factors. Insulin, transferrin and selenit (ITS) are used as basal growth factor supplements and additionally to reduce serum requirements in the media composition. The essential amino acid L-glutamin is important for cell growth and different cell functions and was supplemented to the medium because of its chemical instability. Non-essential amino acids (NE-AA) were supplemented to media to support cell growth and prolong the viability of the B cells. As an additional source of energy Na-Pyruvat was added to the medium. As reducing agent β-Mercapthoethanol was tested, which supports the transport of nutrients into the cell and the reduction of toxic metabolites secreted by the B cells in the medium thereby improving the long term medium conditions for B cells? The different compositions are depicted in table 1.

**Table 1:** *Components of the different media.*
(Pen/Strep = Penicillin/Streptomycin, ITS = Insulin Transferrin Selenit, NE-AA = non essential amino acids)

| Medium | Constitutents part |
|---|---|
| 1 | IMDM |
| 2 | IMDM, 5% human AB serum, 1% Pen/Strep, 2mM Glutamin |
| 3 | IMDM, 5% human AB serum, 1% Pen/Strep |
| 4 | IMDM, 5% human AB serum, 1% Pen/Strep, 50µM ß-Mercapthoethanol |
| 5 | IMDM, 5% human AB serum, 1% Pen/Strep, ITS (10mL/L) |
| 6 | IMDM, 5% human AB serum, 1% Pen/Strep, 1% NE-AA, 1% Na-Pyruvat, 50 mM ß-Mercapthoethanol, ITS (10mL/L), 2mM Glumatin |
| 7 | RPMI |
| 8 | RPMI, 5% human AB serum, 1% Pen/Strep, 2mM Glutamin |
| 9 | RPMI, 5% human AB serum, 1% Pen/Strep |
| 10 | RPMI, 5% human AB serum, 1% Pen/Strep, 50µM ß-Mercapthoethanol |
| 11 | RPMI, 5% human AB serum, 1% Pen/Strep, ITS (10mL/L) |
| 12 | RPMI, 5% human AB serum, 1% Pen/Strep, 1% NE-AA, 1% Na-Pyruvat, 50 mM ß-Mercapthoethanol, ITS (10mL/L), 2mM Glumatin |

The different media compositions had no further supplements, like stimulating additives or cytokines. The cells were cultivated over 10 days and analyzed on day 0, 3, 5, 7 and 10 for survival. Cell death was analyzed by staining with 7-Aminoactinomycin (7-AAD) which intercalates in double-stranded DNA of dead cells. As shown in figure 11a three media compositions lead to a definite high survival rate compared to all other compositions. As shown in figure 11a medium composition of IMDM, 5 % human AB serum, 1 % Pen/Strep, 1% NE-AA, 1% Na-Pyruvat, 50 mM ß-Mercapthoethanol, ITS (10 mL/L) and 2 mM Glumatin resulted in a survival rate of 80 % at day 10 (media composition 6). A slightly lower survival rate was achieved by media 3 which contains 5 % human AB serum and 1 % Pen/Strep with 60 % after 10 days. Nearly the same survival rate was achieved with the medium 12 consiting of RPMI, 5 % human AB serum, 1 % Pen/Strep, 1 % NE-AA, 1 % Na-Pyruvat 50 mM ß-Mercapthoethanol, ITS (10 mL/L) and 2 mM Glumatin. For all following experiments the media composition 6 was used.
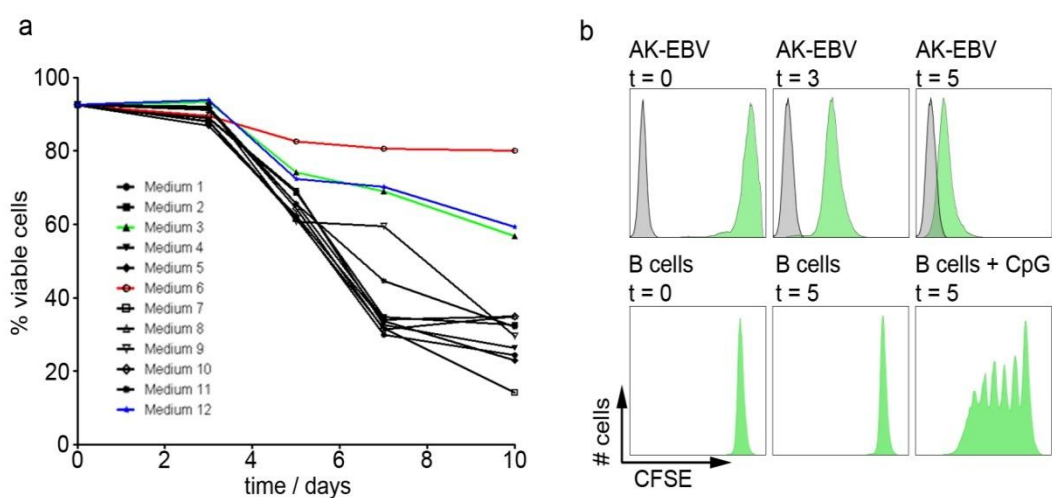


**Figure 11:** *Optimization of the media conditions and detection of proliferation.*
**a) CD19^{+} B cells were isolated from buffy coats as described in chapter 3.1 and cultivated in several media compositions and analyzed for vitality on day 0, 3, 5, 7, and 10. b) Detection of proliferation through measuring of the reduction of intracellular CFSE intensity. Top panel: AK-EBV B cell line cells were labeled with 5 µM CFSE and analyzed for CFSE reduction on day 0, 3, and 5. Bottom panel: Peripheral B cells were labeled with 5 µM CFSE. The B cells were analyzed without stimulation or after CpG stimulation and compared to the B cells on day 0.**

Antigen-specific activation of B cells induces proliferation which can be detected by the dilution of cell labeling dyes, like Carboxyfluorescein succinimidyl ester (CFSE). For the establishment of the method we labeled a continuously proliferating B cell line (AK-EBV B cell line) with 5 µM CFSE and analyzed the reduction of CFSE intensity at several time points. Figure 11b illustrates that the CFSE intensity is continuously decreasing

over time corresponding with the continuously proliferating phenotype of this cell line. To verify that non activated B cells maintain their CFSE intensity peripheral B cells were labeled with 10 µM CFSE and left unstimulated or were stimulated with CpG-ODN$_{2006}$. The proliferations of B cells were analyzed on day 0 and on day 5. The unstimulated B cells show unchanged CFSE intensity on day 0 and 5. In contrast, B cells stimulated with CpG-ODN$_{2006}$ display distinct cell populations with reduced CFSE intensity.

### 3.1.3  Manipulation of B cells by transient introduction of IVT-RNA

Different strategies exist for the introduction of nucleic acids into cells, like chemical-induced transfection, non-chemical methods, particle-based methods, viral methods and other hybrid methods. The electroporation technique is a highly efficient method to introduce RNA, DNA or proteins into cells by inducing transient gaps in the cell membrane. By optimization of several parameters like pulse time, capacitance and voltage the instability of the cell membrane can be minimized, cell viability can be enhanced and the reproducibility and transfection efficiency can be increased. The introduction of genetic information can be achieved by the electroporation with DNA or RNA. The transfection with *in vitro* transcribed RNA (IVT-RNA) allows the transient introduction of foreign nucleic acids into cells. In contrast to DNA RNA only needs to be transfected into the cytosol of cells facilitating the expression of the encoded protein. For that reason RNA electroporation parameters had milder conditions compared to DNA resulting lin ower mortality.
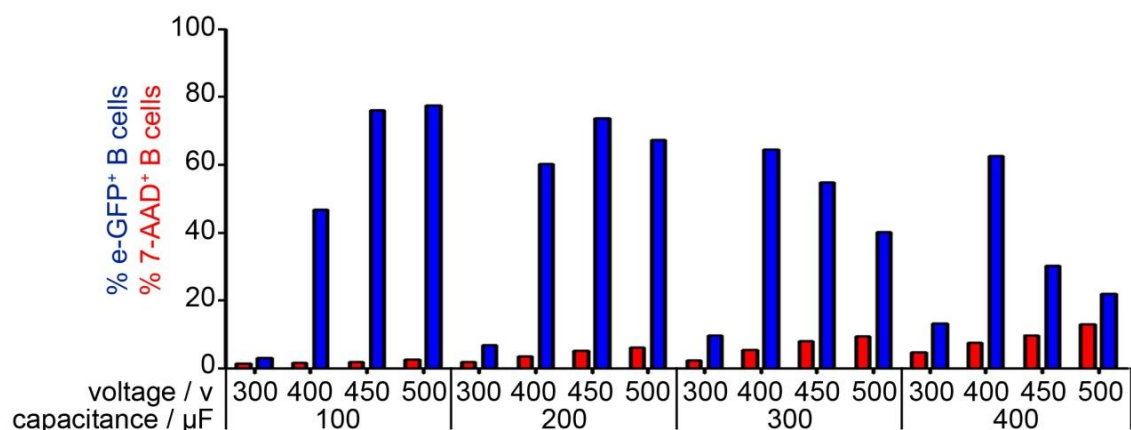


**Figure 12:** *Determination of optimal electroporation parameters.*
**Peripheral B cells were electroporated with 5 µg e-GFP IVT-RNA with different parameters. 24 hours post electroporation the B cells were analyzed by flow cytometry. Electroporation efficiency was represented by eGFP expression (blue bar) and the mortality was analyzed by 7-AAD staining (red bars).**

To find the electroporation parameters with the highest transfection rate in combination with lowest mortality rate the cells were transfected with IVT-RNA encoding e-GFP with different voltages and capacitance parameters. The parameter ranged from 100 µF to 400 µF and 300 V to 500 V. The two parameter voltage and pulse time or capacitance characterize the electroporation process. The parameter must be high enough to induce membrane pores but low enough that the cell can regenerate. After 24 hours the cells were analyzed for e-GFP expression and incorporation of 7-AAD. The electroporation parameters 100 µF and 500 V showed the best e-GFP expression of nearly 80 % together with a survival rate of more than 95 %. All other parameters had a lower transfection rate and a higher mortality (Figure 12).

### 3.1.4 Optimization of cytokine additions for B cell activation

Under physiological conditions the stimulation of B cells mainly takes place in the peripheral lymph nodes and spleen. B cells which are activated by BCR engagement through antigen obtain their secondary CD40 signal through the CD40L expressed on T helper cells in germinal centers in B cell follicles. Besides, T helper cells and other cells express a number of different cytokines in the lymph nodes which are important for activation, class switch and survival of stimulated B cells (Cyster, 2010). Therefore, different cytokines and cytokine combinations were tested and analyzed for the induction of proliferation.

In this experiment the medium was supplemented with several B cell affecting (IL2, IL4, IL6, IL7, IL10, IL12, IL13, IL15, IL21) cytokines and with plate-bounded anti-CD40 antibody or no antibody as negative control. In all combination approximately 30 % – 40 % of the peripheral B cells proliferate, except for IL4 which support proliferation resulting in 90% proliferation of the peripheral B cells (Figure 13 a). For further optimization IL4, IL15 and IL21 were tested individually or in combinations. Addition of IL15 or IL21 to IL4 did not enhance the proliferation of CD19$^+$ B cells (Figure 13 b). But the addition of IL15 and IL21 increased the viability of the main population and lead to a stronger proliferation of CD27$^+$ B cells, which mainly includes memory B cells (Figure 13 c).
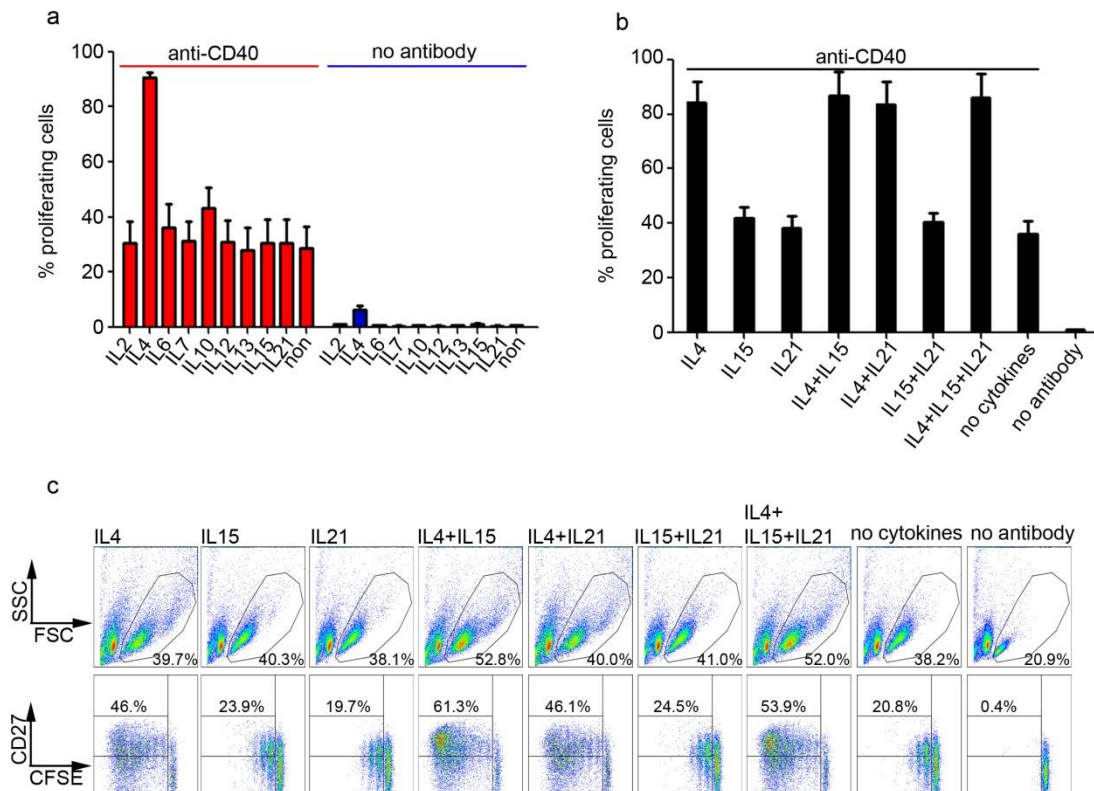
**Figure 13: *Optimization of cytokine conditions for B cell activation.***
**a) Influence of several cytokines on CD40-activated or non-activated CD19⁺ B cells. CD19⁺ B cells**
**were isolated from buffy coats, labeled with CFSE and cultivated for 5 days under different**
**cytokines and stimulating anti-CD40 antibody or not. b) Influence of cytokine combinations on**
**CD40-activated B cells. CD19⁺ B cells were isolated from buffy coats, labeled with CFSE and**
**cultivated for 5 days under different cytokines and combinations. The B cells were activated**
**through plate-bound anti-CD40 antibody. c) Analysis of CD27 (memory B cells marker) expression**
**of CD40 activated B cells. CD19⁺ B cells were isolated stimulated like in b and analyzed for CD27**
**expression.**

## 3.1.5 Different strategies for *in vitro* antigen presentation to B cells

CD40 signaling is induced in B cells by the trimerization of the cell surface CD40
receptor by binding of the CD40 ligand provided by T helper cells (van Kooten and
Banchereau, 2000). To mimic the physiological situation *in vitro* different settings with
anti-CD40 antibodies were tested: soluble antibody, soluble antibody crosslinked with
secondary anti-IgG, dish coated antibody and antibody bound to a constitutively Fcγ-
receptor expressing CHO-K1 cell line. The anti-CD40 antibody simulates the future
antigen of interest which could be also presented in similar settings to the B cells as the
anti-CD40 antibody.

No proliferation was observed in B cells which were either left unstimulated or treated
with anti-CD3 antibody (Figure 14 a). The different applications of anti-CD40 led to
different strength of proliferation. While the stimulation with the soluble anti-CD40
antibody only led to a moderate proliferation of ~ 35 % of the cells, the additional

crosslinking by anti-IgG antibody increased the proliferation to ~ 45 % - 50 %. Higher proliferation rates of ~ 60 % or ~ 90 % of the cells were reached by plate-bound anti-CD40 antibody or presentation of anti-CD40 antibody by CHO-CD32 respectively (Figure 14). Because cell free culture systems increase the reproducibility and are easier to handle, the plate bound antibody system was used for further experiments.
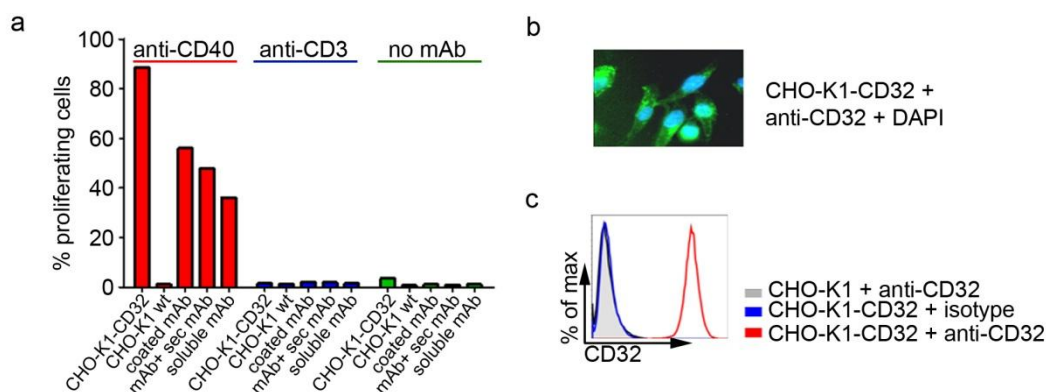


**Figure 14:** *Testing of different strategies for antigen presentation to B cells.*
**a) CD19$^+$ B cells were isolated from buffy coats, labeled with CFSE and cultivated for 5 days with anti-CD40 antibody in different settings: antibody bound to CHO-K1-CD32 cells, dish coated antibody, soluble antibody crosslinked with secondary anti-IgG and soluble antibody. b) Immunofluorescence staining of CHO-K1-CD32 cells. CHO-K1-CD32 cells were cultured on cover slides and stained with DAPI for nuclei staining (blue) and anti-CD32 for CD32 staining (green). c) FACS analysis of CHO-K1-CD32 cells. CHO-K1-CD32 or wildtype cells were stained with anti-CD32 antibody or isotype control.**

### 3.1.6  Summary of chapter 3.1

In this chapter the optimal cultivation, growth and stimulation parameters were analyzed. The selection of the different parameters was based on the simulation of the physiological situations and the feasibility. Taken together, B cells were cultured in IMDM medium supplemented with 5 % human AB serum, 1 % Pen/Strep, 1% NE-AA, 1% Na-Pyruvat, 50 mM ß-Mercapthoethanol, ITS (10 mL/L) and 2 mM Glumatin. For activation experiments the B cell medium was supplemented with IL4 and IL21. IL4, *in vivo* mainly secreted from Th2 cells, has a dramatic effect on the B cells and increase the proliferation from 30 – 40 % up to 90 %. IL21 had no direct effect on the proliferation but augments to CD40-driven B cell survival. A variety of different settings for the presentation of the antibody or antigen was tested. The coupling of the anti-CD40 antibody to the constitutively CD32 expressing CHO-K1 cell line and plate-coated anti-CD40 antibody showed the highest activation pattern. The reduction of complexity and easy handling are important factors and led to the decision to work in a cell free antibody plate-coated setting.

## 3.2 Proteinbiochemical analysis of the ABCos constructs and determination of their ability to heterodimerize with immunoglobulins

To mimic the physiological situation of B cell activation at least two different signals are required, the BCR signal and the CD40 signal. Therefore, the ABCos constructs (**ABCos** = **A**ntigen-specific B cell costimulator) were designed to activate B cells in an antigen-specific way without the need of T cell help to answer immunological questions and to identify antigen-specific B cells in the complete B cell repertoire. Besides the clonal B cell expansion, the constructs were designed to differentiate B cells into antibody secreting plasma cells. For this issue, it is important that the constructs are expressed and show no toxicity in B cells. Moreover, the ability to heterodimerize with endogenous immunoglobulins is fundamental to induce an antigen-specific CD40 signal.

### 3.2.1 Design of ABCos constructs

ABCos constructs were designed for antigen-specific expansion of peripheral B cells initiated by an antigen-specific CD40 signal. To achieve this, the constructs have a domain consisting of the constant domain 2 and 3 from the human IgG1 facilitating the association with the endogenous IgG molecule. ABCos1 and ABCos2 differ in the transmembrane domain: ABCos1 contains the transmembrane domain derived from the CD40 receptor while ABCos2 contains the IgG1 transmembrane domain (Figure 12). The intracellular domain is derived from the human CD40 receptor. For detection and crosslinking the constructs contain a N-terminal marker domain, consisting of two cMyc tags separated by an HA tag. Upstream of the tag the IL2 secretion signal is located which allows the membrane localization of both ABCos constructs (Figure 15). These constructs were cloned into a pst1-A120 vector for the production of *in vitro* transcribed (IVT) RNA. This optimized vector displays several features, like two 3'UTR (untranslated region) derived from human β-globin and a poly A-tail of 120 nucleotides to increase RNA stability and to enhance the translation efficiency (Figure 16).
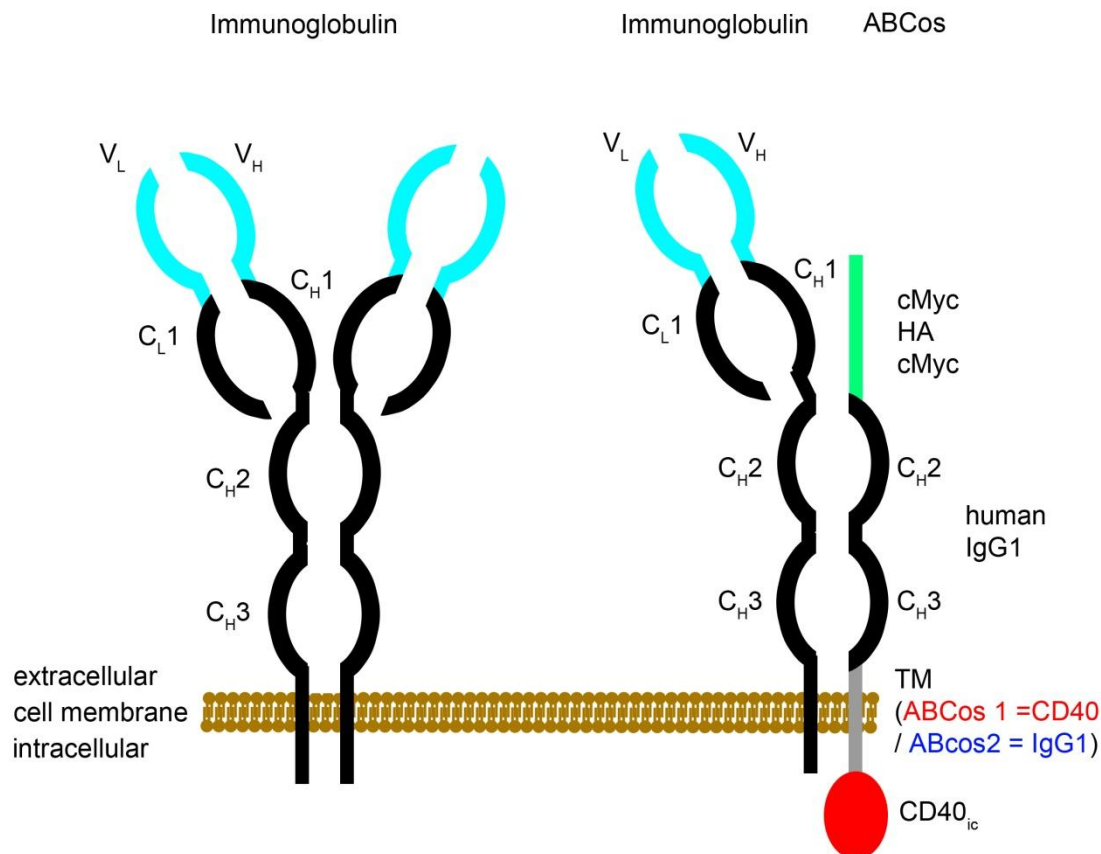
**Figure 15:** *Heterodimerization of the ABCos construct with immunoglobulin.*
**Schematic draft of the BCR and heterodimeric IgG:ABcos complex. $C_H$ = constant heavy chain domain, $V_H$ = variable heavy chain domain, $C_L$ = constant light chain domain, $V_L$ variable light chain domain, TM = Transmembrane domain, ic = intracellular, HA = hemagglutinin**

For the validation of the constructs a digestion with restriction enzymes was performed. Sac1 restriction enzyme cleaves the DNA in the human IgG1 domain and in the 3´untranslated region. The resulting product has a size of 934 bp for ABCos1 and 961 bp for ABCos2. Both specific bands are detectable in the gel as shown in figure 16. The BamH1 and Xho1 digestion produces an 809 bp product by cleaving in the IgG1 association domain in both constructs and the marker domain in ABCos1 and sec-IL2 signal in ABCos2, respectively. All specific signals for the correct ABCos DNA could be detected. No further signals to the specific fragments could be observed (Figure 16 c).
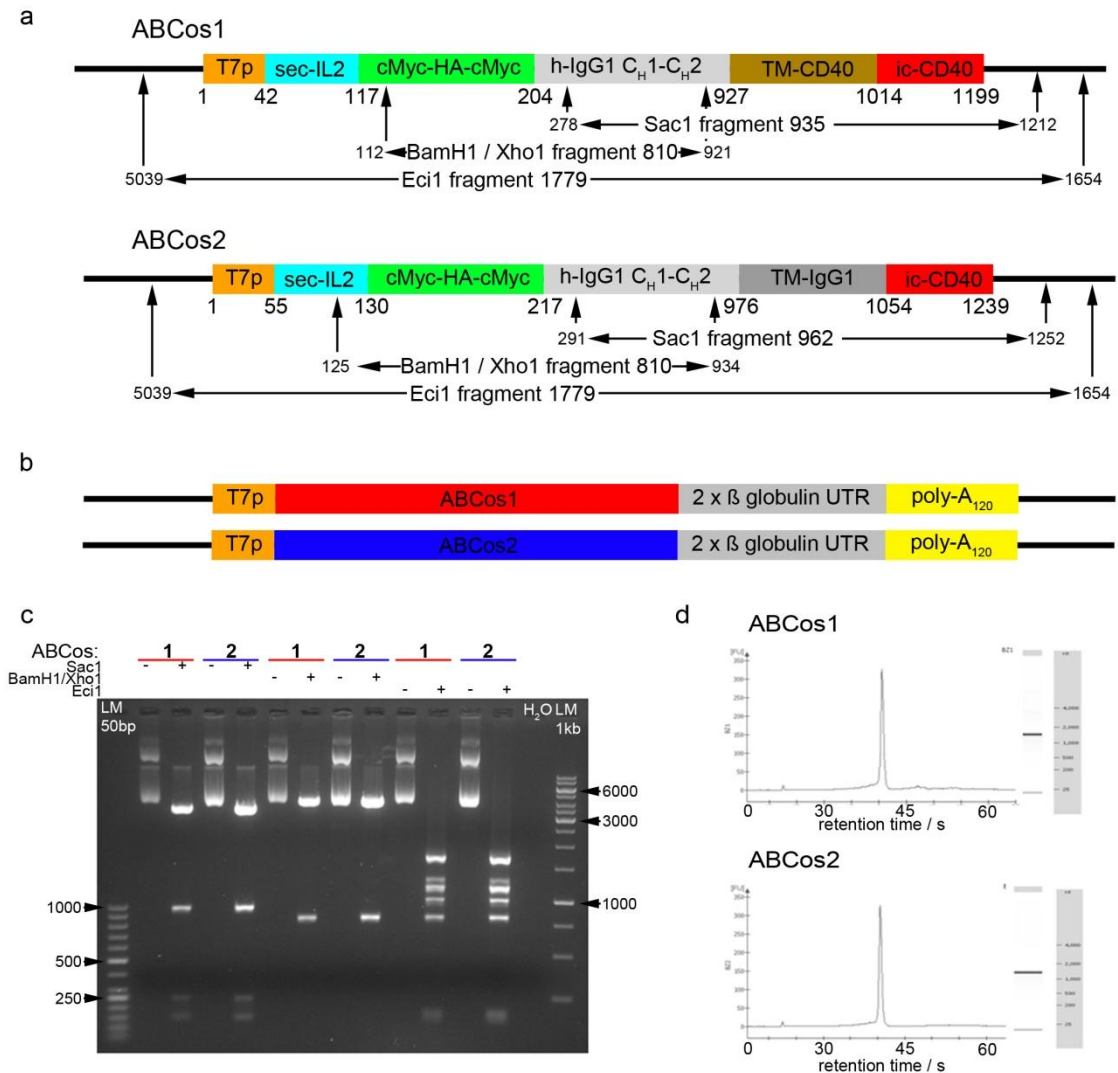
**Figure 16:** *Quality control of ABCos1 and ABCos2 DNA and IVT-RNA.*
a) Schematic draft of ABCos1 and ABCos2 DNA (numbers indicate base pair position or restriction product in base pairs). b) Schematic draft of pst1-A120 vector including ABCos1 or ABCos2 (T7p = T7 promoter, sec-Il2 = secretion signal derived from IL2, TM = transmembrane domain, ic-CD40 = intracellular part of CD40). c) 1.5 % Agarose gel for separation of ABCos1 and ABCos2 DNA and restriction products. Restriction enzymes were used as indicated (LM = DNA length marker, kb = kilo base pairs, bp = base pairs). d) Quality control of ABCos1 and ABCos2 IVT-RNA analyzed with Bioanalyzer®.

For the production of IVT-RNA the circular plasmid has to be linearized by digestion with the restriction enzyme Eci1. Eci1 cuts the plasmid at base pair 5039 and 1654. The resulting fragments include one signal that comprise the complete ABCos construct under a T7 promoter for *in vitro* transcription, the 3`-terminal UTR and the poly A tail (Figure 16 c). To analyze the *in vitro* transcribed RNA, the RNA was electrophoreticaly separated by Bioanalyzer® 2100. The peak at ~ 40 sec in the chromatogram represents the IVT-RNA. Contamination or degradations would be detectable in further signals or in an indistinct peak. The small signal between 20 and 25 seconds represents an internal control signal (Figure 16 d). All further experiments

presented were performed with IVT-RNA which quality was controlled by spectrophotometry, agarose/formaldehyde gel electrophoresis, Bioanalyzer[®] and sequence analysis.

## 3.2.2 Analysis of ABCos expression on the cell surface of B cells
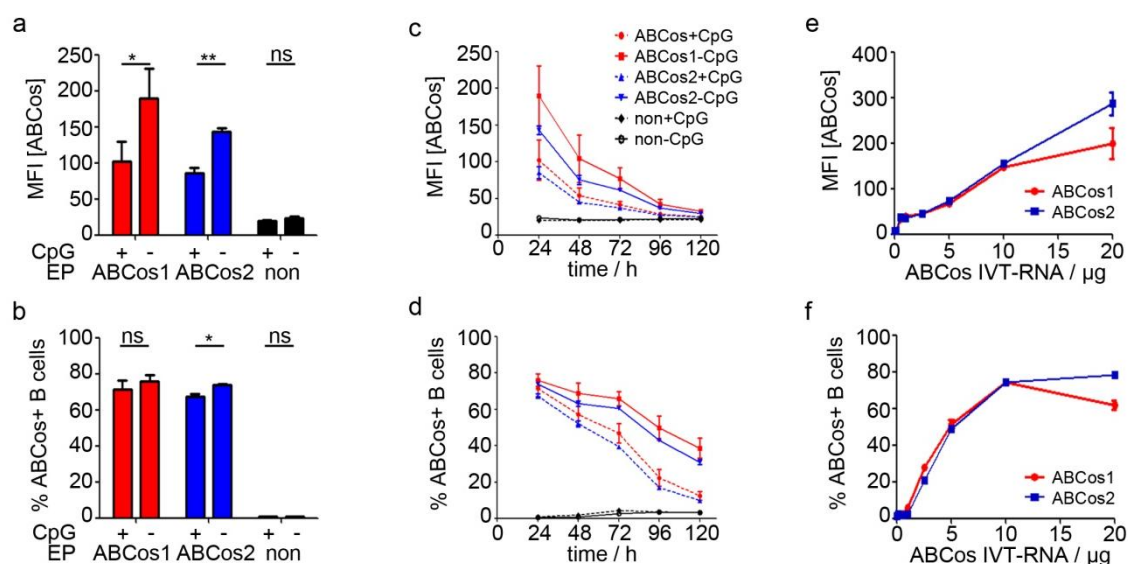


**Figure 17:** *ABCos expression in CD19^{+} B cells.*
**CD19^{+} sorted B cells were transfected with 10 µg IVT-RNA encoding for ABCos1 and ABCos2 in the presence or absence of CpG-ODN$_{2006}$ and analyzed 24 h post transfection (a, b) or over a periode of 5 days (c, d) for ABCos expression by flow cytometry, or B cells were transfected with increasing amounts of IVT-RNA encoding ABCos constructs and ABCos expression was analyzed 24 h post-transfection (e,f). All experiments were conducted with B cells from three different donors (Shown are mean standard deviation).**

To validate the translation and expression of the ABCos constructs on the cell surface of human B cells flow cytometric analysis were performed (Figure 17 a-f). CD19^{+} sorted B cells were transfected with 10 µg IVT-RNA encoding ABCos1 or ABCos2 and stimulated with CpG-ODN$_{2006}$ or not to analyze the expression pattern under activated and non activated conditions. Staining with an anti-cMyc antibody 24 h after electroporation of B cells with ABCos1 or ABCos2 allows the identification of ABCos expressing cells (Figure 17 a, b) and indicates a transfection efficiency of 70 – 80 % which is independent of the concomitant stimulation with CpG-ODN$_{2006}$. In contrast to this the protein amount per cell as represented by the fluorescence intensity is reduced in B cells that were activated with CpG-ODN$_{2006}$. Over the time course of 5 days post transfection of B cells with ABCos constructs the frequency of ABCos expressing cells decreases from 70 – 80 % to 20 – 40 % (Figure 17 c). ABCos protein can be well detected for up to 72 h post transfection and continuously declines until it is only weakly detectable after 120 h (Figure 17 d). The ABCos amount on the B cell surface

after transfection can be easily regulated by titrating the amount of ABCos IVT-RNA. While the frequency of ABCos positive B cells reaches a plateau with 10 µg of ABCos IVT-RNA (Figure 17 f) the ABCos protein amount is proportional to the amount of introduced IVT-RNA and can be increased with higher amounts of IVT-RNA (Figure 17 e).

### 3.2.3  Association of ABCos constructs with endogenous IgG molecules

Induction of an artificial antigen-specific CD40 signal requires the association of the ABCos constructs with endogenously expressed immunoglobulin molecules. To analyze this the constitutively antibody secreting producer cell line CHO-pC15 was transfected with ABCos IVT-RNA and analyzed 24 h post transfection for ABCos and kappa expression.
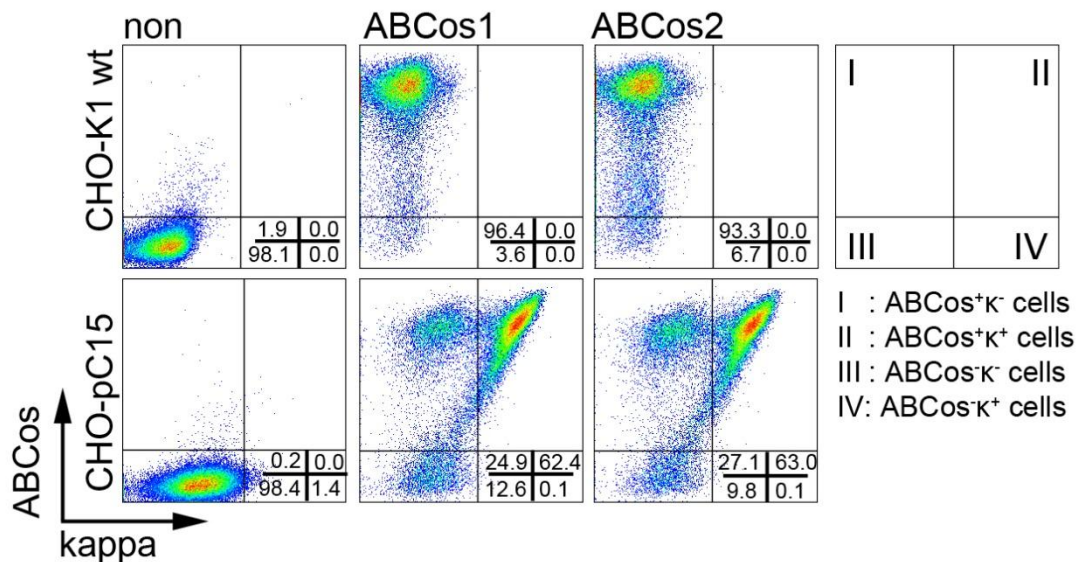


**Figure 18:** *Heterodimerization of ABCos and immunoglobulin in CHO-pC15 model cell line.*
**CHO-K1 and CHO-pC15 cells were transfected with 20 µg ABCos IVT-RNA. The cells were stained 24 hours post transfection for surface expression of ABCos and kappa. Shown are representative results of 3 experiments.**

ABCos transfected CHO-K1 wild type cells show an intense staining with anti-cMyc antibodies but no anti-kappa staining, as expected (Figure 18). The constitutively antibody secreting CHO-pC15 cell line shows no significant anti-kappa surface staining without transfection with the ABCos construct, indicating that all antibodies are secreted. However, the transfection with ABCos IVT-RNA leads to a significant anti-kappa staining in more than 60 % of the CHO-pC15 cells. There is still a kappa negative ABCos positive population of approximately 25 % and a completely negative population of approximately 10 % (Figure 18). This data indicates that the ABCos

constructs are able to associate with immunoglobulins on the surface of antibody secreting cells.
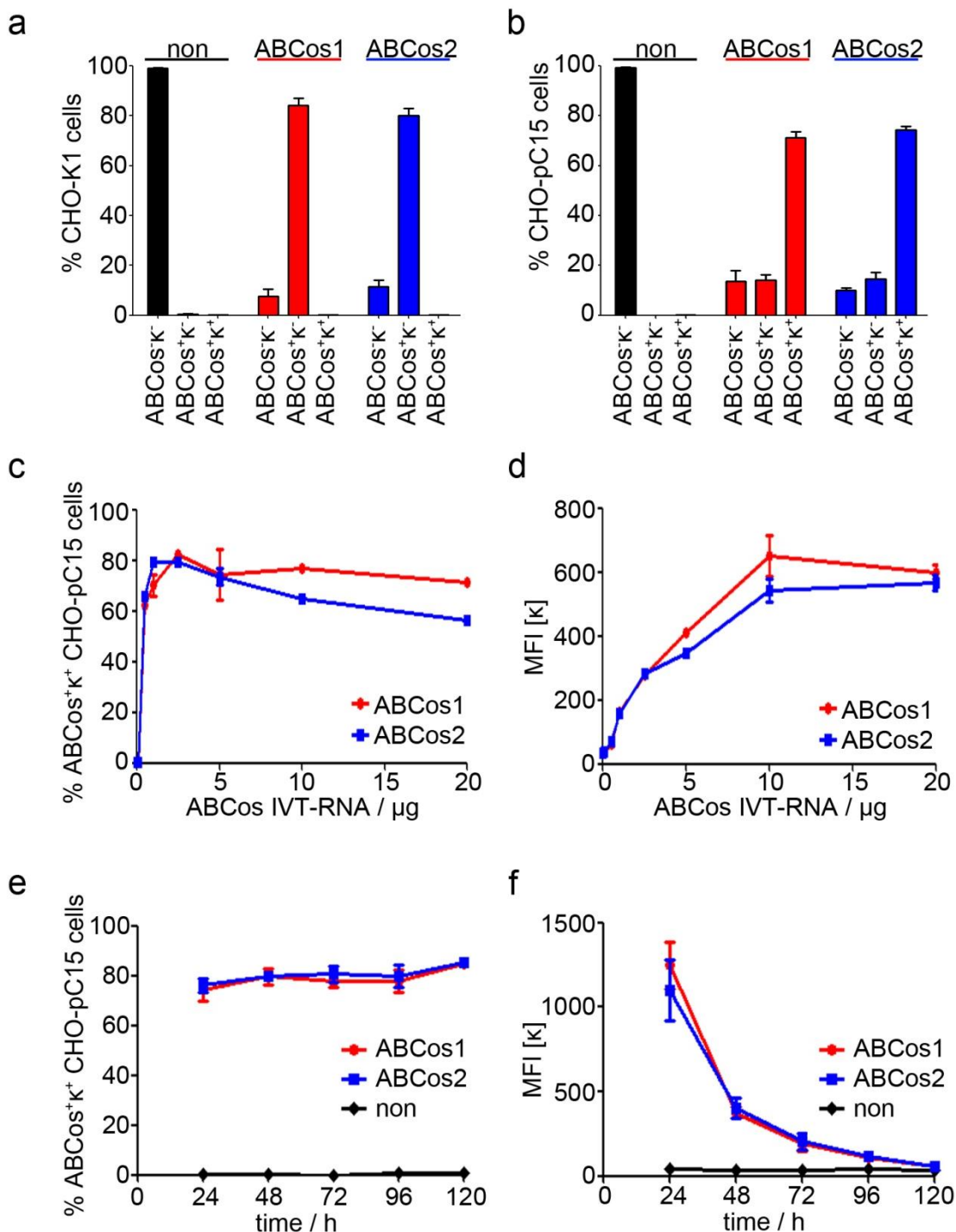


**Figure 19:** *Analysis of the heterodimerization of ABCos and immunoglobulin in CHO-pC15 model system.*
**CHO-K1 and CHO-pC15 cells were transfected with 20 µg ABCos IVT-RNA and analyzed for surface expression of ABCos and kappa light chain by flow cytometry 24 h post transfection (a, b). CHO-pC15 cells were transfected with increasing amounts of ABCos IVT-RNA and the expression of ABCos and kappa light chain was analyzed 24 h post transfection (c, d). CHO-pC15 cells were transfected with 10µg of ABCos IVT-RNA and the expression of ABCos and kappa light chain was analyzed over a periode of 5 days post transfection (e, f). All experiments were performed in three different independent experiments (Shown are mean standard deviation).**

To analyze the characteristics of the heterodimer, the expression and heterodimerization of ABCos constructs and immunoglobulin molecules were analyzed in more detail. Figure 19 shows the results of ABCos transfected CHO-K1 wild type and pC15 cells analyzed for the distribution of ABCos expressed as monomer or homodimer (Figure 19 a) or as a heterodimeric ABCos:IgG complex (Figure 19 b). ABCos can be expressed as homodimer or heterodimer in 80 % of the CHO-K1 wild type cells. In approximately 70 % of the ABCos transfected CHO-pC15 cells the heterodimeric ABCos:IgG complex was present on the cell surface. 10 % of the transfected CHO-pC15 cells were only ABCos positive and further 10 % showed no expression of ABCos or kappa (Figure 19 a, b).

Titration experiments determined that the amount of surface retention of secreted immunoglobulin molecules depend on the amount of IVT-RNA (Figure 19 d). The titratable amount of ABCos:IgG heterodimer is connected with an almost constant ratio of transfected CHO-pC15 cells of 80 % (Figure 19 c). The kinetic experiment shows that the amount of ABCos:IgG complex is diminished within the first 72 hours. The amount of ABCos:IgG rapidly decreases until 96 h and is hardly detectable after 120 h. Nevertheless, the homogenous frequency of ABCos positive B cells is constant by 80 % over the time (Figure 19 e, f).

## 3.2.4  Co-localization of ABCos and IgG on the surface of CHO-pC15 cells

For the visualization of the constructs and ABCos:IgG heterodimer complex on the surface of the CHO-pC15 cells immune fluorescence microscopy experiments were conducted. The CHO-K1 and CHO-pC15 cells were transfected with ABCos IVT-RNA and cultivated for 24 hours on cover slides. Subsequently the slides were stained with anti-cMyc (green staining), anti-kappa (red staining) antibodies and DAPI (blue staining).

CHO-K1 cells transfected with ABCos RNA showed a distinct green surface staining. The expression of ABCos is distributed homogenously on the surface of transfected CHO-K1 cells, indicating no accumulation or intracellular clustering of ABCos construct. The constitutively antibody secreting CHO-pC15 cell line showed also a homogenous surface staining of the ABCos constructs. Notably, there was no kappa staining detectable in ABCos negative CHO-pC15 cells, while ABCos positive CHO-pC15 cells are also positive for kappa staining (Figure 20). These data strongly suggested that ABCos expression resulted in the surface retention of antibody molecules, while the endogenous antibody was directly secreted to the surrounding medium in the absence of ABCos expression.
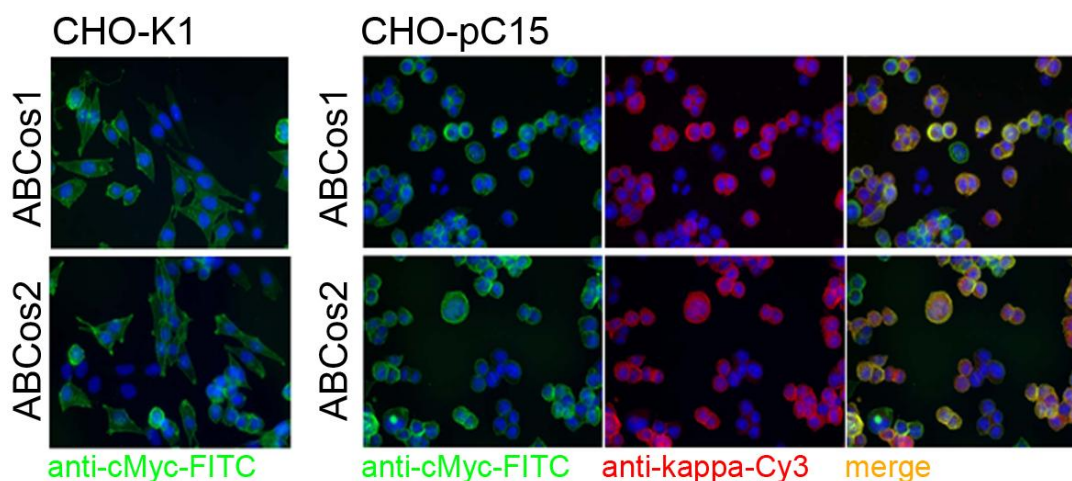
**Figure 20:** *Co-localization of ABCos and IgG on the surface of CHO-pC15 cells.*
**CHO-K1 and CHO-pC15 cells were transfected with 10 µg ABCos IVT-RNA and cultivated for 24 hours on cover slides. To visualize ABCos protein cells were stained with anti-cMyc-FITC antibody (green) or to visualize membrane-associated IgG cells were stained with anti-kappa-Cy3 (red). The blue colour (DAPI) represents the cell nuclei. Co-localization was analyzed by merging both pictures.**

## 3.2.5 Biochemical analysis of ABCos:IgG heterodimer in CHO-K1 pC15 cells

To finally prove that the ABCos constructs are able to associate with immunoglobulin molecules western blot experiments with cell lysates from ABCos1, ABCos2 and non transfected CHO-pC15 cells were performed. The ABCos transfected CHO-pC15 cells were cultivated for 24 hours, thoroughly washed with PBS and pellets lysed under non reducing conditions to analyze the presence of the ABCos:IgG heterodimer. The lysates were separated by non-reducing SDS-PAGE and transferred for immune staining onto a PVDF membrane.

All CHO-pC15 cells, ABCos transfected or not, showed a specific kappa signal with a molecular weight of approximately 150 kDa. These signals represent the immunoglobulin molecules in the cell lysates consisting of two heavy chains and two light chains. However, only CHO-pC15 cells transfected with ABCos1 and ABCos2 showed a specific signal of approximately 119 kDa, which was absent in non transfected CHO-pC15 cells. A cMyc signal of the same molecular weight could also be detected. A strong cMyc signal smaller than 119 kDa was detected on the membrane, that probably represents the homodimeric form of ABCos (Figure 21 a). To finally prove that this band of 119 kDa represent the association of ABCos proteins with IgG molecules the band was dissected and reanalyzed under reducing condition. To recover the protein from the membrane the specific band at 119 kDa was excised and incubated with stripping buffer at 95 °C. The protein solution was separated by SDS-

PAGE and analyzed by western blot. The re-analysis of the excised band under reducing conditions showed a signal of 44 kDa with anti-CD40 antibody as well as anti-cMyc antibody representing the monomeric ABCos construct. A signal of 25 kDa detectable with anti-kappa antibody on the membrane representing the immunoglobulin molecules, which was absent in untransfected CHO-pC15 cells. These results finally prove the ability of ABCos constructs to associate with immunoglobulin molecules (Figure 21 b,c).
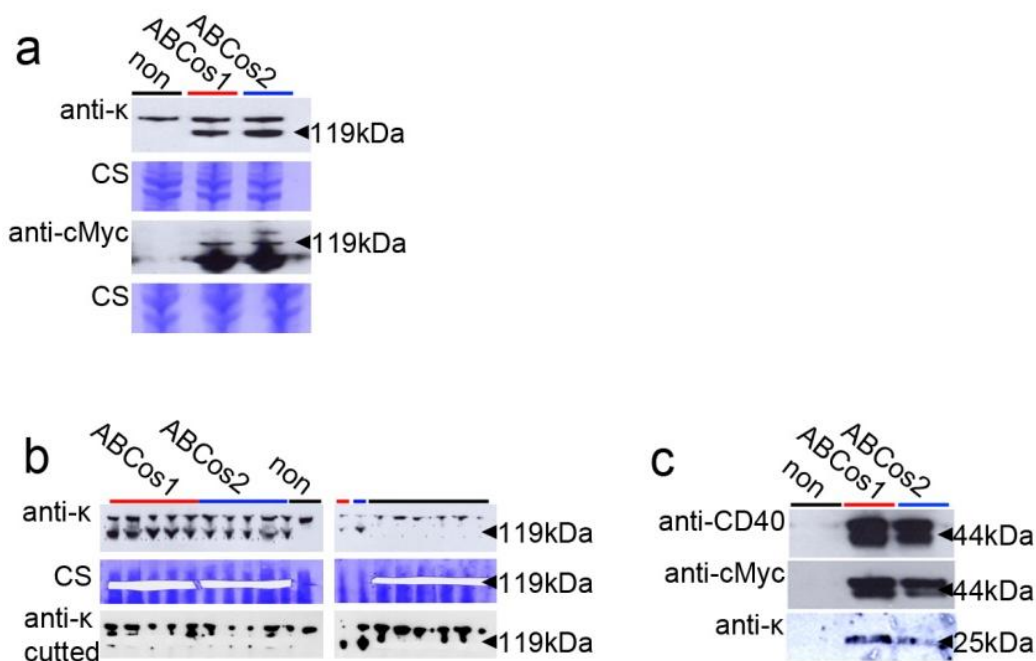


**Figure 21: *Detection of ABCos:IgG heterodimer in CHO-K1 pC15 cells.***
**a) CHO-pC15 cells were transfected with 10 µg ABCos IVT-RNA and cultivated for 24 hours. Subsequently, the cells were harvest and pelleted. The cell pellets were lysed under non reducing conditions, separated by SDS-PAGE and transferred onto a PVDF membrane. For protein amount validation the membrane was stained with Coomassie (CS). The membranes were stained with anti-kappa and anti-cMyc antibody, followed by the detection of primary antibodies with horseradisch peroxidase-conjugated goat-anti-mouse secondary antibody. b) Same preparation like in a) and subsequently excision of the heterodimeric band at approx. 119 kDa. c) The excised protein was resolved from the membrane and analyzed under reducing conditions for the presence ABCos (CD40 and cMyc) and IgG (kappa) protein.**

## 3.2.6 Analysis of the glycosylations of ABCos constructs

A common form of post-translational modification is the glycosylation of membrane proteins. To analyze if the ABCos constructs were post-translationally modified deglycosylation experiments were performed. CHO-K1 cells were transfected with 20 µg ABCos IVT-RNA and cultivated for 24 hours. Cells were harvested, washed and pellets were treated under reducing conditions to generate monomeric ABCos proteins. Subsequently, the lysates were treated with PNGase enzyme which cleaves N-glyosylated proteins in the penta core saccharid.
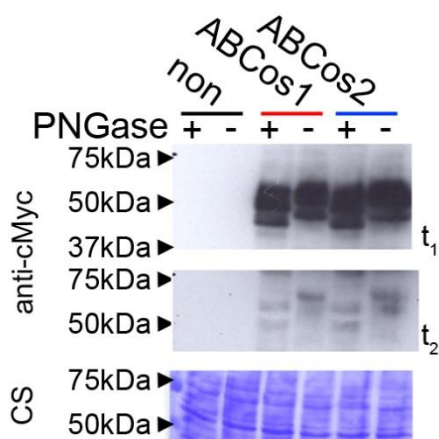
**Figure 22:** *Analysis of the post translational modification of ABCos constructs.*
**CHO-K1 wild type cells were transfected with ABCos IVT-RNA and cultivated for 24 hours. The ABCos transfected CHO-K1 cells were harvest, washed and pelleted. Subsequently, the cell pellets were lysed under reducing conditions and treated with PNGase. The lysate were separated by SDS-PAGE and analyzed for ABCos expression. (Top: anti-cMyc staining, bottom = Coomassie staining, $t_1 \gg t_2$)**

On each lane two signals could be detected. Both signals shifted to a smaller molecular weight after deglycosylation (Figure 22). This results shows that the glycosylation was not the cause for the presence of two signals, but shows that the constructs are intracellularly glycosylated. A second reason for the two signals could be an alternative reading frame on the DNA used for the production of IVT-RNA. Therefore the DNA sequence was analyzed for an alternative start codon and stop codon. No alternative open reading frame could be detected in the DNA sequence upstream and downstream the normal start codon with a fitting molecular weight.

### 3.2.7 Summary of chapter 3.2

The ABCos constructs were cloned into vectors which allow the production of highly stable IVT-RNA with an enhanced translational efficiency. All experiments were conducted with IVT-RNA which was controlled for correct sequence, integrity and poly-A tail length.

Those IVT-RNAs can be easily transferred into CD19$^+$ B cells by electroporation and is translated into proteins which finally locate on the cell membrane. It could be shown that the constructs are able to associate with endogenous IgG molecules. This data are proved by flow cytometry, immune fluorescence microscopy and western blot experiments. The amount of IVT-RNA is determining the amount of ABCos protein on the cell surface and the amount of antibody retention through heterodimeric association allowing the titration of ABCos:IgG amount on the surface of antibody secreting cells.

## 3.3 Functional characterization of ABCos activated versus CD40 activated B cells

### 3.3.1 NF-κB activation by ABCos induced signaling

The NF-κB experiment analyzes the functionality of the CD40 domain in the ABCos constructs to elicit a CD40 like signal. A hallmark of CD40 induced signal transduction is the activation of the transcription factor NF-κB and requires the clustering of the intracellular CD40 domains. Conformational alterations, steric configurations and subcellular localization play an important role in the induction of intracellular signaling and could inhibit the signal transduction by ABCos constructs.

To monitor the induction of ABCos signaling in response to ABCos clustering the in-house generated HEK293 cell line which is stably transfected with luc-pNifty vector was used. The luc-pNifty vector is a reporter plasmid including the luciferase gene under the control of a minimal NF-κB-inducible ELAM1 composite promoter.

To perform NF-κB luciferase assay, HEK293 reporter cells transiently transfected with ABCos constructs were incubated over night in anti-cMyc or control antibody coated 96-well plate. 6 hours before analysis PMA and Ionomycin were added as positive control. PMA and Ionomycin are highly activating compounds through the opening of calcium channels and activation of protein kinase C. Thereby PMA and Ionomycin are able to activate almost all cell types. A mixture of D-Luciferin and ATP-Tris base solution was added to each well and bioluminescence flux was measured using a microplate luminescence reader with 1sec integration time/well.
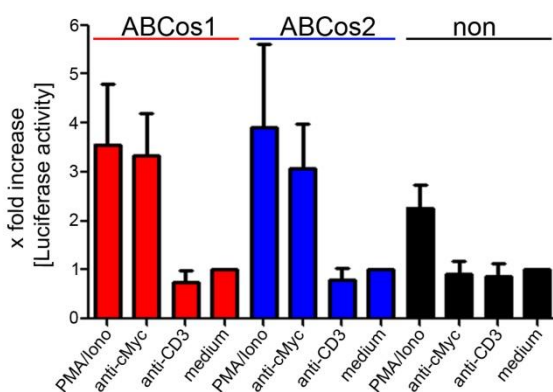


**Figure 23:** *NF-κB activation by ABCos induced CD40 signaling.* **HEK 293 NF-κB reporter cells were transfected with 20 μg ABCos IVT-RNA and incubated over night in anti-cMyc or control antibody coated 96-well plate. 6 hours before analysis PMA and Ionomycin were added as positive control. Bioluminescence flux was measured using a microplate luminescence reader. The results are calculated to the relative luminescence increase based to medium control. Shown are the summaries of three independent experiments (Shown are mean standard deviation).**

Non transfected HEK 293 reporter cells showed very weak bioluminescence (Figure 23). The HEK293 reporter cells transfected with ABCos1 or ABCos2 treated with PMA and Ionomycin showed an increase in the luciferase signal of a factor of three to four. Corresponding, the crosslinking of the ABCos constructs by anti-cMyc antibodies induced a strong signal increase, which is comparable to the PMA and Ionomycin treated cells. These data show that crosslinking of ABCos constructs with anti-cMyc antibodies results in the activation of the NF-κB pathway.

### 3.3.2  Induction of proliferation via ABCos signaling in CD19$^+$ B cells

As mentioned in chapter 3.1.5 different strategies for antibody induced clustering could be useful. To compare the results from the anti-CD40 (natural CD40 signal) activation with anti-cMyc (artificial ABCos signal) a further set of antibody application experiments were performed. Briefly, the antibody was presented by CD32 (Fc-γ receptor) expressing CHO-K1 cell line or soluble form with or without crosslinked by a secondary anti-Fc antibody. Furthermore the antibody was directly coated to the culturing dish. For the activation and expansion of ABCos transfected peripheral CD19$^+$ B cells anti-cMyc antibody was used. The anti-cMyc antibody clusteres the ABCos constructs by binding to the marker tag and simulates an antigen. The CHO-K1-CD32 and CHO-K1 WT cell line were treated with Mitomycin C to inhibit proliferation and loaded with antibody. The B cells were transfected with ABCos IVT-RNA, stained with CFSE and stimulated for 120 h. Subsequently, the cells were analyzed for the reduction of CFSE intensity by flow cytometry.
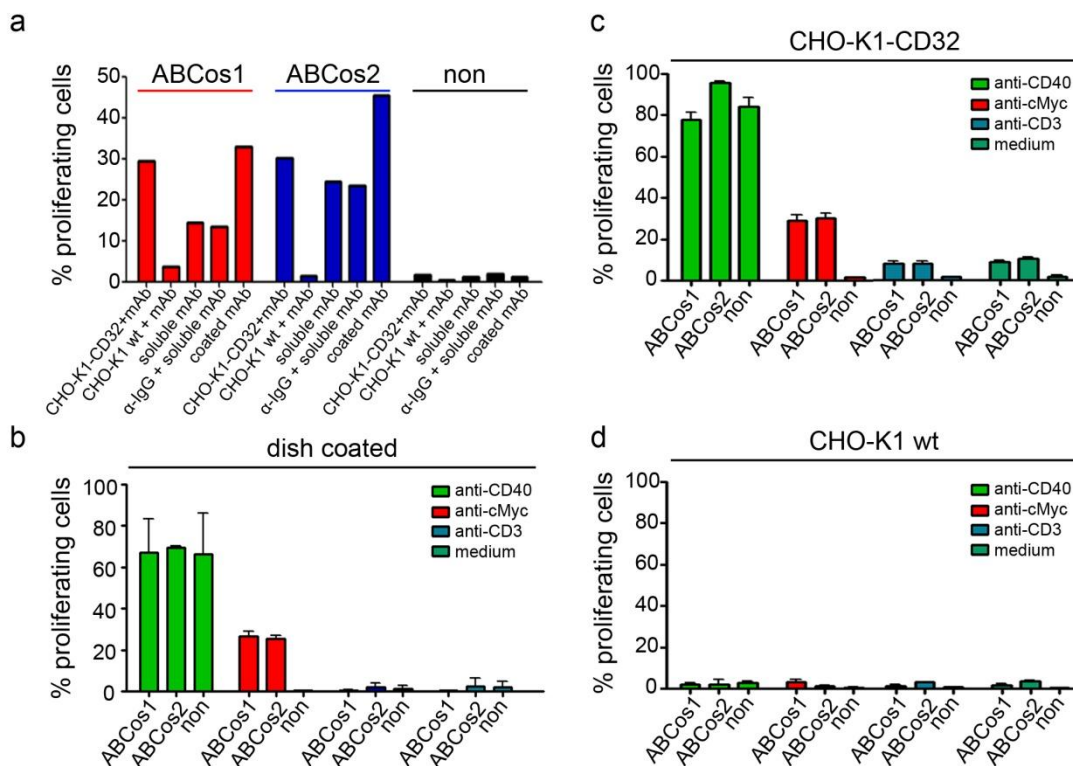
**Figure 24:** *Induction of proliferation via ABCos signaling in peripheral CD19$^+$ B cells.*
CD19$^+$ sorted B cells were transfected with 20 µg ABCos IVT-RNA, stained with CFSE and stimulated for 120 hours. a) ABCos$^+$ B cells were stimulated with anti-cMyc antibodies presented by CHO-K1-CD32, soluble, soluble crosslinked with anti-Fc antibodies or plate-bound. ABCos$^+$ B cells were stimulated with anti-cMyc antibodies presented as (b) plate-bound antibodies or (c) presented on CHO-K1-CD32. d) To proof that the CHO-cells had no influence the B cells were co-cultivated with CHO-K1 wild type cells. The summary of three independent experiments are shown (Shown are mean standard deviation)(b, c, d).

Weak proliferation was detected in untransfected B cells in all 5 application strategies (Figure 24 a), as well as in ABCos transfected B cells cultivated with CHO-K1 cells (Figure 24 a, d). ABCos transfected B cells could be activated by different anti-cMyc applications. The highest proliferation rate was detected by antibody coated to the dish in which case 30 to 40 % of proliferating cells were detected in cells transfected with ABCos1 or ABCos2 IVT-RNA, respectively (Figure 24 a). The induction of proliferation with anti-cMyc antibody presented on CHO-K1-CD32 or soluble anti-cMyc antibody ranged from 25 % to 30 %. Additionally, the crosslinking of soluble anti-cMyc antibody by a secondary anti-Fc antibody had no influence on the activation (Figure 24 a). To validate these observations the experiments were repeated in a greater cohort. The figures 24 b and c show that no strong differences are detected between plate- or CHO-K1-CD32 bound antibody presentations. Due to feasibility and reduced complexity all following experiments were performed with plate coated anti-cMyc antibody.

### 3.3.3 Phenotypic characterization of ABCos- versus CD40 activated B cells

Since CD40 mediated proliferation of B cells is associated with the induction of differentiation (Arpin et al., 1995) we investigated the phenotypic changes induced by ABCos constructs after crosslinking with anti-cMyc antibodies. In germinal center reactions B cells undergo class switch recombination which results in the switch from IgD$^+$ IgM$^+$ B cells to IgG$^+$, IgA$^+$ or IgE$^+$ B cells as described in the introduction (chapter 1.3.1). To prove if this is also the case in the ABCos system the down regulation of IgD (expressed before class switch) was analyzed. For that, CD19$^+$ sorted B cells were transfected with ABCos IVT-RNA, stained with CFSE and stimulated for 5 days. Subsequently, the cells were analyzed by flow cytometry for the reduction of CFSE and down regulation of IgD.

The stimulation of transfected B cells with anti-CD40 results in a strong proliferation signal, as expected. Both IgD$^+$ and IgD$^-$ CD19$^+$ B cell subtypes proliferate in response to CD40 signal (Figure 25). The frequency from IgD$^-$ proliferated B cells displays a small shift from approximately 35 % for untransfected up to 55% in ABCos1 transfected B cells. However, the presence of ABCos constructs affects the number of class switched proliferating cells. While the number of IgD$^+$ is approximately 35 % in the absence of ABCos, these number increase to 50 – 55 % by transfection with ABCos1 or ABCos2. The effect of predominant proliferation of class switched IgD$^-$ B cells is further increased when ABCos transfected B cells are stimulated by anti-cMyc. In this case around 70 % to 80 % of proliferating B cells was negative for IgD expression (Figure 25).
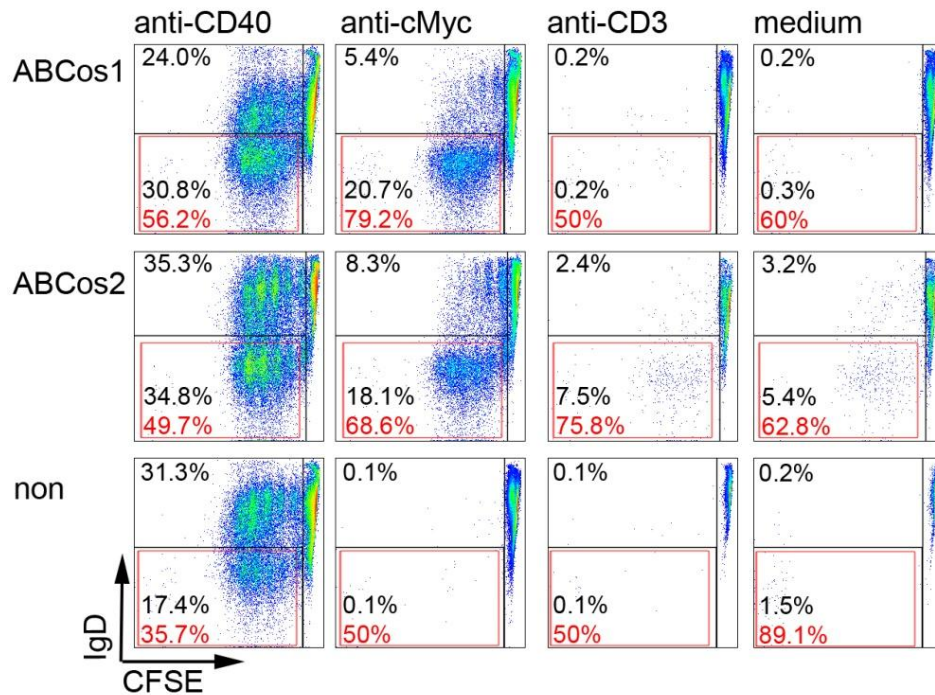
**Figure 25: *Preferential activation of IgD⁻ B cells by ABCos mediated signaling.***
CD19⁺ sorted B cells were transfected with 10 µg ABCos IVT-RNA, labeled with CFSE and stimulated by 10 µg/mL of indicated plate-bound antibody. The reduction of CFSE intensity and down regulation of IgD was analyzed on day 5 post transfection by flow cytometry. One representative experiment of 3 independently performed experiments is shown.
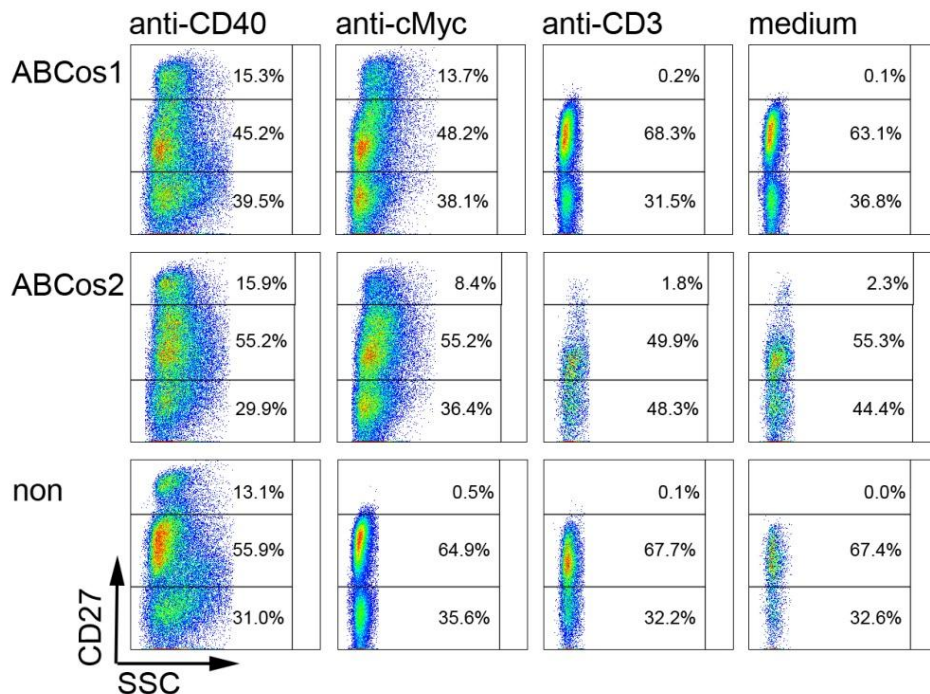


**Figure 26: *B cell subsets distribution in peripheral B cells after CD40 or ABCos stimulation.***
CD19⁺ sorted B cells were transfected with 10 µg ABCos IVT-RNA, labeled with CFSE and stimulated by 10 µg/mL of indicated plate-bound antibodis. The surface expression of CD27 was analyzed on day 5 post transfection by flow cytometry. One representative experiment of 3 independently performed experiments is shown.

The next experiment was performed to analyze the preferential expansion of class switched IgD⁻ B cells in more detail. For that, the CD19⁺ B cells were divided in naïve B cells, memory B cells and plasma blast and plasma cells as a consequence of the expression of CD27 (Odendahl et al., 2000).
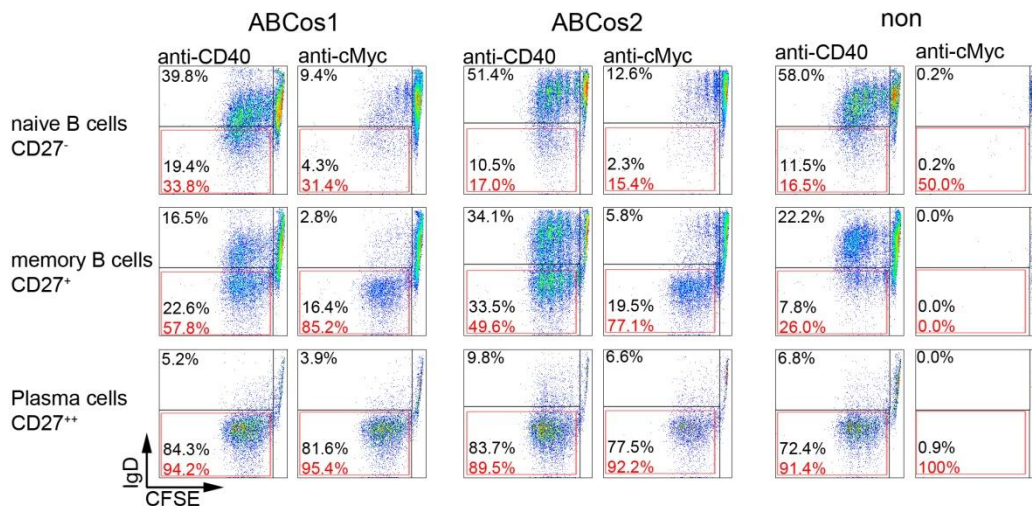


**Figure 27: *B cell subsets distribution in peripheral B cells after CD40 or ABCos stimulation.* CD19⁺ sorted B cells were transfected with 10 µg ABCos IVT-RNA, labeled with CFSE and stimulated by 10 µg/mL of indicated plate-bound antibodies. The surface expression of CD27, the reduction of CFSE intensity and down regulation of IgD was analyzed on day 5 post transfection by flow cytometry. One representative experiment of 3 independently performed experiments is shown.**

The unstimulated peripheral CD19⁺ B cells distributed in 30 to 35 % CD27⁻ B cells, 65 to 70 % CD27⁺ B cells and under 1 % of CD27⁺⁺ B cells (Figure 26). The activation of CD19⁺ B cells with anti-CD40 antibodies had only weakly influenced the CD27⁻ B cell subset. In contrast to the CD27⁺ and CD27⁺⁺ B cell subpopulations which were strongly influenced. As a result of anti-CD40 stimulation the frequency of CD27⁺ B cell was declined from 68 % to 56 % in non transfected B cells and from 68 % to 45 % in ABCos1⁺ B cells. In contrast, the CD27⁺⁺ B cell frequency increased to 15 %. These changes in the B cell homeostasis could be also observed in ABCos activated B cells, with regard to the reduction of CD27⁺ B cells and the increase of CD27⁺⁺ B cells (Figure 26). Besides the isotype specific activation, these data show that the ABCos system is able to induce in addition to proliferation also differentiation. Next the different B cell subpopulations were analyzed for CFSE reduction and IgD down regulation (Figure 27). As mentioned above, the overall distribution of naïve, memory, and plasma cells according to the expression of CD27 after activation of ABCos constructs was similar to B cells stimulated with anti-CD40 antibody (Figure 26). The IgD expression of proliferating CD27⁻ B cells (naïve) shows no difference in cells stimulated by CD40 or ABCos constructs. The ratio of IgD⁻ to IgD⁺ is in both activation

settings very similar, around 30 % for ABCos1 and around 15 % for ABCos2. Furthermore, proliferating CD27$^{++}$ cells (plasma) were nearly completely negative for IgD expression irrespective of the stimulus used (Figure 27). However, stimulation of CD27$^+$ cells (memory) with anti-CD40 or anti-cMyc results in a different IgD expression pattern. IgD$^+$ and IgD$^-$ cells proliferated after the stimulation of CD27$^+$ B cells with anti-CD40 antibodies in an almost equal ration. In contrast, ABCos activation of CD27$^+$ B cells mainly activated IgD$^-$ B cells. This means, this type of activation leads to a different isotype specific activation pattern. While CD40 stimulation induced proliferation in both IgD$^+$ and IgD$^-$ CD27$^+$ B cell populations, ABCos stimulation preferentially activated IgD$^-$ CD27$^+$ B cells (Figure 27).



**Figure 28:** *B cell differentiation into plasma cells mediated by CD40 or ABCos signaling.*
**CD19$^+$ sorted B cells were transfected with 10 μg ABCos IVT-RNA, labeled with CFSE and stimulated by 10 μg/mL of indicated plate-bound antibodie. The surface expression of CD138 and the reduction of CFSE were analyzed on day 5 post transfection by flow cytometry. One representative experiment of 3 independently performed experiments is shown.**
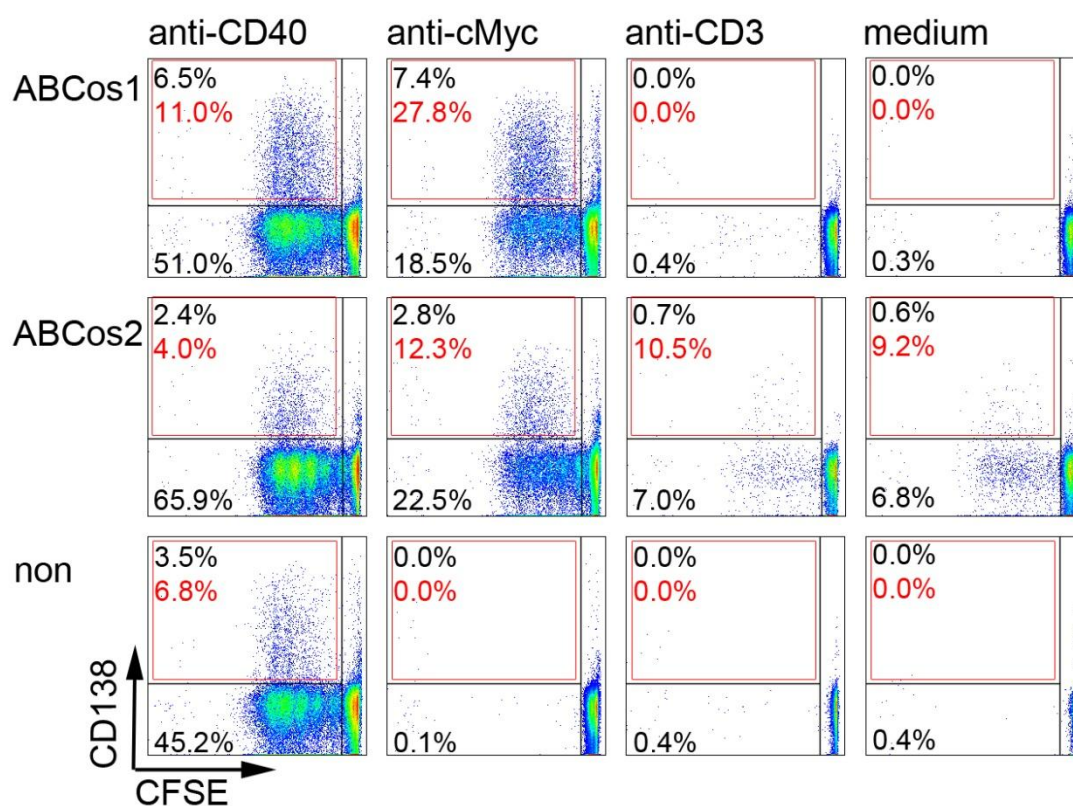
Since activation of memory B cells can lead to differentiation into plasma cells (Agematsu et al., 1998) the question arised, whether the constructs are able to induce plasma cell development. To answer this question the experiment was repeated and the cells additionally analyzed for expression of CD138 (Syndecan-1) which is exclusively expressed on plasma cells (Sanderson et al., 1989). Stimulation of CD19$^+$ B cells with anti-CD40 resulted in the expression of CD138 in 4 to 10 % of proliferating cells (Figure 28). The up regulation of CD138 correlated with the down regulation of

CD20, which is not expressed on plasma cells but on memory B cells (data not shown). This effect was increased by the stimulation of ABCos transfected B cells with anti-cMyc. ABCos clustering induced the differentiation into plasma cells in up to 10 to 30 % of proliferating cells which constitutes a 2 – 3 fold increase (Figure 28). This result shows that compared to a CD40 signal an ABCos mediated signal has a higher ability to induce differentiation of B cells into plasma cells.

### 3.3.4 ABCos specific expansion of a minority of ABCos transfected B cells

The overall goal is to isolate, identify and amplify a small number of certain antigen-specific B cells in a complete pool of peripheral B cells with different specificities. Therefore ABCos stimulated B cells must be able to proliferate even when present at only very low frequencies. To prove the concept, untransfected B cells were labeled with the proliferation dye CPD-eFluor670 and ABCos transfected B cells were labeled with CFSE. Subsequently, decreasing numbers of ABCos transfected CFSE labeled B cells were titrated into untransfected B cells so that the amount of ABCos transfected B cells ranged from 50 % to 1 %. The B cell mixture was stimulated with either anti-CD40 antibody or anti-cMyc antibody and analyzed for specificity of the induced proliferation (Figure 29).

ABCos$^+$ and ABCos$^-$ B cells proliferate equally well after anti-CD40 stimulation as indicated by the reduction of CFSE and CPD-eFluor670 intensity (Figure 29 a). In contrast to this, stimulation with ABCos constructs results in the exclusive proliferation of ABCos$^+$ CFSE$^+$ B cells (Figure 29 a). The crosslinking of ABCos constructs by anti-cMyc antibodies results in the proliferation of about 30 % of CFSE$^+$ B cells, which is approximately half of the amount of anti-CD40 activated B cells (Figure 29 b). The specificity was analyzed with CFSE$^+$ ABCos$^-$ B cells which show no proliferation after co-cultivation with anti-cMyc antibodies. The 30 % proliferation of ABCos activated CFSE$^+$ ABCos$^+$ B cells was unaffected by the number of ABCos expressing cells in a large pool of untransfected B cells (Figure 29). These data suggest that it is possible to activated and indentify low frequencies of ABCos expressing B cells in a large pool of non proliferating B cells.
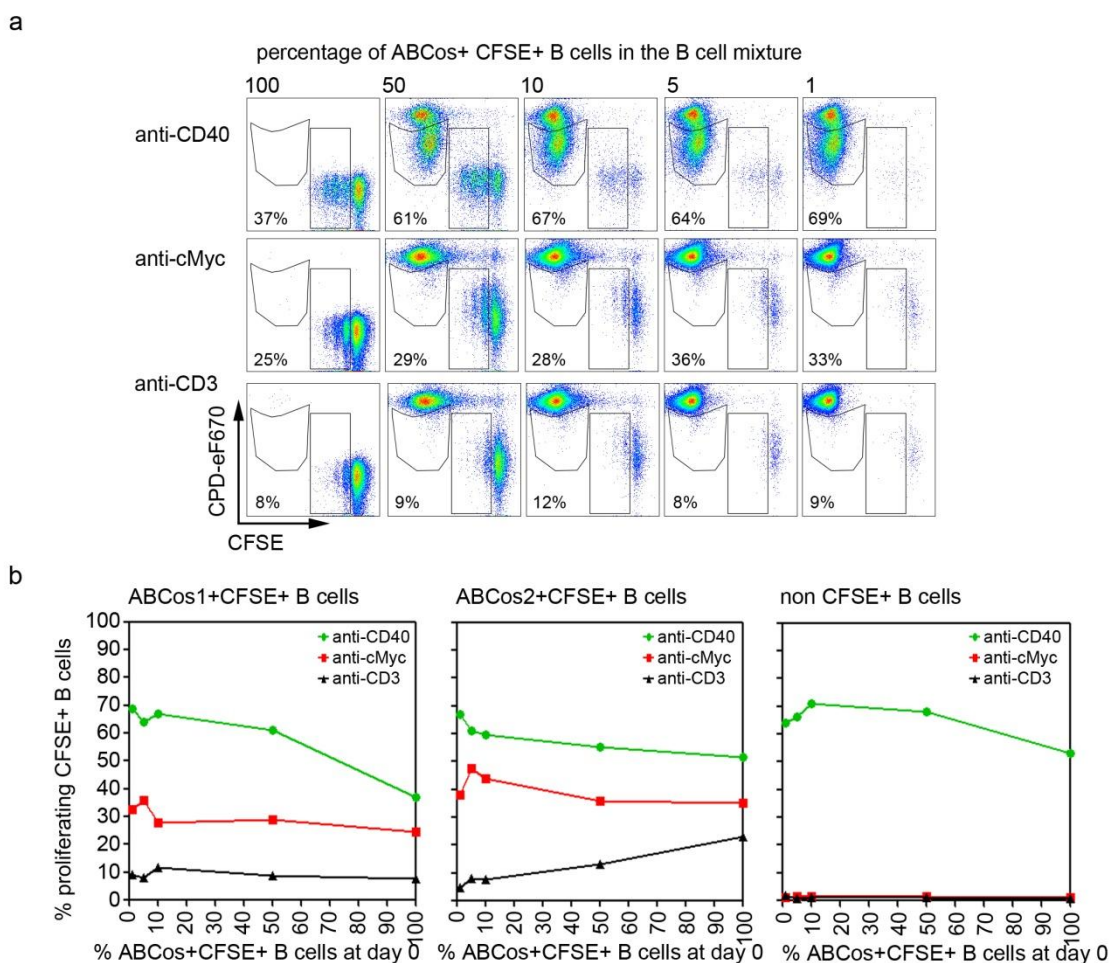
**Figure 29:** *Proliferation of ABCos activated B cells in a large pool of untransfected B cells.*
**CD19+ sorted B cells were either untransfected and labeled with CPD-eFluor670 or transfected with 10 µg ABCos IVT-RNA and labeled with CFSE. B cells mixture, composed of both populations in titrated amounts, were activated by 10 µg/mL of indicated plate-bound antibodies and proliferation was analyzed on day 5 after activation by flow cytometry (a). Numbers indicate percentage proliferating ABCos+ B cells based to all ABCos+ B cells. Panel (b) as in (a), summary of ABCos1, ABCos2 and non transfected B cells.**

### 3.3.5 Summary of chapter 3.3

The ABCos constructs are able to activate the transcription factor NF-κB and induce proliferation after crosslinking in peripheral B cells. In experiments determining the B cells subset and isotype specific activation of ABCos stimulated B cells it was shown that ABCos activation mainly occurs in IgD⁻ B cells, which was in strong contrast to CD40 activation. It appears that the isotype specific activation is not mediated by an isotype switch from IgD⁺ to IgD⁻ but rather by an isotype specific activation of IgD⁻ B cells. Besides the ability of the ABCos constructs to induce differentiation, the plasma cell development was analyzed. The activation of B cells with ABCos construct induced a higher plasma cell differentiation when compared to CD40 activated B cells analyzed by the up regulation of the plasma cell marker CD138. Furthermore, the experiments

show that it is possible to specifically stimulate an extreme minority of ABCos transfected B cells.

## 3.4 Antigen-specific activation of ABCos transfected B cells

### 3.4.1 Antigen-specific activation of ABCos and anti-Plac1 BCR transfected B cells

The activation of antigen-specific B cells *ex vivo* is challenging due to the extremely low frequency of this certain B cell type and the lack of co-stimulatory signals. In this novel system the CD40 co-stimulatory signal is provided by heterodimerized ABCos:IgG complex. To increase the fraction of antigen-specific B cells the isolated peripheral B cells were transfected with a BCR specific for a certain antigen derived from the tumor antigen Plac1. Briefly, CD19$^+$ sorted B cells were transfected with equi-molar amounts of anti-Plac1 BCR light chain and heavy chain or ABCos construct or all three IVT-RNAs. Subsequently, the cells were labeled with CFSE and stimulated with antigenic peptide as indicated in figure 30.

Figure 30 shows the co-expression of the anti-Plac1 B cell receptor and ABCos construct on the cell surface of triple transfected B cells (figure 30 a). For that transfected B cells were incubated with biotinylated peptide specific for the anti-Plac1 BCR and stained with anti-cMyc and strepavidin coupled to APC. Non transfected B cells or B cells transfected with ABCos IVT-RNA showed any staining for streptavidin indicating no unspecific binding of the Plac1 peptide. In contrast, B cells transfected with light and heavy chain for anti-Plac1 BCR were highly positive for streptavidin, as a consequence of Plac1 peptide binding. Furthermore, the triple transfection with anti-Plac1 BCR and ABCos construct was also positive for Plac1 peptide and ABCos construct (Figure 30 a). To prove the specificity the transfected B cells were treated with biotinylated peptide followed by the competition with the 10 time higher amount of unbiotinylate peptide. The recognition of the Plac1 peptide was damped in the competition assay in the single BCR transfected and the two ABCos:BCR transfected setting (Figure 30 a). These data clearly show that the BCR is expressed and able to bind the antigen. For the antigen-specific stimulation two different formulations of antigenic peptide were used, one is the single biotinylated peptide the other is the biotinylated di-peptide. The single transfection of BCR in B cells was not sufficient to induced proliferation after antigenic stimulation (Figure 30 b). The co-transfection of the

BCR together with ABCos constructs allows the antigen-specific expansion of transfected B cells resulting in 3 – 4% proliferating cells after antigen-specific stimulation elicited by both antigenic peptide forms (Figure 30 c). The antigen-specific proliferation was significantly increased compared to control peptide stimulated ABCos transfected B cells and represents approximately twice as much proliferating cells as observable in the unspecific activation (Figure 30 c).
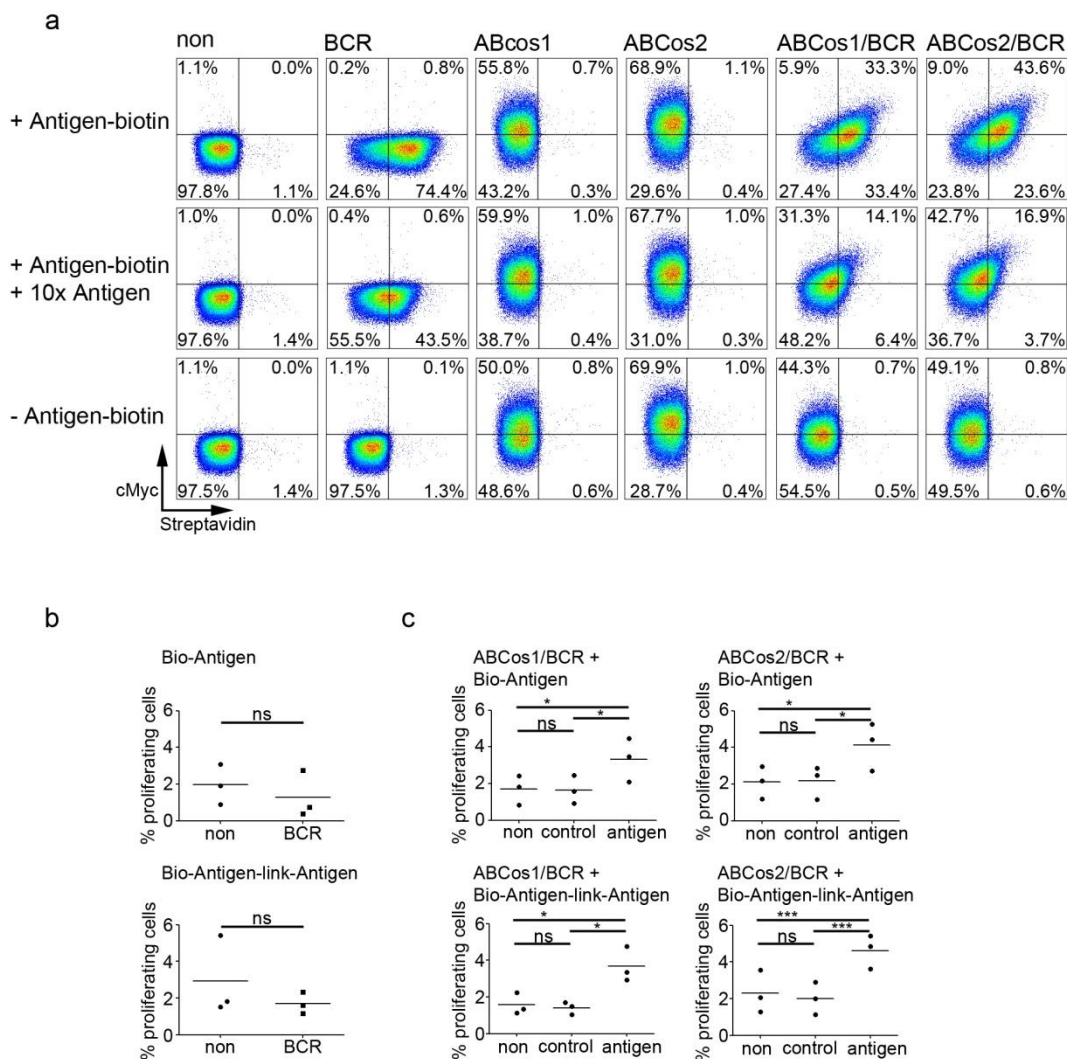


**Figure 30: *Antigen-specific activation of ABCos and anti-Plac1 BCR transfected B cells.***
**a) CD19^{+} sorted B cells were transfected with 10 µg of each ABCos, BCR heavy chain and BCR light chain IVT-RNA and subsequently labeled with CFSE. The expression and functionality of BCR and ABCos was analyzed 24 h post transfection by staining with anti-cMyc and biotinylated-peptide streptavidin-APC complex. One representative experiment of 3 independently performed experiments is shown. (b) and (c) as in (a), cells were stimulated with 10 µg/mL of indicated antigenic peptide coated on a streptavidin coated culture dishs and proliferation was analyzed on day 5 post transfection. The experiment was conducted with B cells from three different donors. (\* indicating significant differences; \* p < 0.05; \*\*\* p < 0.001)**

### 3.4.2 pp150-specific activation of ABCos⁺ IgG⁺ memory B cells from CMV⁺ donors

The final proof of the concept was performed by the pp150-specific activation of peripheral B cells obtained from CMV-infected individuals. For that, IgG⁺ memory B cells were isolated from the total pool of primary peripheral human B cells of three different CMV reactive donors. Subsequently, the B cells were transfected with ABCos and labeled with CFSE. The stimulation was performed with the known CMV antigen pp150 and analyzed 5 days post transfection by flow cytometry. The ABCos constructs were able to induce proliferation in 0.4 to 1.2 % of IgG⁺ memory B cells which represents about twice to thrice as much proliferating cells as in the control (0.2 – 0.6 %) (Figure 31). This result confirms the functionality of the ABCos constructs and their ability to induce an artificial antigen-specific CD40 signal which allows the identification and expansion of antigen-specific primary human B cells.
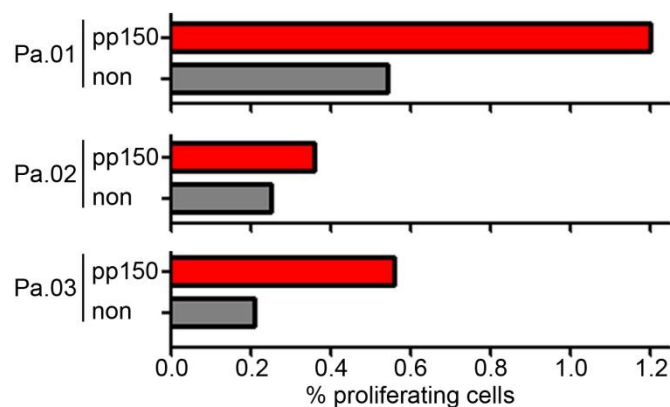


**Figure 31:** *pp150-specific activation of ABCos+ IgG+ memory B cells from CMV⁺ donors.*
**IgG⁺ sorted memory B cells were transfected with 10 µg ABCos1 IVT-RNA, labeled with CFSE and stimulated with 10 µg plate-bound antigenic peptide as indicated. The proliferation was analyzed on day 5 post transfection. The experiment was conducted with B cells from three different CMV⁺ donors.**

### 3.4.3 Summary of chapter 3.4

The ABCos constructs demonstrate their ability to induce antigen-specific CD40 signals in two different experiments. The first experiment was an artificial system in which the BCR was transfected into the B cells. Only ABCos and BCR double positive B cells proliferate after stimulation with the BCR specific peptide. Neither BCR single transfected B cells nor ABCos single transfected B cells were activated by the antigenic peptide. The second experiment was performed with peripheral blood from CMV infected individuals. The pp150 peptide was able to induce antigen-specific proliferation in ABCos transfected IgG⁺ memory B cells. Both experiments clearly demonstrate the

functionality of the ABCos constructs, their ability to induce antigen-specific CD40 signal and to identify antigen-specific B cells in the repertoire of peripheral B cells.

# 4    Discussion

## 4.1    Historical background, socio-economic and scientific interest of the study

Since Edward Jenner 1796 developed the immunization against smallpox a multitude of human lives were saved by vaccination technology. Today two kinds of different vaccinations are used to protect human lives, the active and passive vaccination. According to the method developed by Edward Jenner live, attenuated or deadened pathogens will be inject into patients and thereby generate mainly a humoral immune response against the pathogen leading to a protecting antibody titer which is present for several years (Sallusto et al., 2010). Otherwise, Emil von Behring shows 1890 that the direct transfer of anti-Diphtheria antibodies induce protection against this disease for a short time. This kind of vaccination is today called passive immunization. Both strategies protect against live-threatening infectious diseases through the activation of the host-own immune system (Reichert, 2007). While the combat of infectious diseases was partially successful in the western world, it was replaced by cancer induced death. Today, cancer is one of the leading causes of death worldwide. More than 7.5 million people died of cancer in 2008. Without novel strategies against these kinds of diseases the incidence will drastically increase to about 11 million in 2030 (www.who.int/en/). At the moment no protective immunization against cancer is available. The treatment of cancer is traditionally based on the surgical intervention, radiation therapy or systemic application of chemotherapeutics. A variety of reasons limits these strategies leading to a low success rate. Recently, the treatment of cancer by the application of therapeutic antibodies was mentioned as a sunrise therapy. 1975 Köhler and Milstein discovered a platform technology to produce antibodies against a virtually unlimited arsenal of target structures. The hybridoma technology based on the immunization of rodent animals resulting in the generation of a humoral immune response against the target structure (Kohler and Milstein, 1975). The antigen-specific B cells were isolated and immortalized followed by the characterization and selection of an ideal therapeutic antibody. A variety of produced rodent antibodies or genetically modified antibodies were used to treat diseases ranging from autoimmune diseases, cancer to infections. The genetic modifications from rodent to chimeric or humanized antibodies reduced the human immune response against the non-human antibody but did not completely

eradicate it (Stein, 2002). Since then research has addressed to generate fully human antibodies. At the moment two possibilities have been developed, the combinatorial phage display technology (Hanes and Pluckthun, 1997; Huse et al., 1989; McCafferty et al., 1990) and the genetically modified mice technology (Kuroiwa et al., 2000; Lonberg et al., 1994; Mendez et al., 1997). The phage display technology based on the random expression of different human variable domains selected against a certain antigen. By the transgenic mice technology the murine immunoglobulin genes were replaced by the corresponding human heavy- and kappa light-chain minilocus. The transgenes rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation in response to antigen stimulation. Both technologies produce human-like antibodies with reduced side effects as a human therapeutic. Nevertheless, it is predicted that fully human antibodies obtained from human beings are without or with negligible side effects (Stein, 2002). Therefore, high therapeutic doses over a long period of time could be used in clinical therapies which will have beneficial impact on the patient outcome. But, the generation of fully human tumorantigen-specific antibodies suitable for anti-tumor therapy is laborious and difficult to achieve. One source for those antibodies could be autoreactive B cells expressing those antibodies, which are detectable in cancer patients (Sahin et al., 1995; Sahin et al., 1997; Tureci et al., 1997). However, the isolation and cultivation of this cell type is challenging. Through the breakdown of the technology barriers a novel class of tumor therapeutic antibodies based on tumor-autoreactive human antibodies will become an essential part of future cancer treatment.

## 4.2   Novel aspects of the study

As mentioned above, the isolation of human autoreactive antigen-specific B cells is extremely difficult as well as their cultivation and the maintenance of physiological cell behavior. One possibility to isolate those B cells is the labeling with fluorescence-coupled antigen followed by fluorescence activated cell sorting (Leyendeckers et al., 1999; Leyendeckers et al., 2002). The antigen-specific B cells have to be immortalized for long-term cultivation, which could be induced by the introduction of viral genes or the transduction with viruses. For example, the transduction of human B cells with Epstein-Barr virus is a common tool to induce immortalization (Traggiai et al., 2004; Beltramello et al., 2010; Simmons et al., 2007; Macagno et al., 2010; Corti et al., 2010; Collarini et al., 2009). Another possibility to generate antigen-specific B cells is the immortalization of the complete B cell repertoire followed by single cell dilution. Both

technologies are limited by their permanently artificial and time consuming nature. This study presents a technology which overcomes these limitations and presents a multitude of different opportunities which will be discussed later.

B cell development results in antibodies which are generated by a random gene rearrangement process with tremendous number of specificities. However, this random process could also result in antibodies which are self-reactive (Wardemann et al., 2003). Those antibodies reactive against several human targets could be used as diagnostics as well as in therapies against different human diseases like cancer, cardiovascular disease, inflammatory diseases, macular degeneration, transplant rejection, multiple sclerosis, and viral infections. To produce antibodies B cells need at least three different signals. One signal is given by the recognition of a certain antigen by the B cell receptor (Bretscher and Cohn, 1970). These interactions result in the clustering of the BCR, initiating intracellular signaling followed by the internalization of the BCR antigen complex. The antigen is intracellularly processed to peptides which will be presented via MHC-II molecules on the cell membrane. Cognate CD4$^+$ T helper cells are able to recognize the peptide-MHC-II complex via their specific TCR and become activated. Subsequently, the T cells will express secondary co-stimulatory molecules, like CD40 ligand (CD154). The interaction of CD40 receptor on B cells with the CD40 ligand provided by T cells in addition to a certain cytokine milieu will induce full activation of B cells resulting in proliferation, differentiation and antibody production (Elgueta et al., 2009; Quezada et al., 2004).
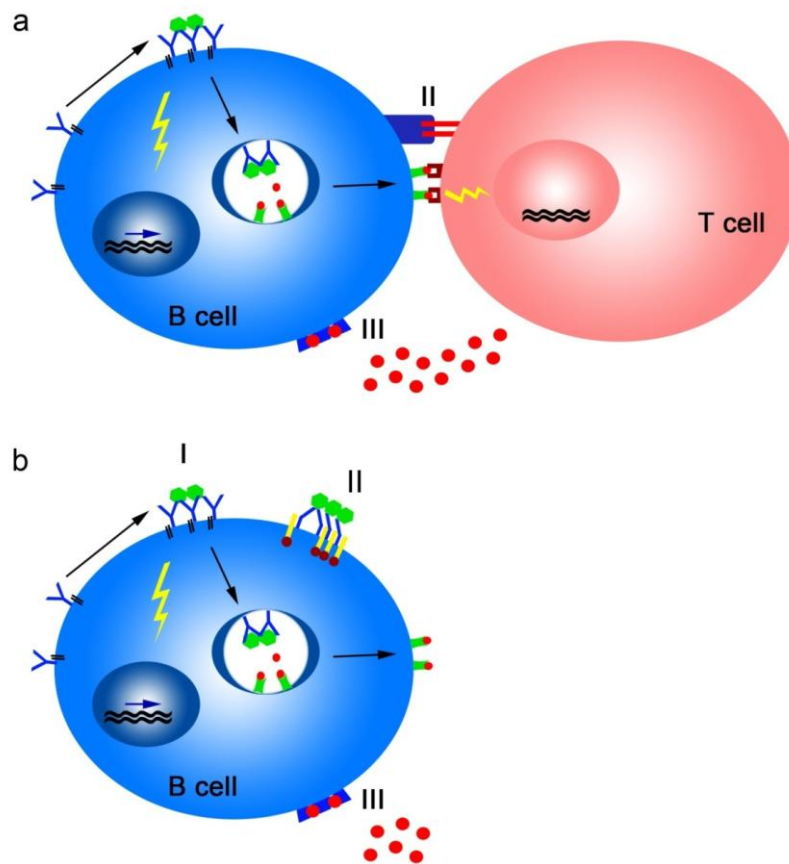
**Figure 32:** *Schematic draft of the antigen-specific activation via ABCos constructs.*
**a) For full B cell activation at least three different signals are required, (I) antigen-specific signal results from BCR clustering by antigen recognition. (II) Antigen independent CD40 signal and (III) stimulating cytokines are provided by cognate T helper cells. b) To mimic the *in vivo* system of B cell activation B cells were transfected with ABCos construct which transform the antigen-independent signal into an antigen-specific one (II). Both signals (I) BCR and (II) CD40 are induced by recognition of a certain antigen and can be supplement by different (III) cytokines.**

To mimic these complex processes *ex vivo* a completely novel technology was developed. The isolation of the entire B cell repertoire including the desired specificity from patients is easy to achieve, but the antigen-specific induction of both stimulatory signals is not. To achieve this, the novel technology was designed to transform the antigen-independent CD40 signal into an antigen-dependent one. This system is based on the expression of an artificial fusion protein called ABCos. The ABCos is composed of an N-terminal marker domain, followed by a $C_H2$-$C_H3$ fusion domain, a transmembrane domain and the intracellular domain of the human CD40 domain. The ABCos constructs are designed to associate with the endogenous IgG molecules thereby generating an artificial novel antigen-specific CD40 signal inducer (figure 32 and 33).
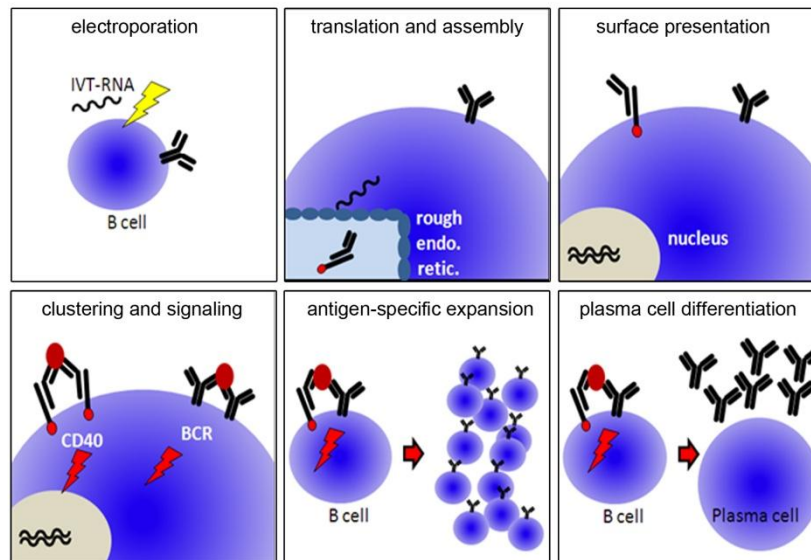
**Figure 33:** *Novel techology for the antigen-specific activation of peripheral B cells.*
**B cells were electroporated with IVT-RNA encoding for ABCos protein. The protein assembled**
**intracellularly with the endogenous immunoglobulin and was subsequently presented on the cell**
**surface. Recognition of the antigen led to the clustering of the immunoglobulin and thereby**
**generating the BCR signal and the ABCos signal. Both signals together led to the antigen-specific**
**expansion and plasma cell differentiation. (BCR = B cell receptor, rough endo. retic. = rough**
**endoplasmic reticulum, IVT-RNA =** *in vitro* **transcribed RNA)**

Artificial fusion proteins for synthetic signaling have proven its importance since more than 25 years (Pellman et al., 1985; Davey et al., 1985; Godowski et al., 1988; Lee et al., 1989). However, ligand binding domains used for induction of artificial signals are limited to a certain receptor. In contrast, the usage of B cell encoded immunoglobulin molecules as ligand binding domains would generate a virtually unlimited repertoire of signal inducing agents. The practical application of this approach, however, has not been described until now.

## 4.3 Key achievements

### 4.3.1 Protocol optimization

The novel strategy is based on the antigen-specific activation of a certain B cell subpopulation which remains all other B cells quiescent. For that, all steps of the process were optimized for highly-pure B cell isolation, survival and non unspecifically activating conditions. The designed process includes the isolation of different B cell subsets from human peripheral blood. This was done by Ficoll-Hypaque density centrifugation, followed by the magnetic activated cell sorting in CD19$^+$ B cells or IgG+ memory B cells. As shown in figure 11 the received B cells are still non-activated

resulting in conserved CFSE intensity. For the manipulation of B cells the electroporation method with IVT-RNA was selected. In contrast to DNA RNA needs milder electroporation parameters. Mild electroporation parameters induce gaps into the cell membrane which are required for passively transport of the nucleic acids into the cytosol. The RNA can be translated directly in the cytosol and transferred to the endoplasmatic reticulum. For DNA it is necessary to be transferred into the cell nucleus. In dividing cells the nuclear membrane is degraded during the cell division and the nucleus is accessible for the transfected DNA. In non dividing cells, like quiescent B cells, the electroporation parameters have to be hard enough to introduce gaps into the nuclear membrane but mild enough for the cell regeneration. The mild optimized IVT-RNA electroporation parameters result in a high viability, high transfection rate and reproducibility. Together with the optimized medium and newly created cytokine mixture a strong basic foundation is build for the isolation, manipulation and cultivation of peripheral human B cells.

As described above the strategy aimed at mimicking the *in vivo* process of B cell activation, including the presentation of antigens for clustering of BCR and heterodimerized IgG:ABCos molecules. For that it was highly important to test different strategies for the antigen presentation ex vivo. To test and validate the different strategies of antigen induced activation of B cells an anti-CD40 antibody was used for simulation. Different settings for antigen presentation are possible like soluble antibody with or without crosslinking by a secondary Fc-specific antibody or antibody coated to the dishes or to a continuously Fc-γ receptor II (CD32) expressing CHO-K1 cell line. As resting B cells hardly respond to soluble anti-CD40 antibodies, crosslinking by immobilized antibody drastically increases the response. Both immobilized forms, coated to the dish or to the CD32 expressing cell line, are highly activating. But the reduction of complexity and manageability of the cell free system were factors which led to the selection of the plate coated strategy. These findings correlate with findings of other groups (Batista et al., 2001) and it is becoming increasingly obvious that B cells recognize their antigen in a membrane bound fashion. The CD40 receptor ligand interaction takes place in a membrane associated fashion as CD40 ligand is provided as a membrane protein by T cells. Furthermore, the BCR and the CD40 receptor are activated by clustering and the spatial steric convergence which is in a coated version easier to achieve.

### 4.3.2  ABCos constructs analysis in human peripheral B cells

The converted CD40 signal from an antigen-dependent into an antigen-independent one is achieved by the association of the ABCos construct with the endogenous immunoglobulin mediated by the $C_H2$-$C_H3$ domain. Expression analysis in B cells, CHO-K1 wild type and CHO-K1-pC15 cells shows the fundamental functions and the highly interesting characteristics of the constructs. The IVT-RNA can be easily transferred into B cells by electroporation and is translated into the protein which is correctly located in the cell membrane. By different experiments the production of the constructs and their ability to heterodimerize with immunoglobulin molecules were shown, like flow cytometry, immune fluorescence microscopy and western blot experiments. The protein folding machinery in the endopasmatic reticulum is not able to discriminate between immunoglobulin and ABCos construct resulting in the associated ABCos:IgG complex. All antibody domains are able to fold autonomously (Feige et al., 2004; Feige et al., 2008), except $C_H1$ and $C_H3$ which depend on the dimerization with a light chain or a second heavy chain (Feige et al., 2009a; Isenman et al., 1979; Thies et al., 2002). The $C_H2$ domain folding is an autonomous one step process which does not require the intra chain disulfide bond for correct folding. The intra chain disulfide bond only accelerates the folding process. This means that folding of $C_H2$ can take place without assistance of chaperons like BIP (Feige et al., 2004). The $C_H3$ domain folding is an autonomous two step process. The folding process first builds an obligate homodimer, which needs for the final folding a second $C_H3$ domain (Isenman et al., 1979; Thies et al., 2002), which can be provided by a second heavy chain or an ABCos construct. The ABCos:IgG complex was detectable on the cell surface. Moreover, this tight binding is stable for more than 72 hours and detectable with high and low ABCos IVT-RNA amounts. Since the ABCos:IgG heterodimer amount on the cell surface is adjustable by the titration of the introduced IVT-RNA amount this allows the fine-tuning of the antigen-specific CD40 signal. This opens the possibility to manipulate transfected B cells by the reduction of ABCos:IgG heterodimer amount on the cell surface and thereby leading to the preferential activation of high-affinity B cells. B cells with receptors specific for a certain antigen would compete according to their affinity and a repetitive transfection with reduced amount of ABCos IVT-RNA will possibly enrich B cells with high affine BCRs.

Furthermore, immune fluorescence experiments have shown that the transfection with ABCos IVT-RNA leads to a homogenous and distinct surface staining, indicating no accumulation and clustering, which could be explain the extremely low adverse activation of transfected B cells. Unchallenged ABCos clustering of transfected B cells can lead to undesired activation due to the intracellular spatial accumulation of the

CD40 receptor. This would decrease the sensitivity and the antigen-specific isolation would be barely possible.

### 4.3.3 Validation of the function of the ABCos constructs

#### 4.3.3.1 NF-κB signaling

B cells are able to present antigens to cognate T cells, which provide the CD40 ligand. The CD40 ligand interacts with CD40 receptor which activates resting B cells. Besides, the interleukin IL4, also provided by T cells, directly binds to the corresponding receptor on B cells. Through this complex interaction B cells become activated resulting in cell division, isotype switch and differentiation into antibody secreting plasma cells or memory cells (MacLennan, 1994).

The intracellular signaling transduction of CD40 is started by the activation of different adaptor molecules like TRAF members or JAK3. In the end the phosphorylation of κB-inhibitory protein initiates its degradation and the release of the protein complex NF-κBp50/p65. One of the 5 different NF-κB proteins are known is NF-κBp50/p65 which is the most important for proinflammatory signaling (Kawai and Akira, 2007; Karin, 1999). The NF-κBp50/p65 complex enters the nucleus and activates different genes responsible for cell survival, cell division, class switch and others. To verify the ability of the constructs to induce a CD40-like signal NF-κB activation experiments were performed. For that a reporter cell line was generated and analyzed for NF-κB signaling. The pNifty plasmid was stably integrated into the host genome. The NF-κB sensitive promoter of the pNifty plasmid is derived from the endothelial cell leukocyte adhesion molecule gene (ELAM-1) containing three NF-κB binding sites. For the NF-κB specificity the full length promoter was truncated for AP1/CREB binding site (Schindler and Baichwal, 1994; Jensen and Whitehead, 2003). The ABCos constructs induce NF-κB in similar strength as the highly activating compounds PMA and Ionomycin. These data show that the crosslinking of the construct is able to induce a CD40-like signal which results in proliferation as shown in B cell activation experiments. If the conformational differences and steric alteration reduce the NF-κB signal was not part of this study. Nevertheless it could be possible that the heterodimerized ABCos construct induce weaker signal as wildtype CD40 receptors due to the spatial changes by IgG association. This would explain the weaker proliferation of ABCos stimulated B cells compared to anti-CD40 stimulated B cells.

### 4.3.3.2    *Ex vivo* germinal center reaction

B cells activated in physiological manner by BCR and CD40 in lymph nodes undergo a germinal center reaction (MacLennan, 1994). Five attributes are characteristic for germinal center reactions: massive proliferation, isotype switching of immunoglobulin, plasmablast differentiation, affinity maturation of the immunoglobulin after repetitive contact with a specifc antigen and the formation of memory B cells (Banchereau and Rousset, 1991).

In proliferation experiments it was shown that the ABCos activation induces strong cell cycling. CD40 or ABCos activated B cells show similar numbers of cell division. The high proliferation rate allows the clonal expansion of antigen-specific B cells *in vivo* and is thereby a powerful amplification tool providing a fast immune response against pathogens (MacLennan et al., 1990). *In vivo* the proliferation is accompanied with somatic hypermutation. The mutations will introduce in immunoglobulin genes which are responsible for antigen binding and thereby produce immunoglobulin molecules with no target specificity and some with high affinity resulting in an antibody titer with increased affinity over time (Kim et al., 1981). If the activation of B cells by ABCos constructs is able to induce mutation into the immunoglobulin genes was not addressed. Future experiments should address this interesting question. If this is the case, it will be possible to produce antibodies with a defined affinity. Furthermore, germinal centers are the place where the isotype switch takes place. In this process the constant part of IgD and IgM is replace by IgG, IgE or IgA depending on different physiological conditions like cytokines (Cerutti et al., 1998). To analyze class switch induction proliferation assays were conducted and B cells examined for the IgD expression. In these experiments it was shown that ABCos activation mainly occurs in IgD$^-$ B cells, which was in strong contrast to CD40 activation. It appears that the isotype specific activation is not mediated by an isotype switch from IgD$^+$ to IgD$^-$ but rather by an isotype specific activation of IgD$^-$ B cells. For this phenomenon three different explanations are possible. On the one hand it could be depend on the IgG nature of the ABCos constructs. Maybe the IgM$^+$ B cells recognize the non IgM nature of the ABCos constructs and become anergic or apoptotic. Or second, the massive proliferation of the ABCos stimulated B cells is mediated by ABCos homodimer and heterodimer, which induce beside the CD40-like ABCos signal a BCR-like signal. This signal is only present in IgG$^+$ B cells because the non heterodimerization ability of ABCos constructs with IgM or IgD. The third possibility is that the main activation of IgG$^+$ B cells depends on the signal strength. Naïve IgM$^+$ B cells require a more intensive signal than IgG$^+$ memory B cells (Neron et al., 2005). It seems to be the case that the ABCos constructs induce a weaker signal as natural CD40. Perhaps, the strength of the ABCos

constructs is only strong enough to activate IgG$^+$ memory B cells and not naïve IgM$^+$ B cells. In contrast the strong activating anti-CD40 antibody is able to activate both B cell subtypes.

The final outcome of the germinal center reaction is on the one hand the effector plasma cell (Kosco et al., 1989) and on the other hand the memory B cell (Coico et al., 1983; Klaus et al., 1980). If the constructs are able to induce differentiation into memory B cell lineage was not analyzed. But the potential to differentiate B cells into plasma cells was clearly shown by the up-regulation of CD138 and the down regulation of CD20. The amount of differentiated plasma cells from all proliferated B cells was even higher by ABCos activation compared to CD40 activation. This unexpected observation is really interesting and may be explained by the formation of short lived plasma cells requiring a weaker activation signal, whereas stronger signals induced differentiation into memory B cells or long lived plasma cells. However, further experiments are required to analyze signal strength dependent activation of IgG$^+$ memory B cells. These findings correlate with data from Néron and colleagues (Fecteau et al., 2009).

Furthermore, the specific stimulation of an extreme minority of ABCos transfected B cells was shown. In this experiment ABCos transfected B cells were spiked into untransfected B cells and the proliferation was analyzed through two different cell proliferation dyes. The stimulation via CD40 or ABCos resulted in a constant activation from around 60 % or 30 % proliferating cells, respectively. These finding had a huge impact, because the antigen-specific B cells in the patient B cell pool will be a minority, too.

## 4.3.4 Induction of antigen-specific CD40 signal in peripheral B cells

It is very difficult to analyze antigen-specific B cells *ex vivo* due to their low frequencies. For the validation of the antigen-specific activation a completely novel system was developed. CD19$^+$ B cells were co-transfected with ABCos construct and a B cell receptor specific for a peptide derived from the tumor antigen Plac1. With this approach different settings were analyzed, like stimulation of single ABCos transfected, single BCR transfected or double transfected B cells with antigenic peptide. The results obtained from this study clearly show that the hypothesis of antigen-specific CD40 signaling through a novel fusion protein works. The antigenic peptide was not able to induce proliferation in BCR or ABCos single transfected B cells, meaning that the recognition of the peptide through the BCR alone is not sufficient to induce proliferation

similarly to the stimulation of single ABCos transfected B cells. Double transfection without antigenic challenge did not induce proliferation. Only double transfected B cells stimulated with the antigenic peptide proliferated due to the simultaneous antigen-specific induction of a BCR and an ABCos mediated CD40-like signals. An additional experiment to prove the concept was performed with peripheral blood from CMV infected individuals. The pp150 peptide was able to induce antigen-specific proliferation in ABCos transfected IgG$^+$ memory B cells. With this approach it was possible to induce proliferation in up to 1.2 % of IgG$^+$ memory B cells which represents about twice to threefold as much proliferation as observed in the control. These results demonstrate the fundamental functionality of ABCos constructs to induce an antigen-specific CD40-like signal.

## 4.4 Possibilities of the technology and possible modifications of the constructs

This study generates a platform technology with a virtually unlimited repertoire of receptors. By exchanging the intracellular and extracellular domains this approach opens up the avenue for a versatile platform of new synthetic antigen-specific signal inducing proteins.

There is a plethora of possibilities for the use of these constructs and modification for other applications. The ABCos constructs can be altered by modifications concerning the isotype of the immunoglobulin fusion construct, the intracellular signaling domain and the donor species, as well as the introduction method to transfer the genetic information into the cells. It is also imaginable to tailor this technology in a way to induce antigen-specific signals in other cell types, like T cells or NK cells.

The constructs used in this study are derived from the human IgG1 immunoglobulin. The study shows their ability of antigen-specific activation of peripheral B cells. As model antigen pp150 demonstrated the capability of the constructs to activate virus specific B cells. IgG is mainly secreted during the secondary immune response against a variety of pathogens, like bacteria, fungi and viruses (Janeway et al., 2005). All these different pathogens are potentially immunogenic and different antibody arise from their interaction with the immune system. By the ABCos technology B cells specific for different target structures can be identified and the antibodies become available. On the one hand this technology is useful to generate diagnostic or therapeutic antibodies or analyze pathogen-specific antibodies. On the other hand the technology provides a way to analyze the pathogen-specific B cells. Besides their potency to act against

pathogens, IgGs have shown to be important in the development of autoimmunity (Yurasov et al., 2006; Tiller et al., 2007b; Mietzner et al., 2008) and in tumor biology (Scanlan et al., 1998; Tureci et al., 2005; Chapman et al., 2007; Tureci et al., 2006; Chapman et al., 2008). In both research fields the constructs can be used for different questions. Future experiments will address if the IgG subtype are affected by the constructs. It is not clear if the ABCos:IgG1 constructs activate B cells expressing IgG1, IgG2, IgG3 and IgG4 in a similar manner or not. The answer will be important for immunological research with ABCos constructs, if observations are induced by an immunological phenomenon or by the different potency of the ABCos constructs in different IgG subtypes.

Additionally, the switch of the constructs to other isotypes could be interesting for other applications. For example, ABCos constructs derived from IgM could be used to analyze the progress of the primary immune responses in viral infections or vaccinations. Infectious diseases are still a serious problem for developing and modern countries. A better understanding of the vaccination will influence future strategies. Also the understanding of the physiological role of the IgM co-expressed receptor IgD is incomplete (Geisberger et al., 2006). Some data predict a role in microbial defense and in the B cell homeostasis (Ohta and Flajnik, 2006; Chen et al., 2009). The analysis of the naïve B cells through ABCos constructs derived from IgD would be very interesting.

Furthermore both of the other subtypes IgA and IgE are possible targets for ABCos constructs. It would be interesting to understand the role of both subtypes in mucosal immunity (Fagarasan and Honjo, 2003; Suzuki and Fagarasan, 2009). Moreover, the identification of IgE epitops in allergy and asthma could be a valuable source for diagnostic and therapeutic antibodies and the determined epitops are maybe a starting point for allergization (Gould et al., 2003).

In addition to the extracellular fusion domain also the intracellular signaling domain represent a site of modification. The CD40 receptor domain can be replaced by other domains derived from related molecules like BAFF-R (Thompson et al., 2001), TACI or BCMA (Gross et al., 2000). This could address the question of the requirement of different B cell subpopulations for specific co-stimulatory signals. By the transfection of different B cell subpopulations with ABCos constructs carrying different signal transduction domains the importance of these receptors for the homeostasis of antigen-specific cells can be addressed. Such an approach could also be helpful to analyze the different niches required for the survival of B cell subpopulations in the organism.

Future experiments will address the prospects of an *in vivo* application and the necessity to clone specific animal constructs, like murine, rabbit or other ABCos constructs or if the human IgG ABCos construct are able to associate with murine IgG. More than the production of diagnostic or therapeutic antibodies the transfer into different species could be extremely interesting to produce different antibodies for the detection of other molecules for basic research. Highly affine antibodies are useful for several research applications, like flow cytometry, western blots, immunofluorescence microscopy and others. The production of antibodies with a defined affinity from low to high for a specific target would be an interesting tool for signaling analysis. There is an ongoing debate about the impact of different signal strength on a certain target. For example, the CD40 signal strength impacts the cell fate to differentiate into plasma cell or memory cell lineage (Neron et al., 2005). Also the basic research in animal models could be addressed by this technology. For example, transgenic cells or animals specific for the induction of fluorescence by a specific construct-dependent signaling could be important for analysis of signaling and cell behavior *in vivo*. Furthermore, the constructs can be used for xenotransplantations of hematopoietic cells. The transfer of human B cells into an animal is impaired by the lack of secondary signals (Shultz et al., 2007). In the described system for example, a CD40 signal could induced by the antigen without the help of the animal specific T helper cell. This could be one solution for some ongoing problems encountered in humanized mice.

As described, the introduction of genetic material by electroporation of IVT-RNA is a transient modification. Besides the transient introduction it could be very interesting to stably integrate this information. This could be achieved by the viral transduction of B cells (Frecha et al., 2010). The infection of B cells and the stable genetic integration into the host would induce a permanent expression of the ABCos construct and thereby a permanent presentation of ABCos:IgG. The subsequent reduction of provided antigen could also be used for the enrichment of highly affine antibodies.

A more future vision is the modification of T cells and NKT cells signaling. Both cell populations express similar receptors as B cells. Nevertheless, T cells including the CD1d restricted NKT cells need both chains for one antigen recognition, either α and β or γ and δ. But the receptors are connected to CD3 and zeta chains which could be modified and used as association domain (Janeway et al., 2005). In principle all described modifications for B cells are transferable to T cells. These possibilities make the ABCos construct to an extremely diverse and versatile platform technology with broad spectrum of applications.

## 4.5   Future impact and outlook

Taken together, the novel technology provides an artificial, antigen-specific signal inducing system for B cells which represents the basis for a multifunctional platform for a wide range of applications ranging from the analysis of B cell immunology to the development of therapeutic antibodies. Antibody therapy has demonstrated their potential in the clinic for a variety of diseases ranging from autoimmunity, infections to cancer. It will be a major goal for the next generation of therapeutic antibodies to obtain fully human antibodies with a desired specificity and defined affinity against a large group of targets (Oldham and Dillman, 2008). To achieve this, the presented study provides a platform technology, which not only competes to hybridoma technology, phage display and transgenic mice but also adds to synthetic signaling molecules for different aspects ranging from basic signaling research, homeostasis to retargeting of effectors cells. The pros of the ABCos constructs against phage display and transgenic mice are described above. But further experiments are needed to expand the potential of the constructs. A main part will be the improvement of the technology. The activation potency of the constructs is dependent on several factors and has to be increased for future optimization. For example, genetic modifications in the association domain which will prohibit the homodimerization are planned. Furthermore, protocol optimization will improve the technology. Therefore the cytokine mixture will be tested for high activation potential specific for certain B cell subpopulations, like $IgG^+$ memory B cells, $IgM^+$ memory B cells or naive B cells. Also the modifications of the antigen presentation are planned. Besides the peptide presentation, the presentation of proteins or cell lysates is imaginable. Additionally, the supplementation with other stimulatory molecules could be useful to improve the protocol. Further experiments to test whether pre-stimulation with polyreactive stimuli concomitant with antigen-specific BCR and ABCos stimulation to isolate and expand antigen-specific B cells are required.
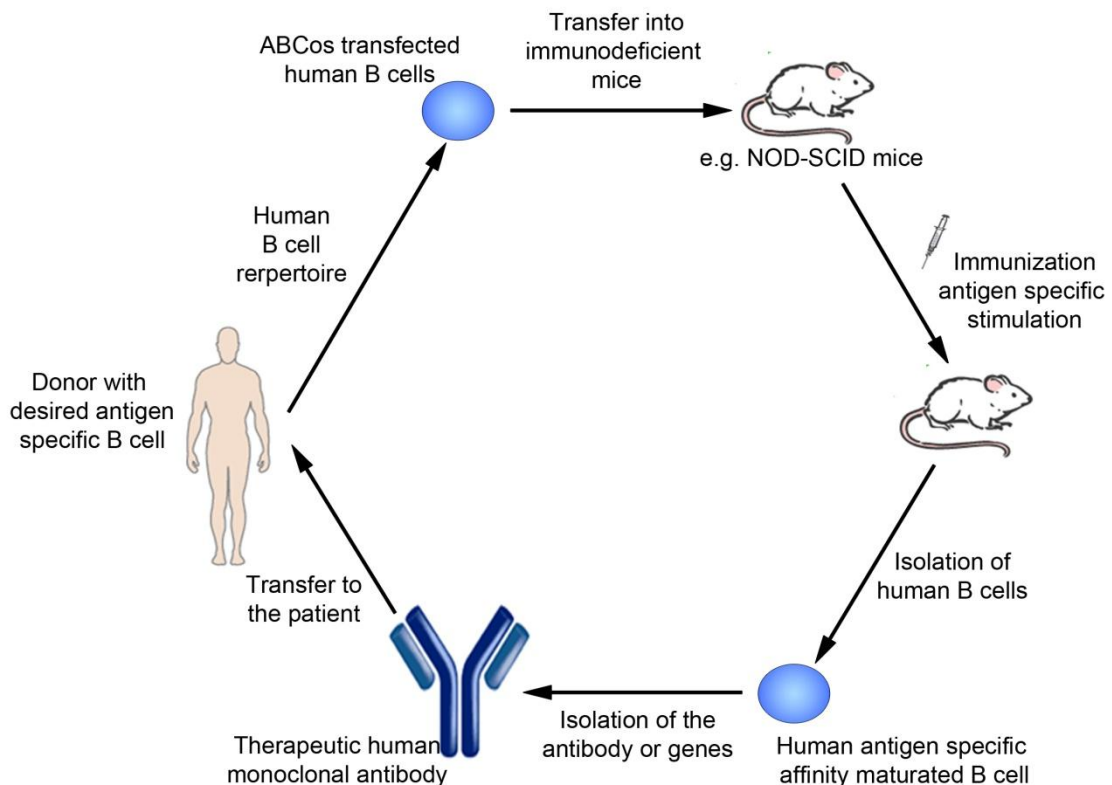
**Figure 34:** *Novel system for the generation of fully human antibodies.*
**Complete B cell repertoire is obtained from desired antigen sero-positive cancer patients and either transfected with ABCos IVT-RNA or infected with ABCos specific virus. B cells encounter their antigen either *in vivo* injected into a mouse or *in vitro* in culture system. Either the reduction of ABCos on the cell surface by the reduction of introduced RNA or the reduction of antigen amount by constant ABCos amount on the cell surface will lead to amplification of highly affine antibodies. Mature B cells expressing high affine fully human antibodies will be used either directly for adoptive B cell transfer or as a source for the corresponding antibody.**

The next milestone will be the isolation and testing of antigen-specific B cells. As shown in figure 34 the overall goal is the therapeutic application of fully human antibodies. For that the complete B cell repertoire of antigen sero-positive cancer patients will be isolated. By the transfection with ABCos constructs and different stimulation protocols high affinity B cells will be isolated. These B cells are a source for, either antigen-specific B cells for adoptive B cell transfer or fully human antibodies for therapeutic applications. At the moment it is not clear if the construct are able to induce affinity maturation through somatic hypermutation or if the reduction of antigen or ABCos RNA is able to select highly affine B cell clones. However, the resulting antibodies are highly attractive for a variety of applications as described above.

In summary, we have developed an artificial, antigen-specific signal-inducing system for B cells which represents the basis for a multifunctional platform for a wide range of applications ranging from the analysis of B cell biology to the development of therapeutic antibodies.

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# 6    List of figures

# 7 Curriculum vitae

# 8    Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbstständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Ugur Sahin an der III. Medizinischen Klinik und Poliklinik der Universitätsmedizin der Johannes Gutenberg-Universität Mainz betreut worden.

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Mainz, im Juni 2011                                                     Torsten Seppmann

Teilaspekte der vorliegenden Arbeit wurden bereits veröffentlicht:

**Publication**

Seppmann T. et al., 2011: Identification of antigen-specific B lymphocytes by induction of a synthetic antigen-dependent CD40 signal.
in preparation (manuscript attached)

**Patents**

U. Sahin, Ö. Türeci, T. Seppmann, H. Hoff, J. Schumacher (2009): Clonal Expansion of B-Cells: Europäische Patentanmeldung EP 2 316 920 und Internationale Patentanmeldung WO 2011/050985

**Oral presentations and Posters**

**Poster (1st Place: Poster Award)**
9th CIMT Annual Meeting,25/5 – 27/5.2011,Mainz, Germany
"Antigen-specific expansion of human B cells mediated by an artificial CD40 signal"

**Oral Presentation**
Sixth Annual Reatreat of the Research training Group "International Graduate School of Immunotherapy" Johannes Gutenberg University of Mainz, 29/10-30/10.2010, Königstein, Germany
"Antigen-specific induction of the CD40 pathway in IgG+ memory B cells"

**Poster**
5th ENII EFIS/EJI Immunology Summer School 2010, European Network of Immunology Institutes, Capo Caccia, Sardinia, Italy
"Functional characterisation and validation of several monoclonal antibodies against the tumor specific antigen Claudin 18.2"

**Poster**
3rd Internatinal Symposium Crossroads in Biology, 04/02-05/02.2010, Köln, Germany
"Activation and expansion of tumorantigen-specific B lymphocytes by antibody induced CD40 signaling"

**Oral Presentation**
Fifth Annual Retreat of the Research training Group "International Graduate School of Immunotherapy" Johannes Gutenberg University of Mainz, 30/10-31/10.2009, Seeheim, Germany
"Stimulation of primary human B cells with antigen-specific CD40 signal"

**Oral presentation**
Fourth Annual Retreat of the Research training Group "Antigen-specific Immunotherapy" Johannes Gutenberg University of Mainz, 14/11 – 15/11.2008, Koblenz, Germany
"In-vitro Cultivation and CD40-Signal Mediated Expansion of Peripheral Blood Isolated B Lymphocytes"

**Poster**
DFG-Evaluation of the Research training Group "Antigen-specific Immunotherapy" Johannes Gutenberg University of Mainz, 24/09.2008, Mainz, Germany
"Characterization of human B-lymphocytes producing autoantibodies against cancer/germline antigens"

**Oral presentation**
Third Annual Retreat of the Research training Group "Antigen-specific Immunotherapy" Johannes Gutenberg University of Mainz, 30/11 – 01/12.2007, Koblenz, Germany
"Characterization of human B-Lymphocytes producing autoantibodies against cancer/germline antigens"

# 9 Acknowledgment