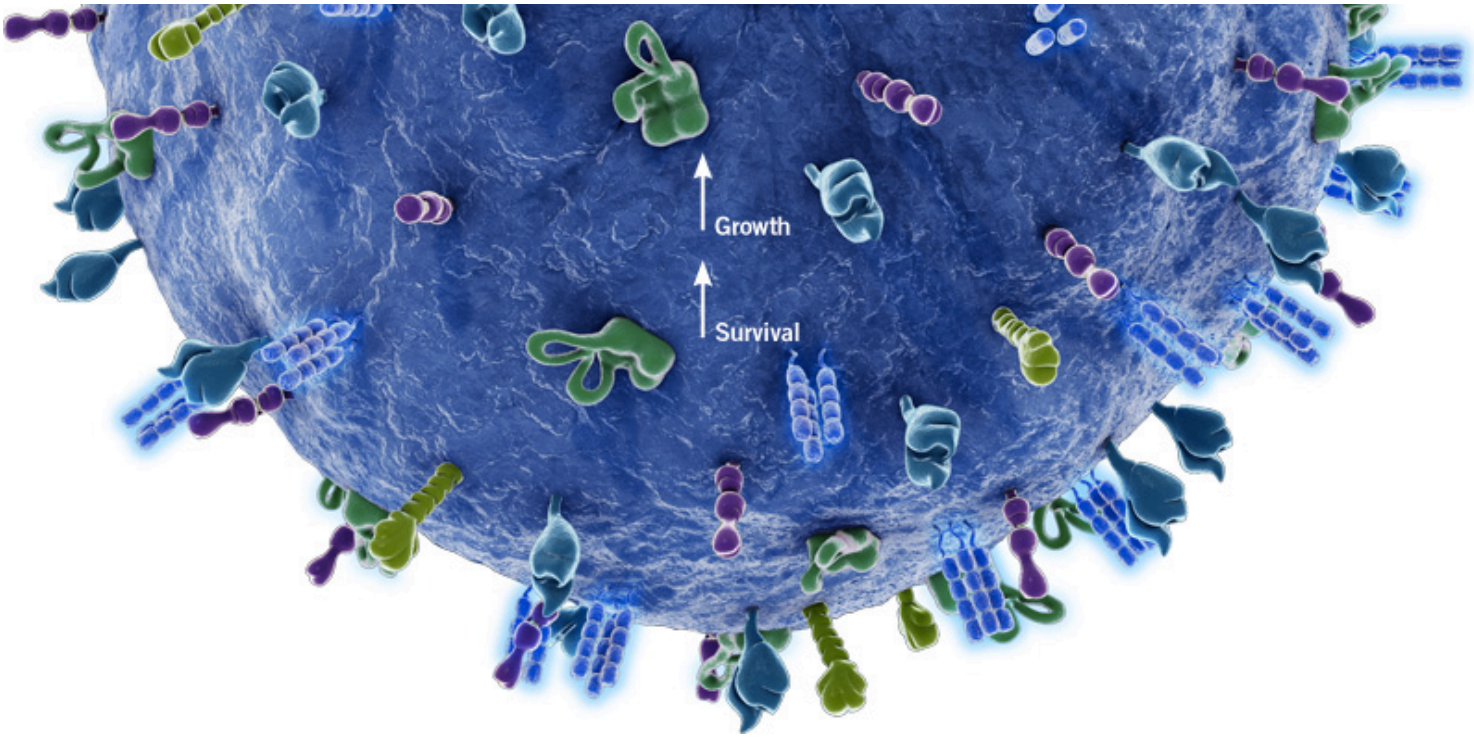

Evaluation of vaccine efficacy *in vitro* using B cells: A valid approach?

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Abstract. Influenza virus is the cause of annual "flu" epidemics and occasional pandemics such as the H1N1 pandemic in 2009. Treatment of influenza is difficult, which is why vaccination offers the best form of protection. Vaccines confer immunity mainly by the induction of a humoral response. This humoral response is facilitated by B cells, which proliferate and differentiate to memory B cells and antibody-secreting cells upon antigen exposure. Antigen-specific antibodies are able to protect the host from an infection by binding to surface proteins on the virus. Since influenza viruses alter their surface proteins frequently, there is a need for novel vaccines that protect from diverging influenza strains. Vaccine candidate and adjuvant testing is currently performed in animal models such as mice. However, animal experiments are laborious, expensive and translation to humans has always been a struggle. Here, the validity of an approach of testing vaccine efficacy in an *in vitro* B cell model will be discussed. To this end, the current status of research of *in vitro* B cell activation and possible readouts will be reviewed and related to the B cell response following infection and vaccination in humans.

List of abbreviations

Abbreviation	Meaning
AID	Activation-induced cytidine deaminase
APC	Antigen-presenting cell
ASC	Antibody-secreting cell
BAFF	B cell activating factor
BCR	B cell receptor
CSR	Class switch recombination
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immuno spot assay
HA	Hemagglutinin
HAI	Hemagglutinin inhibition
Ig	Immunoglobulin
IIV	Inactivated influenza vaccine
IL	Interleukin
LAIV	Live attenuated influenza vaccine
NA	Neuraminidase
PB	Plasma blast
PC	Plasma cell
RT-qPCR	Reverse-transcription real-time quantitative polymerase chain reaction
SHM	Somatic hypermutation
TCR	T cell receptor
TD	T cell dependent
TI	T cell independent
TIV	Trivalent inactivated vaccine
TLR	Toll-like receptor
TNF	Tumour necrosis factor

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1 Introduction

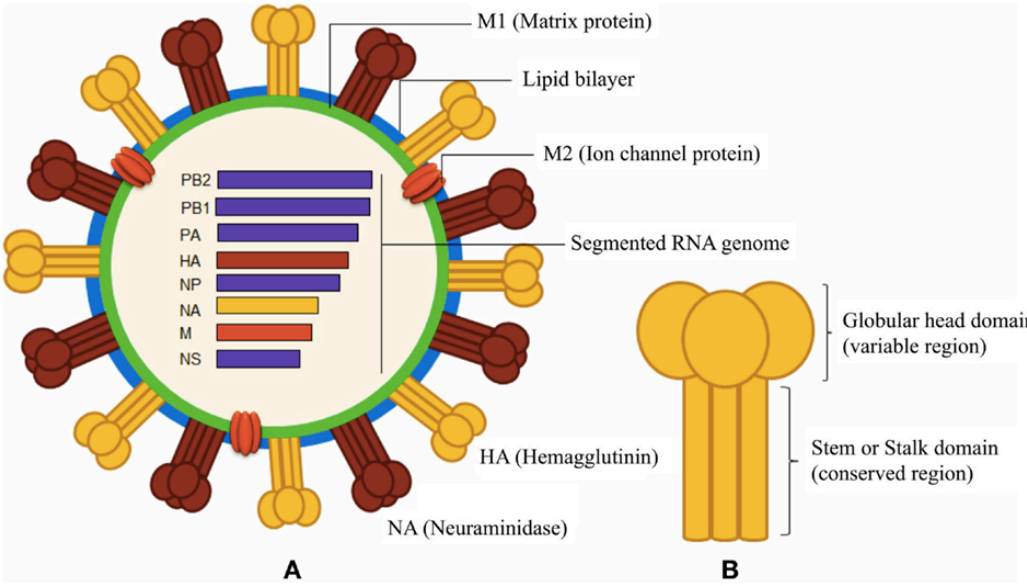
Influenza virus causes severe respiratory disease in humans and is the cause of the annual "flu" epidemics.[1] Different strains of the influenza virus exist, which are defined by differences in their surface proteins hemagglutinin (HA) and neuraminidase (NA) (Fig. 1).[2] The virus possesses a negative-strand RNA genome, which is separated into eight segments (Fig. 1). Replication of the RNA genome is prone to errors, which causes rapid antigenic drift leading to minor alterations in the surface proteins.[2] Moreover, the genome segments of two distinct influenza strains can recombine to form a novel strain. This process is called reassortment and leads to antigenic shift, which is the cause for occasional pandemics such as the H1N1 pandemic (Swine flu) in 2009.[3] The treatment of influenza infections is difficult, which is why vaccinations provide the most reliable form of protection.[4]

Influenza vaccines consist of virus-derived antigens that stimulate the adaptive immune system. Inactivated influenza vaccines (IIV) and live attenuated influenza vaccines (LAIV) are most commonly used (Box 1).[5] These vaccines mainly target the humoral immune response, causing production of antibodies specific for HA or NA. Antigen-specific antibodies are able to bind the viral surface proteins, thereby inhibiting entry of the virus into host cells, or facilitating the clearance of virus-infected cells.[6]

The humoral response is mainly driven by B cells, which are able to differentiate into antibody-secreting cells (ASCs) upon antigen exposure.[7] After development in the bone marrow, naïve B cells can be found circulating in blood or associated with lymphoid tissues, such as lymph node or spleen. When naïve B cells encounter an antigen, they will get activated in presence or absence of T helper cells.[8] T-independent (TI) activation causes differentiation into plasma cells (PCs), whereas T-dependent (TD) activation leads to differentiation into memory B cells and PCs.[9] Before activation, B cells produce the non-switched antibodies IgM and IgD. During differentiation, B cells undergo class switch recombination (CSR), which leads to expression of the switched antibodies IgG, IgA or IgE.[10] These antibodies differ in function. IgG, for example, is the predominant Ig isotype found in the body, whereas IgA is the main secretory antibody and therefore important in mucosal immunity.[10,11] IgE protects from parasitic worm infections and is associated with allergic reactions.[11] Furthermore, B cells experience somatic hypermutation (SHM) during differentiation in order to develop high affinity antigen-specific antibodies.[10] Antigen-specific memory B cells are maintained in the blood and the bone marrow and can rapidly expand and differentiate into PCs after secondary antigen stimulation.[7,12]

Currently used influenza vaccines facilitate antibody-mediated protection against the variable head domain of HA or NA.[5] However, seasonal antigenic drifts and occasional antigenic shifts create a need for the development of novel vaccines that generate long lasting and broad immunity. To date, animal models such as mice are mainly used to evaluate the functionality of new vaccine candidates or adjuvants. However, animal experiments are expensive, laborious and translation from mice to humans has been proven difficult.[13] Therefore, a human model that investigates the efficacy of new vaccines is highly desirable. Naturally, the possibilities to test vaccine candidates in humans are restricted, due to ethical and safety reasons. A different approach could be the use of primary human cell cultures. Since B cells and the humoral response are the

main targets of vaccines and the main readouts for vaccine efficacy, establishing an *in vitro* method using B cells could advance vaccine development.
Albeit, such a B cell culture method for testing vaccine efficacy has not been described or established yet. Therefore, this review will discuss whether this approach could offer a valid form of vaccine testing. For that purpose, the role of B cells during natural influenza infections and vaccinations in humans will be reviewed. Moreover, the current status of research concerning *in vitro* B cell activation and readouts for activation will be addressed. Based on the findings, the capability of vaccine-mediated B cell activation *in vitro* will be discussed, regarding vaccine testing in humans and possible readouts.



Lofano *et al.* 2015

Figure 1. Structural composition of the influenza A virus. (**A)** The influenza A virus possesses a negative-sense RNA genome, which is divided into eight segments. Among other proteins, these segments encode for viral surface proteins hemagglutinin (HA) and neuraminidase (NA). **(B)** HA contains a variable head domain and a conserved stem domain.

Box 1. Inactivated influenza vaccine and Live attenuated influenza vaccine
Inactivated influenza vaccine (IIV)
Inactivated vaccines are produced by growing viruses in embryonated chicken eggs and killing them using physical or chemical processes.[14] This inactivation method renders the virus non-infectious. Usually, trivalent or quadrivalent vaccines are used. This means that the vaccine consists of two different influenza A strains (H1N1 and H3N2) and one or two different influenza B strains.[9] The use of different strains for vaccines is necessary to protect against the strain that circulates in a season.[14] IIVs are injected intramuscularly.

Live attenuated influenza vaccine (LAIV)
As the name illustrates, live attenuated vaccines consist of living viruses, which have been altered in order to reduce their virulence.[14] Just like IIVs, LAIVs usually come as trivalent or quadrivalent vaccines. In contrast to IIVs, LAIVs are administered intranasally, thereby mimicking the route of a natural influenza infection.

2 B cell response to infections and vaccinations in humans

2.1 B cell response during influenza infection

The B cell response during an influenza infection in humans can be divided into three main reactions: an innate-like response, a TI response and a TD response.
The innate-like response is mainly facilitated by secretion of natural antibodies by B1 cells (Fig. 2A).[15] B1 cells are innate immune cells that do not differentiate to become effector B cells. These innate cells secrete polyreactive IgM antibodies, which are antigen-nonspecific, but are able to recognize a variety of different microbial structures including viral surface proteins. However, the clearance of viruses by natural antibodies is not very efficient, which is why an adaptive immune response is needed to develop virus-specific antibodies.[15]
The adaptive B cell response can be triggered in a TI and TD manner.[9] The process of TI activation of naïve B cells usually starts before TD activation and yields the first antigen-specific antibodies. TI activation of B cells can be facilitated through cross-linking of the BCR e.g. by repetitive polysaccharides.[16] Such repetitive polysaccharides can be found in LPS and the influenza HA protein.[16,17] Moreover, B cells can get activated by toll-like receptor (TLR) stimulation (Fig. 2B), e.g. by single-stranded RNA or by unmethylated CpG DNA.[16] TLRs are pattern recognition receptors that detect microbial substances.[16] During TI activation, B cells can rapidly differentiate into PCs, which produce low affinity antibodies. TI activation however, does not lead to differentiation into memory B cells (Fig. 2B).[9]
TD B cell activation leads to the induction of highly antigen-specific antibodies (Fig. 2C).[9] T helper cells get activated in the lymph node by dendritic cells that carry a viral antigen. Activated T cells migrate into the B cell follicle, where they interact with B cells that are specific for the same antigen. This interaction consists of binding of the T cell receptor (TCR) to the MHC class II molecule, and CD40L to CD40 (Fig. 2C).[9] After encountering T cell help, CSR takes place in follicular naïve B cells. This allows B cells to alter their antibody isotype from IgD or IgM to IgG, IgE or IgA.[10] Furthermore, T cell help induces SHM in variable regions of immunoglobulin DNA regions, leading to the development of high affinity antibodies, specific for the viral antigens.[10]
The previously described processes that happen during an influenza infection are well established, but mainly based on animal experiments. In general, it is difficult to study the mechanisms of influenza infections in humans because of ethical and safety reasons. However, studies have made an effort to profile the cytokine and antibody responses of subjects suffering from a natural influenza infection, which can give an insight into the mechanisms of the human immune response.[18] It was shown that during the acute phase of an influenza infection, subjects developed fever and exhibited increased interferon (IFN) γ and interleukin (IL) 6 responses.[18] IFNγ primes the immune system against viral infections and specifically induces B cells to undergo class switching to IgG-secreting cells.[19] IL6 is a B cell stimulating cytokine that induces B cell proliferation and differentiation.[19] Furthermore, the amount of antigen-specific antibodies was measured.[18] About 10 days after the onset of fever, antigen-specific IgM, IgA and IgG antibodies simultaneously and rapidly increased. The antibody titres peaked 2-3 days later.[18] Within the next 40 days, antibody titres

gradually decreased, but remained elevated compared to initial values.[18] In most subjects, antigen-specific IgA titres were lower compared to IgM and IgG.
Summarized, the B cell response during influenza infections is a process that involves several steps. Innate-like B cells can offer a first line of defence by secreting natural antibodies, followed by antigen-specific low affinity antibodies during T-independent activation. Germinal centre reactions finally result in highly specific memory B cells and ASCs, which secrete high affinity antibodies responsible for clearance of the infection. In infected humans, an increase in inflammatory cytokines and antigen-specific antibodies can be observed after the onset of fever.

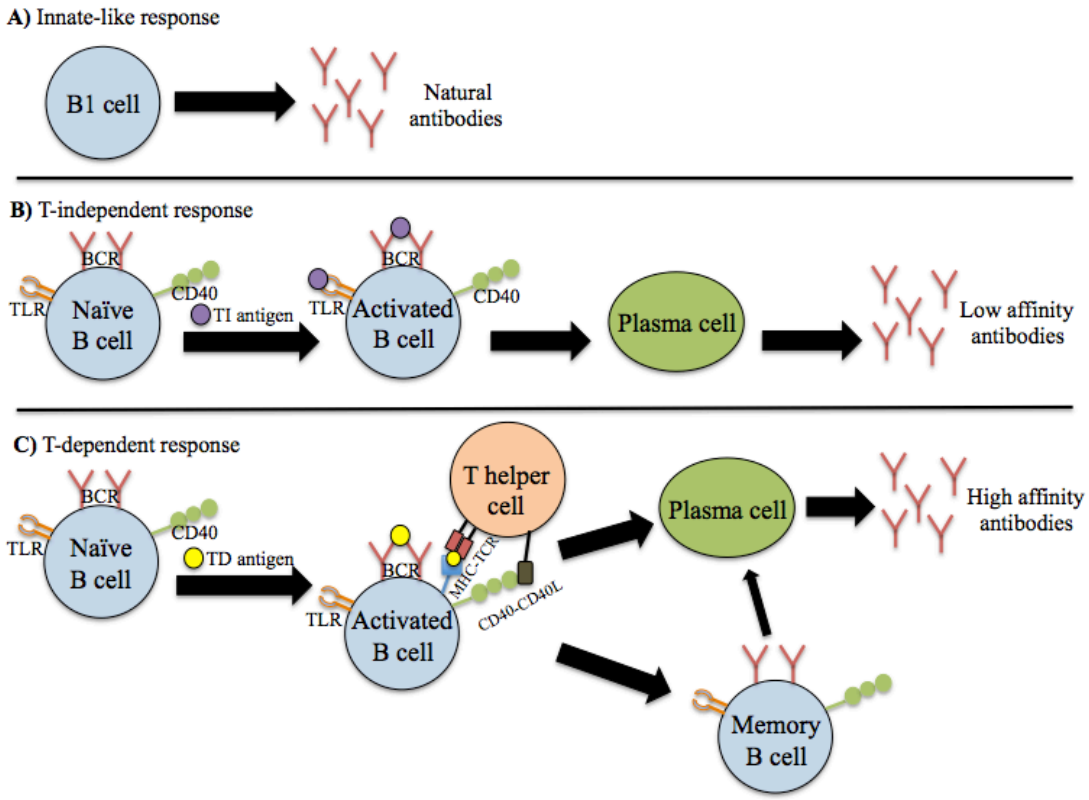


Figure 2. B cell response during influenza infection. A) B1 cells are innate-like B cells, which secrete natural antibodies. These natural antibodies serve as a first line defence against intruding viruses. **B)** The T-independent (TI) response is driven by specific TI-antigens, such as highly repetitive hemagglutinin (HA) sequences. These sequences are able to crosslink the B cell receptor (BCR) to activate B cells. Other TI signals include Toll-like receptor (TLR) agonists, which are able to activate B cells. These cells will not form memory B cells, but plasma cells (PCs) that secrete low affinity antibodies. **C)** The T-dependent (TD) response is facilitated by protein antigens, which bind the BCR. T helper cells will give an activation signal to B cells through MHC-T cell receptor (TCR) interaction and CD40-CD40L interaction. Activated B cells will differentiate to form memory B cells and PCs that secrete high affinity antibodies. TLR = Toll-like receptor, BCR = B cell receptor, TI = T-cell independent, TD = T-cell dependent, TCR = T cell receptor, MHC = Major histocompatibility complex.

2.2 Vaccination-mediated B cell response

Influenza vaccinations attempt to mimic a natural infection in humans, thereby inducing an adaptive immune response that protects the host.[2] This vaccine-mediated immune response is mainly driven by antigen-specific antibodies, which originate from B cells. Several studies investigated the humoral response following vaccination. These studies mainly aimed to profile the expansion of B cells and the production of antigen-specific antibodies. Here, the findings of *ex vivo* studies are presented and associated with the mechanism of vaccination and possible readouts to determine vaccine efficacy.

Ex vivo studies have shown that prior to vaccination, adults and children already exhibit low percentages (>1%) of influenza-specific IgG memory B cells.[20] After vaccination with LAIV or TIV, all subjects showed a significant increase in IgG ASCs and some developed an IgA ASC response. IgA is of particular interest, since it is the major mucosal antibody and therefore the first line of defence against intruding influenza viruses.[10] Furthermore, vaccination increased titres of neutralizing antibodies significantly. Neutralising antibodies are able to bind viruses directly to facilitate their clearance or inhibit entry into host cells.[10] These findings indicate that a low number of influenza-specific memory B cells exists prior to vaccination, which can expand rapidly to form ASCs.[10,20] Furthermore, antigen-specific and neutralising antibody titres appear to be an indicator to assess vaccine efficacy.

A different study investigated the clonal expansion of B cells following vaccination.[21] Here, ELISPOT assays and flow cytometry showed that antigen-specific IgG ASCs have their origin in a few antigen-specific B cell clones that are present prior to vaccination. Although antigen-specific ASCs arise rapidly after vaccination, their presence is only transient. Specific memory B cells peaked approximately one week after ASCs arise and were maintained for several weeks after vaccination, shown by antigen-specific IgG ELISA.[22] Measuring memory B cells over a time period of 17 years revealed that some memory B cells are maintained several years following an infection.[22]

Besides antibody titres and B cell differentiation, gene expression profiles were found recently to be a valid measure and predictor of vaccine efficacy. It was shown that mRNA and protein expression profiles of single B cells change upon vaccination.[23,24] The authors used microarray datasets of PBMCs derived from vaccinated subjects. For analysis, the fold change in expression was calculated using the ratio between expression at day 0 (pre-vaccination) and day 7 (post-vaccination). Generally, immunoglobulin and proliferation gene sets were enriched among PBMCs after vaccination, shown by single sample gene set enrichment analysis (ssGSEA). Moreover, up-regulation of these gene sets in B cells that were isolated after vaccination successfully predicted vaccine efficacy based on hemagglutinin inhibition (HAI) titres in the subjects.[24]

In summary, there are various factors that contribute to efficient vaccine-mediated protection from infection. Vaccines induce a rapid and transient proliferation of antigen-specific IgG ASCs, and a later and more stable increase in specific memory B cells. Furthermore, antigen-specific antibody titres can be used to assess vaccine efficacy. Interestingly, the ability of a vaccine to induce a humoral response can be predicted using the expression profiles of B cells. Therefore, this approach could be used as a predictive method to evaluate vaccine efficacy.

3 *In vitro* B cell activation

3.1 Naïve B cell activation

To evaluate whether an *in vitro* B cell model can be used as method to test vaccine efficacy, the current status of research on B cell activation was investigated. Although the role of B cells in infection and vaccination in humans is largely understood, only a small number of studies has examined the activation of B cells *in vitro*.

Lanzavecchia and colleagues published several studies that shed light on the mechanisms of naïve B cell activation in culture.[25-27] In the late 20th century, the authors established a first model of *in vitro* naïve B cell activation. According to this study, a CD40L protein that mimics the T cell signal for B cell activation induced moderate naïve B cell proliferation.[26] This proliferation was largely increased by BCR engagement using anti-Ig antibodies. Based on these results, a two-signal model of naïve B cell activation was put forward, consisting of CD40L and BCR stimulation.

Several years later, the authors found that upon BCR activation naïve B cells up-regulated the expression of TLR9 and TLR10, which was measured using RT-qPCR.[25] Based on their findings, Lanzavecchia and colleagues investigated the role of TLR signalling in naïve B cell activation.[27] Proliferation assays using flow cytometry suggested that BCR stimulation and co-stimulation by either T cells directly or by using CD40L mimics gives a signal for initial proliferation, but not for survival and differentiation.[27] After BCR stimulation, TLR agonists and T cell cognate help are needed in order to drive extensive survival and proliferation of naïve B cells. Thus, BCR stimulation seems to be a requirement for TLR expression and stimulation in naïve B cells. Naïve B cells respond to stimulation of TLRs 1,2,6,7 and 9, which is in line with the TLR expression by human B cells. TLR3/4 agonists however, fail to induce B cell proliferation.[27] Nevertheless, B cells can also be stimulated indirectly by using supernatants of DC cultures. Here, supernatants of TLR3/4 stimulated DCs (poly:IC and LPS) induced B cell proliferation.[27] The addition of neutralizing antibodies against IL6 and IL12 abrogated the stimulatory effect of DC supernatants. Furthermore, the addition of IL6 and IL12 increased B cell proliferation, indicating that cytokines secreted e.g. by DCs can simulate B cells.[27]

In contrast to the previous findings, other studies suggest that naïve B cells can be activated *in vitro* in the absence of BCR stimulation.[28,29] The results of these studies indicate that naïve B cells exhibit a constitutive TLR9 expression, independent of BCR stimulation.[28] Hence, the authors were able to activate naïve B cells in the presence of CD40L and the TLR9 agonist CpG alone.[28,29] ELISA measurements showed that IL6, IL10 and tumour necrosis factor (TNF) α secretion was enhanced in naïve B cells stimulated with CD40L and CpG. Additionally, stimulation of naïve B cells with the TLR7 agonist resiquimod led to weak proliferation and strong induction of IgM and IgG secretion, measured by ELISA.[30] This suggests that TLR7 is constitutively expressed as well and that TLR7 stimulation is also sufficient for B cell activation.

The opposing findings can be explained by a different discrimination of naïve B cell populations and by the use of different readouts for B cell activation. Lanzavecchia *et al*. isolated CD19+ B cells and used CD27, IgG and IgA for a double FACS sorting, which yielded an extremely high purity of naïve B cells of

>99.99%.[27] In contrast, other studies used a negative selection procedure employing magnetic beads and thus worked with a naïve B cell purity of >98%. This means that the naïve B cell population could have been contaminated with other cells such as memory B cells. A contamination with memory B cells would explain the findings of constitutive TLR expression in naïve B cells. Furthermore, Lanzavecchia and collegues employed proliferation assays rather than measuring cytokine or antibody secretion to determine naïve B cell activation.
Although naïve B cell activation *in vitro* has not been studied extensively, several publications indicate that TLR signalling plays a crucial role in proliferation, differentiation and survival of naïve B cells. In summary, upon BCR stimulation naïve B cells up-regulate TLRs. Antigen-specific B cells then need to get further signals from T cells (CD40L) and TLR agonists, which leads to survival, proliferation and differentiation (Box 2) (Fig. 3). In absence of BCR stimulation, TLR9 agonists and CD40L treatment results in production of cytokines, due to a constitutive TLR9 expression in naïve B cells (Fig. 3). Moreover, TLR7 stimulation increases antibody secretion by naïve B cells (Fig. 3).

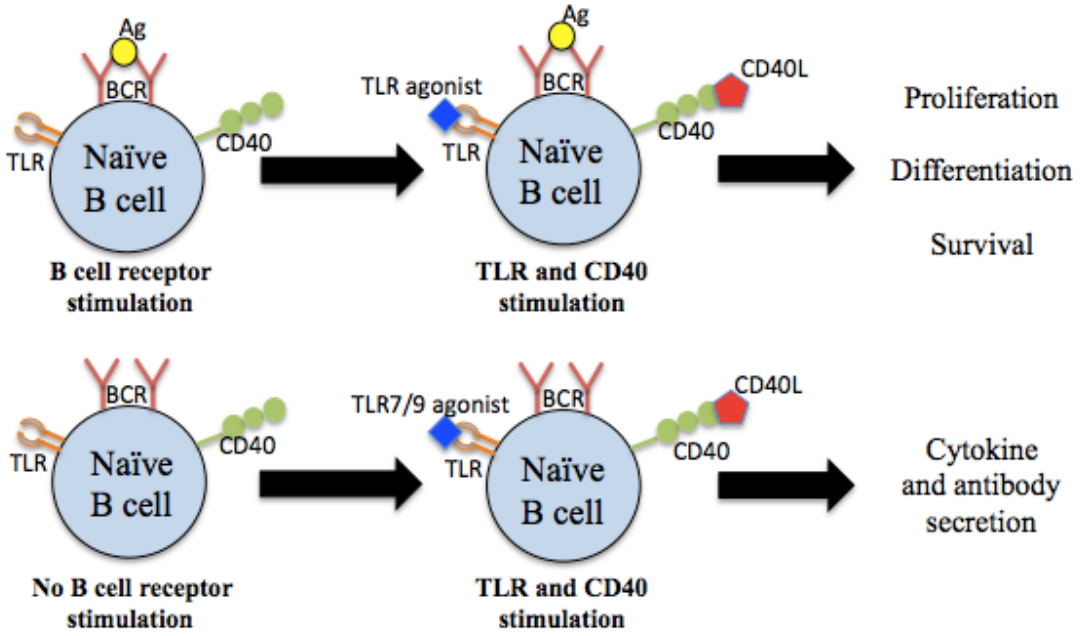


Figure 3. Naïve B cell activation *in vitro*. In presence of BCR simulation, TLR agonist and CD40L treatment leads to proliferation, differentiation and survival of naïve B cells. In absence of BCR stimulation, TLR7 agonist or TLR9 agonist and CD40L alone are able to induce an antibody or cytokine response, respectively. TLR = Toll-like receptor, Ag = Antigen, BCR = B cell receptor.

3.2 Memory B cells, plasma cells and plasma blasts

In contrast to *in vitro* naïve B cell activation, memory B cells have different activation requirements in culture. Memory B cells are antigen-specific B cells, which can expand rapidly in the presence of an antigen. It is also worth mentioning that most studies focused on using the TLR9 agonist CpG DNA to study *in vitro* B cell activation. Therefore, mainly CpG-mediated B cell activation will be discussed in the following chapters.
Just like naïve B cells, memory B cells respond to CpG initially by secreting IL6.[28] In contrast to naïve B cells however, RT-qPCR revealed that memory B cells

express TLRs constitutively, which is therefore independent of BCR stimulation.[25,28] Thus, memory B cells started proliferating in the presence of CpG only, without addition of anti-Ig, which was determined by proliferation assays using flow cytometry.[25]
Further studies tried to elucidate the role of TLR signalling in memory B cells. In a study from 2011, the differentiation potential of memory B cells in response to CpG and CD40L was investigated.[31,32] Culturing memory B cells in the presence of CpG gave a strong activation signal, leading to differentiation into PCs, which was shown by flow cytometry and ELISPOT.[31] Moreover, CpG stimulated IgM memory B cells to proliferate in the absence of other activation signals, which was assessed using flow cytometry.[31,32] Flow cytometry also revealed that in a memory B cell culture, CpG induced differentiation into PCs and plasmablasts (PBs) more efficiently than CD40L.[32] In addition, flow cytometry and expression profiles indicated that CD40L treatment yielded classical circulating CD20- PCs and PBs, whereas CpG promoted differentiation to resident CD20+ PCs and PBs.[32] Jung *et al.* focused on the effect of CpG DNA on different types of human B cells *in vitro.*[33] They also observed that CpG (in presence of CD40L) is able to promote memory B cell differentiation into PCs. However, they also studied the effect of CpG on PCs in culture and found that PCs proliferate in response to CpG, IL2 and IL10.
In summary, the activation requirements for memory B cells are less stringent compared to those for naïve B cells, since memory B cells react strongly to CpG by proliferating and differentiating (Box 2). This could be explained by the constitutive expression of most TLRs in memory B cells. Interestingly, CD40L-mediated memory B cell activation promotes differentiation into circulating PCs and PBs, whereas CpG generates resident PCs and PBs. Therefore, studying both circulating and resident PCs and PBs is possible using memory B cell cultures.

3.3 Antibody response

Previously, it was shown that memory B cells can be activated *in vitro* using CpG and/or CD40L, which leads to proliferation and differentiation into PCs and PBs. The main task of activated and differentiated memory B cells is the secretion of antibodies. Therefore, it is essential to study and profile the *in vitro* antibody response, which could serve as an indicator for B cell activity.
Using an ELISPOT assay, Bernasconi *et al.* established that memory B cells respond to CpG by secreting IgM, IgG and IgA.[31] In contrast, naïve B cells seem to require BCR stimulation in addition to CpG for secretion of IgM and IgG. However, Glaum *et al.* found that naïve B cells respond to a TLR7 agonist (resiquimod) by secretion of IgM and IgG.[30] These opposing findings could be explained by diverging expression of different TLRs in naïve B cells, or by contamination with memory B cells as described previously. In general, memory B cells seem to have a higher antibody secreting potential compared to naïve B cells.
Since memory B cells seem to respond differently to CD40L and CpG as explained before, another study examined whether this difference also applies to antibody secretion.[32] Here, ELISA revealed that memory B cells secrete significantly more IgG, IgA and IgM in response to CpG compared to CD40L. They also investigated

the antibody secretion of PBs that where generated from CD40L-treated memory B cells and were therefore classical circulating CD20- PBs. Culturing those PBs in the presence of CpG significantly increased the secretion of IgM, induced a minor IgA increase, and a decrease in IgG secretion.[32] Thus, CpG seems to induce PB differentiation into IgM secreting PCs rather than IgA or IgG secreting PCs.[32] Furthermore, these studies indicate that memory B cell activation and differentiation can easily be assessed using ELISA to measure antibody secretion.
A different study investigated the effect of CpG on transitional B cells.[34] Transitional B cells are similar to naïve B cells, yet they feature a more immature phenotype. Upon CpG stimulation, transitional B cells, which express IgM and IgD, are able to differentiate into plasma cells that secrete IgM and IgG. These findings are valuable because IgM and IgD are secreted by non-switched B cells, but in order to produce IgG, IgA or IgE, a B cell has to undergo CSR. Thus, in the absence of T cell help, B cells seem to be able to induce CSR *in vitro*. Therefore, the following chapter will elucidate the cellular mechanisms and signalling cascades involved in Ig class switching in B cells *in vitro.*

3.4 Class switch recombination and somatic hypermutation

In humans, naïve B cells that encounter an antigen will undergo CSR in germinal centres to achieve isotype switching from IgM or IgD to IgG, IgA and IgE.[10] This process is usually performed in the presence of T-cell help, however TI activation of B cells can also lead to CSR.[10] Furthermore, these differentiating B cells will generate SHM in order to produce high affinity antigen-specific antibodies. SHM describes the process of generating mutations in the Ig variable regions on the DNA, which increases antigen-specificity and affinity.[10] Both SHM and CSR are facilitated by activation-induced cytidine deaminase (AID), which causes a DNA nick. This nick is either repaired by an error-prone polymerase or leads to a double-strand break (Fig. 4).[35] Error-prone repair causes SHM, whereas a double-strand break leads to CSR (Fig. 4).[35]
As described previously, CpG seems to drive class switching of B cells in order to generate IgG-secreting PCs.[34] Therefore, class switch recombination can probably be achieved *in vitro* in the absence of T cell help. The same study measured mRNA levels of AID using RT-qPCR, which is an enzyme essential for CSR (Fig. 4). They found that transitional B cells up-regulate AID upon CpG stimulation, which could explain the induction of IgG-secreting PCs. The results of a different study support these findings.[36] Here, the authors additionally showed that B cell activating factor (BAFF) substantially increases transitional B cell proliferation, differentiation and CSR.
However, not only transitional B cells have been studied for the presence of CSR and AID expression, but also activated naïve B cells. Lanzavecchia *et al.* found that when naïve B cells are treated with a combination of anti-Ig, CD40L and CpG to induce differentiation, they start to express AID four days after stimulation.[27] In this study, the authors also revealed that TLR stimulation is essential for AID expression in naïve B cells and also for successful isotype switching. Naïve B cells treated with all three signals were stained for surface IgG and IgA, and subsequent flow cytometry showed that activated naïve B cells express IgG and IgA *in vitro*.

Interestingly, none of the studies mentioned above was able to detect IgE in *in vitro* class switched B cells, although class switching to IgE is also dependent on AID expression. The answer to this finding is provided by a study from 2003.[37] The results of this publication show that CpG inhibits IgG1 and IgE class switching at least in mouse-derived purified B cells.

A recent study specifically addressed AID-mediated CSR in naïve B cells in a TI model with special focus on signalling pathways that are used.[16] The authors found that BCR signalling synergizes with TLR signalling to induce AID expression. In combination, BCR-TLR signalling activates the non-canonical and canonical NF-κB pathways in a TI manner, which leads to AID expression and consequently CSR. In this model, TLR1/2, TLR7 or TLR9 signalling successfully induced CSR. Furthermore, LPS as a TLR4 ligand induced CSR, since it is able to stimulate the BCR as well as the TLR4 receptor.[16] In the absence of BCR stimulation, TLR signalling alone was able to induce CSR inefficiently, which was shown by measuring surface IgG. Therefore, the results underline the synergy of BCR and TLR signalling.

Besides for CSR, AID is also responsible for SHM in B cells. However, SHM in activated B cells *in vitro* has not been studied extensively yet. One study investigated the ability of CpG to induce SHM during differentiation of transitional B cells *in vitro*.[38] In response to CpG, transitional B cells proliferated and differentiated as discussed earlier. Additionally, CpG introduced SHM in the variable regions of the heavy chain of Ig. SHM was determined by sequencing the VH region and comparing it to sequences in the ”ImMunoGeneTics” information system. The authors also compared these SHM to naturally occurring SHM in human peripheral blood-derived memory B cells and found that mutation frequency and type are comparable.

Thus, it is possible to activate naïve B cells and transitional B cells *in vitro* which leads to proliferation and differentiation. Furthermore, it is interesting to see that *in vitro* activation also induces CSR and SHM in B cells, which leads to expression of most Ig isotypes and to the differentiation into switched memory B cells, PCs and PBs.

Box 2. *In vitro* B cell activation: An overview

Naïve B cell activation

Naïve B cells can be activated *in vitro* either in presence or absence of BCR stimuli. In absence of BCR activation, CD40L and TLR9 agonists will induce naïve B cells to secrete cytokines such as IL6.[28,29] After BCR stimulation, naïve B cells will up-regulate TLRs and respond strongly to CD40L and TLR agonists by proliferating and differentiating.[27] Furthermore, *in vitro* activation of naïve B cells will cause them to undergo CSR and SHM.[16]

Memory B cell activation

In contrast to naïve B cells, memory B cells have less stringent activation requirements. Memory B cells will proliferate and differentiate to PCs or PBs in the presence of either CD40L or TLR agonists, or a combination of both.[25] Memory B cells that undergo CSR during differentiation are able to secrete IgG and IgA.[31] Interestingly, treatment with CD40L or TLR agonists yields different types of PCs and PBs: classical circulating CD20- or resident CD20+ PCs and PBs, respectively.[32]

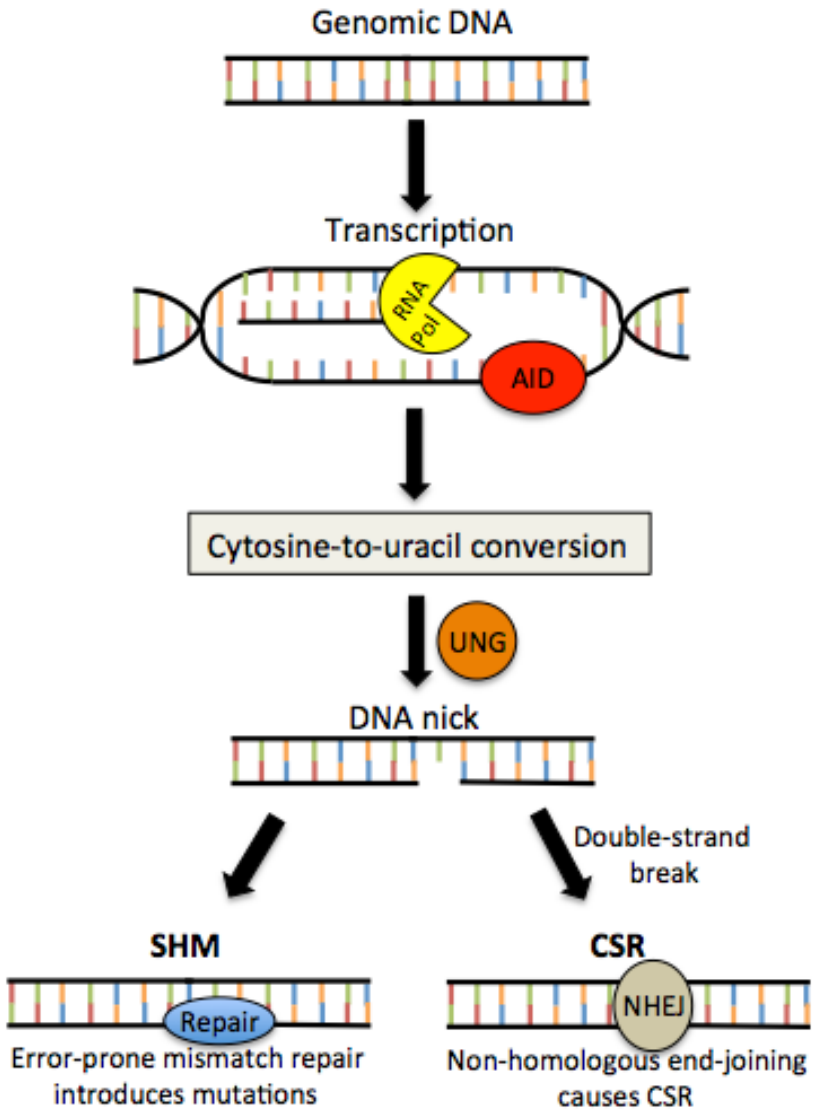


Figure 4. Mechanisms of class switch recombination and somatic hypermutation. During transcription, activation-induced cytidine deaminase (AID) converts cytosine to uracil on single-stranded DNA. Uracil is recognised as a wrong base and excised by Uracil N-glycosylase (UNG), leading to a single-strand nick. The nick can be repaired by error-prone mismatch repair, which results in somatic hypermutation (SHM). The nick can also cause double-strand DNA break, which is repaired through non-homologous end-joining (NHEJ), leading to class switch recombination (CSR). AID = Activation-induced cytidine deaminase, UNG = Uracil N-glycosylase, SHM = somatic hypermutation, NHEJ = non-homologous end-joining, CSR = Class switch recombination.

4 Towards evaluating vaccine efficacy using B cell cultures

To answer the question whether evaluating vaccine efficacy by using B cell cultures is a valid approach, it is essential to consider several aspects. The ability of *in vitro* B cell activation to mimic the effect of vaccination in humans must be examined. Furthermore, it is essential to discuss possible readouts and determine a valid model of a B cell culture system. It is important to note that to

date, no studies have been published that attempted establishing a B cell *in vitro* model for vaccine candidate testing.

Vaccine efficacy in humans can be assessed generally in two different ways. First, a double blind, randomized, clinical controlled trial can assess variables such as infection incidence and hospitalisation. Evaluating the disease attack rate of vaccinated compared to unvaccinated subjects consequently reflects the ability of a vaccine to confer immunity against an infection. Naturally, this approach cannot be used in B cell cultures. Second, a serological evaluation of vaccine efficacy offers a method that can be applied to *in vitro* systems as well. Serological assays include measuring antigen-specific antibody titres such as IgG, IgM and IgA directly.[3,20] Moreover, HAI assays can be used, which determine the amount of HA-specific antibodies in a functional assay.[24,39] Therefore, assessing vaccine efficacy using antibody responses offers a valid form of testing immunogenicity *in vitro*.

Recently, a different strategy of anticipating vaccine efficacy has been described.[24] The authors used two existing microarray datasets, which measured gene expression profiles of PBMCs following vaccination. Here, they found that Ig and proliferation gene sets are up-regulated in PBMCs 7 days after vaccination. Furthermore, they found a strong association of expression of these gene sets in B cells with HAI titres in vaccinated subjects. Up-regulation of Ig and proliferation gene sets in B cells was therefore predictive for high HAI titres and vaccine efficacy.[24] Thus, in addition to serological assays, expression profiles of single B cells could be used to determine vaccine efficacy, which is applicable to B cell cultures as well.

Having discussed the possible readouts and assays that could determine vaccine efficacy in B cell cultures, it is important to examine potential models of *in vitro* B cell activation. First of all, when establishing a B cell culture, primary B cells derived from human peripheral blood should be used preferentially. Obtaining primary B cells from various individuals would allow testing vaccines in a highly heterogeneous population with distinct genetic and environmental background. An optimal approach would be to immortalise these cells to form cell lines. Primary cells only divide a definite number of times after which they will go into senescence. By introducing mutations or viral genes (e.g. Epstein-Barr Virus), primary B cells can be immortalised. This means that that these cells can divide infinitely without entering senescence.[40] Of course, this would make vaccine testing easier, since the same donors could be used for each test and B cells would not have to be isolated from blood repeatedly. However, it is known that immortalisation of cells will change their genetic composition and therefore behaviour. Thus, immortalised B cell lines should be extensively evaluated for their use in vaccine testing.

Second, a suitable type of B cell has to be chosen, which could be naïve B cells and/or memory B cells. Using naïve B cells would be advantageous in terms of antigen-specific activation, since BCR stimulation is required for activation. Therefore, naïve B cells could be stimulated *in vitro* using an antigen (or vaccine) and would proliferate and differentiate following CD40L and TLR agonist treatment.[27] However, studies have only used anti-Ig to stimulate the BCR so far and not antigens, which is why antigen-specific activation of naïve B cells would have to be investigated beforehand. Due to the ability of B cells to undergo CSR and SHM *in vitro*, it is very likely that upon antigen stimulation naïve B cells will

differentiate into antigen-specific memory B cells. The presence of antigen-specific B cells could easily be determined by measuring antigen-specific antibody titres or surface antibodies using ELISA and/or flow cytometry. However, antigen-mediated naïve B cell activation would probably be much weaker compared to anti-Ig activation, since only a small fraction of antigen-specific B cells would respond.

Activation of naïve B cells could be assessed using various different approaches. For example, proliferation assays using flow cytometry and ELISA to measure secretion of IL6 or TNFα could be employed to determine naïve B cell activation.[28,29] Since naïve B cells secrete IgM and IgG following activation, ELISA could also be used to detect these antibodies as a readout.[30] Additionally, naïve B cells express TLRs only after BCR stimulation, so that up-regulation of TLRs would be an indicator for antigen-specific B cell activation. It has also been shown previously that *in vitro* activation of naïve B cells leads to CSR and SHM.[16,27] Therefore, the expression of AID could be measured using RT-qPCR as a readout for naïve B cell activation, CSR and SHM.

Another suitable approach would be the use of memory B cells. As explained earlier, upon vaccination a small fraction of pre-existing antigen-specific memory B cells proliferates and differentiates in response to the antigen in humans.[21] Isolating the whole population of memory B cells from blood of subjects and stimulating these memory B cells *in vitro* would lead to an extensive response including antigen-specific antibody secretion. Readouts for this response could be the use of proliferation assays and flow cytometry to determine the degree of differentiation into PCs and PBs. Furthermore, the amount and antigen-specificity of antibodies could be measured to examine the efficacy of vaccines. A possibility would also be to use both naïve B cells and memory B cells from one donor and access vaccine efficacy separately in both. This approach could give information about the mode of action of the vaccine. For example, if naïve B cells do not react strongly to a vaccine, whereas memory B cells do, this could be evidence for a strong TLR-activating component of a vaccine.

Interestingly, a human-derived *in vitro* model that should enable researchers to test vaccine efficacy was introduced recently.[41] VaxDesign, a biotechnology company, established a technology called Modular IMmune *In vitro* Construct (MIMIC). In this approach, DCs and T cells are used in a co-culture together with B cells. The culture system is ought to resemble a germinal centre in the lymph node. Specifically, the use for influenza vaccine testing has been examined in a case study clinical trial. In this case study, antibody titres in response to the vaccine *in vitro* almost resembled findings in humans.[41] This system illustrates that *in vitro* modelling of the human immune system is possible to some extent. It will be interesting to examine the potential use of primary B cells only, since the antibody response to a vaccine should be evaluable without the use of DCs and T cells.

Based on this discussion, the use of primary B cells to evaluate vaccine efficacy could be a valid approach. However, establishing such as model will be difficult, since many variables have to be regarded.

5 Conclusion

The initial question was whether evaluating vaccine efficacy *in vitro* using B cells could be a valid approach. On the basis of the preceding discussion, such an *in vitro* B cell system could probably be established to allow vaccine efficacy testing.

Establishing a primary B cell *in vitro* system to evaluate vaccine efficacy of candidates can have several advantages. Apparently, the use of *in vivo* models could potentially be reduced, which is beneficial as animal experiments are laborious and expensive. Moreover, translation from animals to humans has always been a struggle. Thus, many vaccine candidates (or adjuvants) that were successful in animal studies turned out to be inapplicable to humans in clinical trials. Therefore, the use of a human-based *in vitro* system might smoothen the step from animal experiments to human trials. Another advantage would be that B cells from many different subjects could be used. Consequently, vaccines could be tested in a highly heterogeneous population with distinct genetic and environmental background. Furthermore, *in vitro* systems allow rapid screening of many different vaccine candidates and adjuvants, which is difficult to address in animal models.

Of course, testing vaccine efficacy in B cell cultures would have limitations. The biggest limitation would be that *in vitro* systems never reflect *in vivo* conditions perfectly. The human immune system is highly versatile and is characterised by the interaction of a variety of immune cells. Therefore, an *in vitro* system is probably an oversimplification and thus cannot replace *in vivo* models. Additionally, it will be difficult to establish a valid *in vitro* system, especially because there is a lack of studies, which investigated an antigen-specific activation of human B cells in culture.

Albeit, an *in vitro* B cell model should not be regarded as a replacement for animal models or clinical trials. More likely, this approach has the capability to smoothen the step between animal testing and human clinical trials.

In conclusion, the potential use of B cells *in vitro* to test vaccine efficacy should be pursued, since it offers great advantages and will also shed light on the mechanisms underlying B cell activation and functionality.

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