Anti-viral humoral responses and beyond
New insights into B cell functions provided by vesicular stomatitis virus-specific B cells

Author(s):
Fink, Katja

Publication Date:
2006

Permanent Link:
https://doi.org/10.3929/ethz-a-005128660

Rights / License:
In Copyright - Non-Commercial Use Permitted
Anti-viral Humoral Responses and Beyond:
New Insights into B Cell Functions Provided by Vesicular Stomatitis Virus-specific B Cells

A dissertation submitted to the
FEDERAL INSTITUTE OF TECHNOLOGY ZURICH
for the degree of
Doctor of Natural Sciences

presented by
KATJA FINK
dipl. biochem, University of Zurich
born 6.3.1975
citizen of Mosnang SG

accepted on the recommendation of
Prof. Hans Hengartner, examiner
Prof. Rolf M. Zinkernagel, co-examiner
PD Dr. Burkhard Ludewig, co-examiner

2006
Summary ............................................................................................................................... 6
Zusammenfassung .................................................................................................................. 8
Foreword .................................................................................................................................. 10
Introduction .............................................................................................................................. 11

1. The onset of a B cell response following viral infections .................................................. 11
2. The humoral immune response following vesicular stomatitis virus (VSV) infection ............................................................................................................................. 11
   2.1 Characteristics of an anti-VSV B cell immune response ............................................... 11
   2.3 Gene-targeted VI10 mice to study VSV-specific B cell responses ................................. 12
3. Effector functions of B cells following viral infection ....................................................... 13
   3.1 Plasma blasts and plasma cells and how they are identified ....................................... 13
   3.1.1 Plasma blasts ........................................................................................................ 13
   3.1.2 Plasma cells ......................................................................................................... 15
   3.2 B cells as APCs ......................................................................................................... 16
   3.3 Memory B cells ......................................................................................................... 17
4. Routes of B cell responses ................................................................................................... 18
   4.1 Extrafollicular responses .......................................................................................... 18
   4.2 Germinal center responses ....................................................................................... 19
   4.3 What decides over extrafollicular versus follicular response? ................................... 20
5. Chemokines in B cell responses ........................................................................................ 21
6. Peripheral maturation and selection of B cells ................................................................ 22
   6.1 Maturation stages of peripheral B cells .................................................................... 22
   6.2 Selection of transitional B cells ............................................................................... 23
   6.3 Peripheral B cell subsets in VSV-tg mice .................................................................. 24
7. Aim of the thesis .................................................................................................................. 25

Early Type-I interferon-mediated signals on B cells specifically enhance antiviral humoral responses in mice ............................................................................................................. 31

Abstract .................................................................................................................................... 32
Introduction .............................................................................................................................. 32
Results ...................................................................................................................................... 34

IFN-α: a "second" signal to drive specific T cell independent B cell activation .................... 34
INF-α/β receptor expression on virus-specific B cells as a requirement for efficient activation ............................................................................................................................................. 36
Poor expansion of IFN-α/βR-deficient specific B cells, but retention of the capacity to differentiate to plasmablasts .............................................................................................................. 38
Summary

In the presented thesis the induction and regulation of B cell responses in mice following infection with vesicular stomatitis virus (VSV) was investigated. As a model antigen, VSV is interesting for two reasons: firstly, it is a live virus and thus represents a situation of physiological relevance, i.e. where the initiation of an appropriate immune response determines protection or disease; secondly, VSV induces both an early extrafollicular B cell response resulting in the production of large amounts of IgM antibodies (Abs), and a germinal center response which is required for the generation of isotype-switched antibodies and the formation of memory B cells. The VSV infection model thus enables the analysis of both aspects of the humoral response, and importantly, the analysis of factors required to trigger one or the other B cell differentiation pathway.

To study B cell responses following VSV infection, VSV-specific B cells of a gene-targeted mouse (VI10), which expresses the rearranged V_{H},D_{J_{H}},variable region of a VSV-neutralizing Ab (monoclonal Ab VI10), were analyzed. In addition, T cell receptor (TCR) transgenic mice specific for a VSV-epitope presented on major histocompatibility complex MHC class II were used to study the impact of specific T cell help on the humoral response.

This thesis describes four projects, aiming at identifying B cell intrinsic mechanisms of the antibody response following VSV infection.

In the first project, we have revealed an important direct action of the antiviral cytokine interferon-α (IFN-α) on B cells to augment activation and early differentiation of specific B cells to plasma cells. Moreover, we found that the induction of IFN-α was dependent on Toll like receptor (TLR)7 in a dose-dependent manner, and that TLR7 triggering in B cells increased generation of early VSV-neutralizing antibodies.

In the second project we found that chemokine receptor CCR7 expression on VSV-specific B cells was required to guide activated B cells from the B cell follicles to the T cell zone to enable efficient contacts with T helper cells. We found that T-B contacts augmented early plasma cell formation and Ab production. Furthermore, we showed that B cells induced T helper cell proliferation at the T-B border of the splenic white pulp and that these T-B contacts were independent of the presence of dendritic cells.

For the third project, peripheral B cell development of VI10 mice was investigated. VI10 mice have an increased population of marginal zone B cells, and these mice generate high titers of "natural" VSV-neutralizing antibodies. We revealed differences in the peripheral development of specific B cells in VI10 weanlings depending on the presence or absence of specific (VI10) maternal antibodies. From these results we propose that self-antigens are
responsible for positive selection of marginal zone B cells and that these self-antigens can be masked by maternal VI10 antibodies.

In the fourth project we investigated germinal center formation of VSV-specific B cells. In a situation with restricted specific T cell help, B cell intrinsic mechanisms appeared to control the onset of a germinal center response. Homozygous VI10 B cells exclusively differentiated into extrafollicular plasma cells following infection, whereas heterozygous VI10 B cells formed both extrafollicular and germinal center responses. When sufficient specific T cell help was available, both homo- and heterozygous B cells formed equal germinal center responses, demonstrating a dominant effect of Th cells on B cell differentiation into germinal center founder B cells.

In summary, we identified VSV-specific B cells as dominant players during an anti-viral response, influencing the response of T cells. VSV-specific B cells produced high amounts of IgM and IgG following infection, whereby isotype switch strictly required T cell help. Moreover, B cells acted as APCs and supported T cell expansion very early after infection, setting the stage for an optimal T cell dependent phase of the antibody response.
Zusammenfassung


Um die B-Zell Antwort nach VSV-Infektion studieren zu können, wurden gezielt genetisch veränderte Mäuse analysiert, welche die rearrangierte variable VHDJH-Region eines VSV-neutralisierenden Antikörpers (monoklonaler Ak VI10) produzieren. Zusätzlich wurden VSV-spezifische T-Zell-Rezeptor-transgene Mäuse eingesetzt, deren T-Helfer (Th)-Zellen ein VSV T-Zell Epitop erkennen, welches auf dem Histokompatibilitäts-Antigen MHC Klasse II präsentiert wird.

Die vorliegende Arbeit umfasst vier Projekte, deren Ziel es ist, B-Zell-spezifische Mechanismen einer Immunantwort gegen VSV genauer zu untersuchen.

Im ersten Projekt haben wir entdeckt, dass das antivirale Zytokin interferon-α (IFN-α) direkt auf B-Zellen wirkt und die Aktivierung und Differenzierung von spezifischen B-Zellen zu Plasmazellen entscheidend verbessert. Die Induktion von IFN-α war abhängig von der Virus-Dosis und der Expression des Toll like Rezeptors TLR7, wobei die TLR7-Ligation in B-Zellen die IgM-Antwort direkt verbesserte.


Im dritten Projekt wurde die B-Zell-Entwicklung in der Peripherie untersucht. VI10 Mäuse weisen eine vergrößerte Population von Marginalzonen-B-Zellen auf. Zudem produzieren
Zusammenfassung


Foreword

This thesis consists largely of three separate projects written in manuscript form, each with its own introduction, result part and discussion. A chapter about the GC response of VSV-specific B cells, containing data not complete enough for publication, follows the manuscripts. As a general introduction, I have tried to cover aspects of B cell immunology which are associated with the projects, and which may help to understand the manuscripts. It should therefore not be considered as a complete overview of B cell ontogeny and effector functions of B cells.
Introduction

1. The onset of a B cell response following viral infections

When an organism is invaded by a pathogen, specific B cells recognize the pathogen and produce antibodies (Abs) to neutralize and clear the invader. Viruses and bacteria generally induce a strong Ab immune response. One reason for this high immunogenicity is the binding of pathogens to so called pathogen recognition receptors (PRRs) on immune cells of the host (1, 2). Triggering of PRRs evokes an inflammation in the infected area and in draining lymph nodes. Toll like receptors (TLRs), which have been extensively studied during the last few years (3-6), represent a large group of PRRs. Triggering of these receptors directly on B cells can increase activation of the cell and thus act as a co-stimulatory signal (7-9). Binding of pathogens to PRRs has mostly been studied in dendritic cells (DCs) because these cells express high levels of PRRs. Ligation of PRRs on DCs induces the production of inflammatory cytokines and augments presentation of the antigen (Ag) on Major histocompatibility complexes (MHCs). Activated DCs present the Ag to T helper cells and thereby accelerate the humoral B cell response indirectly. Furthermore, activated DCs produce various cytokines supporting the B cell response. However, some viruses such as VSV can induce rapid production of antibodies independent of T cell help, and probably even independent of help by DCs, as discussed in the manuscript "Extrafollicular plasma cell formation and isotype switch after viral infection is enhanced by CCR7-dependent traffic to the T-B cell zone border".

2. The humoral immune response following vesicular stomatitis virus (VSV) infection

2.1 Characteristics of an anti-VSV B cell immune response

Vesicular stomatitis virus VSV is a negative sensed, single stranded RNA virus belonging to the family of rhabdoviridae (10). Infection of mice with VSV elicits a strong immune response. VSV triggers the production of high amounts of type I IFNs in infected cell. Type I IFNs are a first defense mechanism of the host to stop proliferation and systemic spread of the virus, which is decisive for the protection of the mice. Infection of the central nervous system (CNS) results in a lethal encephalitis. The pathology of the disease is not entirely clear, though. A cytolytic effect of the virus most likely contributes to the observed neurological disorders. The neurotoxic effect of IFN-α may indeed be worse than the actual cell loss (11, 12).
T cell independent production of high titers of VSV-neutralizing IgM starts very early and can be measured in the sera of wild-type mice between day three and four after infection (13) (Fig. 1). As early as four days after infection, isotype switched Ab-production starts (14). Germinal center (GC) are formed around day six, peaking at day twelve following infection. Some VSV-specific GCs seem to be long-lived and may contribute to the generation of memory B cells (15). Germline neutralizing antibodies are already very high affine, constituting binding avidities of up to $10^9$ M$^{-1}$, and hypermutation of VSV-neutralizing antibodies does not significantly increase the avidity of neutralizing Abs (14, 16).

To dissect virus-specific factors of the B cell response from B cell activation by the glycoprotein of VSV in a non-infectious immunization, mice can be immunized with VSV-G, which is available in the following forms (13):

- VSV-G protein expressed in vitro by recombinant baculovirus-infected insect cells forms micelles with a low degree of glycoprotein organization (17).
- VSV-G expressed by a recombinant vaccinia virus (vacc-G) is expressed in a random distribution on the membranes of host cells following productive infection (18).

The type of antibody response (TI-1, TI-2, TD) elicited by live virus and VSV-G seems to depend on the organization of the glycoprotein, but also on the antigen dose and route of infection (19, 20).

![Fig.1: Humoral immune response after VSV infection. Live virus in organs and in the serum is measurable for only a short time. The kinetics of the onset of IgM and IgG antibodies are shown.](image)

### 2.3 Gene-targeted VI10 mice to study VSV-specific B cell responses

Monoclonal Ab VI10 was generated by fusing splenocytes of balb/c mice twelve days following infection with VSV and a second infection at day nine (14). The recombined H-chain variable region of mVI10 was used to generate gene-targeted mice by replacing all endogenous H-chain J ($J_H1 - 4$) segment and one D segment (21). The replacement of the
variable H-chain region by homologous recombination conserves the ability of VSV-specific B cells to switch isotypes. Monoclonal Ab 35.61 is an anti-idiotypic Ab that recognizes the original H-L chain variable region of mVI10. In VI10 mice, where the light chain is of endogenous origin, the transgenic H chain seems to pair preferentially with the same L chain family as in mAb VI10. The variable region of the VI10 H and L chain combination is recognized by mAb 35.61 (22). In VI10 mice, the combination of tg H and endogenous L chain recognized by 35.61 represents the VSV-neutralizing population (21, 22).

With mAb 35.61, the VSV-neutralizing part of transgenic B cells in VI10 mice can thus be followed in flow cytometry and histology.

VI10 mice express high titers of pre-immune VSV-neutralizing Abs. Following intravenous infection with VSV, VI10 mice generate a more moderate antibody response than C57BL/6 mice, since a large proportion of the virus is captured by pre-immune VSV-neutralizing Abs (Fig. 2). Therefore, VI10 mice were usually infected with a high dose of virus to trigger Ab titers comparable to C57BL/6 mice values.

Fig. 2: VSV-neutralizing total (IgM and IgG) and IgG Abs measured in the sera of VI10 (open symbols) and B6 mice (filled symbols) at indicated time points following infection with 2x10^6 pfu of VSV.

3. Effector functions of B cells following viral infection

3.1 Plasma blasts and plasma cells and how they are identified

3.1.1 Plasma blasts

The most obvious function of a B cell is to produce antibodies following invasion of a pathogen to protect the host. Antibodies neutralize the pathogen directly, or they can activate the complement system. Furthermore, antibodies form complexes with antigens (immune complexes) and thereby enhance clearance of the antigen. Accumulation of immune complexes in secondary lymphoid organs may help to trigger a strong immune response
In order to become an antibody-forming cell, B cells need at least two activating signals. The first signal is the specific binding of antigen to the B cell receptor (BCR), and a second signal may be provided by T helper cells, which requires tight interaction with B cells (26, 27). Alternatively, pathogen-recognition-receptors on B cells can be ligated by viruses, bacteria or fungi, providing a strong non-specific second activation signal for B cells (5, 28, 29). Cytokines are key in further supporting differentiation and proliferation of activated B cells (30).

Thus, two or three signals are required to reach activation above a certain threshold. Once activated, the B cell eventually starts to proliferate and to differentiate. In the proliferation phase, the B cell is called B cell blast or plasma blast. Plasma blasts can already produce and secrete antibodies (31, 32). In histology, plasma blasts can be identified as enlarged cells with increased granularity, packed with intracellular antibodies. Blasting cells down-regulate B220 and surface expression of the BCR. Additionally, MHC class II, CD19, CD21 and CD22 are down-regulated upon activation of B cells (33). BCR down-regulation of specifically activated B cells can be detected best in tg models, i.e. at high frequencies of B cells with the same specificity. Figure 3 shows plasma blast differentiation, i.e. B220-downregulation of activated BCR-transgenic (tg) VI10 (34)B cells. Spleens of infected VI10 mice were removed and cells were stained with B220 and anti-idiotypic antibody (Ab) 35.61 for detection of VSV-specific B cells before (naïve) and one day after infection (Fig. 3A). Plasma blasts are larger than naïve cells and show an increased granularity, visible in flow cytometry by the increased forward- and sideward scatter (Fig. 3B).

**Fig. 3**

A) Upon infection of VI10 mice, VSV-specific B cells are activated and down-regulate B220 and surface Ig, which can be detected with Ab 35.61. B) Plasma blasts (grey) of the gated area shown in A) were back-gated in a FSC-SSC-graph to ungated splenocytes (black) to show the larger size and increased granularity of these cells compared to naïve cells. Plasma blasts are still much smaller than fully differentiated plasma cells, as shown in the right graph for comparison.
3.1.2 Plasma cells

Some blasting B cell reach a final differentiation state and do not proliferate anymore, but produce and secrete large amounts of Abs. At this stage, B cells are called plasma cells. Various changes in gene and protein expression accompany plasma cell differentiation (35). The most prominent alterations on the nuclear level are neo-expression of transcription factors XBP-1 and BLIMP-1. Blimp-1 is a transcriptional repressor which drives the terminal differentiation to plasma cells by suppressing the expression of genes required for BCR signaling, immunoglobulin class switch and somatic hypermutation. The transcription factor XBP-1 (X-box-binding protein-1) is specifically required for terminal differentiation to plasma cells. (36, 37). For ex vivo analysis of humoral immune responses, however, surface markers are more attractive. In contrast to blasting cells, different surface markers have been discussed to identify plasma cells. Best accepted in the literature may be Syndecan-1 or CD138, a proteoglycan expressed exclusively on plasma cells among hematopoietic cells. (33). Recently, Ly6C has been shown to be specifically expressed on plasma cells and to be absent on naïve B cells (38). However, this marker is also expressed on T cells, macrophages and NK cells. Multi-parameter analysis is therefore required for an unequivocal identification of plasma cells with Ly6C.

Together with surface B220 and intracellular Ig, surface CD138 is a reliable marker for the identification of plasma cells in histology and flow cytometry. B220 is useful if a differentiation from plasma blasts is required since plasma cells are B220-negative and CD138 positive, whereas plasma blasts are B220 low/negative and CD138 negative, as shown in figure 4. Spleens of V110 mice were analyzed at different time points after infection and stained with B220 and 35.61 (intracellular) (Fig.4A). One day after infection, plasma blasts were detected as B220 low cells, as indicated by the thin lined gate. These plasma blasts were CD138-negative (Fig. 4C), whereas fully differentiated plasma cells observed at day two in the thick lined gate were CD138-positive (Fig.4A and C). Fig. 4B shows that surface 35.61 is down-regulated after activation, whereas a population with high expression of intracellular 35.61 appears at day two, representing plasma cells producing soluble Ab 35.61 for secretion. The formation of early plasma blasts and plasma cells is transient and peaks at day two in V110 mice. Already at day four, most of the plasma cells have died or have left the spleen.
Activated VSV-specific B cells and their differentiation to plasma cells.

A) Plasma cells appear later than plasma blasts in the course of an anti-viral response. V110 mice were infected with $10^9$ pfu VSV and the spleens were analyzed at different time points after infection. Gates in the graphs for day 1 and day 2 following infection represent the appearance of plasma blasts (thin line gate) and plasma cells (thick line gate), respectively. Ab 35.61 detects VSV-specific B cells (anti-V110). B) Intra-cellular and extra-cellular expression of V110 was directly compared with differently labelled 35.61. C) CD138 expression of plasma blasts (thin line) and plasma cells (thick line) as gated in A).

3.2 B cells as APCs

DCs are more efficient antigen presenting cells (APCs) than B cells when analyzing their capacity to induce T cell proliferation. Nevertheless, the Ag-presenting capacity of B cells may be important in situations where DCs are not (yet) available or have not yet processed the antigen for presentation on MHC II. Following intravenous administration of the Ag, the
spleen filters big amounts of Ag mostly in the marginal zone (MZ) (39). B cells are activated in the MZ area and start to migrate towards the T cell zone. Only four to six hours following their activation in the MZ, B cells can then interact with Th cells at the T-B border (40, 41). It has also been observed that MZ B cells are better APCs than follicular B cells (42). The restricting factor in this B cell-Th cell interaction may be the frequency of specific T cells at early time points of an immune response. It is conceivable that B cells already arrive in the spleen as activated cells or that they are efficiently activated in the MZ and start then to differentiate and to present the Ag. B cells are thus not the cells to be helped for initial activation and rather represent the “active” part, inducing Th cell activation and expansion (43). This sequence of events has been established based on studies with B cells specific for haptens and bacteria in BCR-tg or knock-in models (41, 42, 44-46). It remains to be elucidated whether this model is applicable to endogenous responses as well, since precursor frequencies of B cells are much lower in this case. At least, endogenous B cells are able to present Ag only two hours after its systemic administration (47).

3.3 Memory B cells
Activated B cells clonally expand and differentiate into two principal effectors, plasma cells and memory cells. It is not entirely clear which signal(s) predispose B cells to one or the other direction. Spatial requirements, antigen-structure and the amount of antigen, stimulation via BCR and the nature of co-stimulation might influence the B cell fate. It has become clear recently that transcription factors are critically involved in plasma cell differentiation (48). In contrast to the genetic requirements for plasma cell differentiation, less is known about memory cells. They express no or low levels of Blimp-1 and do not express XBP-1. Regarding expression of surface molecules, memory cells are not easy to identify in mice. CD27, a TNF receptor family member which characterizes human memory B cells, is down-regulated on mouse memory B cells. Activated B cells acquire CD27 at the centroblast stage and lose it progressively upon further differentiation (49). CD27 on B cells can ligate CD70 on T helper cells and has been suggested as a costimulatory signal supporting plasma cell differentiation (50, 51). Somehow contradictory, ligation of CD27 on B cells has also been shown to promote GC formation (49) and memory B cell responses (52).

The lab of M. McHeyzer-Williams has made big efforts to unequivocally show the generation of memory B cell on a cellular level in mice following immunization with the hapten 4-hydroxy-3-nitrophenyl (NP) (37, 53). McHeyzer-Williams and Co-workers have mainly used the immunizing antigen to visualize isotype switched specific B cells in a sandwich stain (37).
More recently, they have suggested surface marker CD79b for identification of memory B cells. However, this stain has not (yet?) reached general acceptance in the literature. Specific memory B cells may be identified by their switched isotype. Functionally, primed mice produce higher titers of IgG antibodies following secondary infection. Moreover, the generation of IgG occurs faster. These two features are generally attributed to the presence of memory B cells in primed mice, although it is only an indirect indication.

4. Routes of B cell responses

4.1 Extrafollicular responses

Following activation by antigen, B cells can potentially enter two differentiation routes, whereby one leads to extracellular plasma cell formation, whereas the other results in the generation of GCs. The extrafollicular plasma cell response is efficiently induced upon strong B cell stimulation, e.g. with T cell independent, but also with T cell dependent Ags (reviewed by MacLennan (54)). Extrafollicular plasma cells are formed within only two or three days. Fast production of protecting Abs is indeed crucial for the defense of harmful pathogens. It therefore makes sense that many viruses, bacteria or fungi strongly trigger this extrafollicular differentiation path in B cells, either by extensive cross-linking of the BCR (25), or, more likely, by additional BCR independent signals via pattern recognition receptors such as TLRs (8). Given such strong B cell activation by the antigen, B cells may start to expand in the absence of T help, and specific T-B interactions may only be required for the formation of germinal center and memory B cells, as discussed in the next paragraph (43). Histologically, extrafollicular plasma blasts and plasma cells are located in bridging channels, thus at the interphase of B cell follicle, T cell zone and red pulp (Fig. 5). These cells produce high amounts of antibodies and die rapidly (see Fig. 4) (32, 55, 56).
4.2 Germinal center responses

In contrast to extrafollicular responses, follicular or germinal center responses aim at establishing a long-lasting, high-affine humoral response. It has been shown in transgenic mice having defects in T cell-B cell interaction or in mice lacking T cells that Th cells are not required for the onset, but for the maintenance of germinal centers (45, 57, 58). Models for GC B cell differentiation in mice have almost exclusively been established with haptens systems such as (4-hydroxy-3-nitrophenyl)acetyl (NP), p-azophenylarsonate (Ars) and phosphorylcholine (PC) (59). The experimental advantage of haptens is a clonally restricted B cell response compared to oligo- or polyclonal activators like sheep red blood cells (SRBC) or viruses. B cell clones can be tracked genetically and studies with haptens have provided invaluable information about kinetics and location of somatic hypermutation (SHM) and class switch recombination (CSR). However, it also became obvious that substantial differences between GCs induced by haptens compared to those induced by viruses exist, i.e. GCs induced by viruses seem to be long-lived whereas those induced by haptens disappear more rapidly (59). Nevertheless, markers for the identification of GC B cells are universally valid. Figure 6 shows a characterization of mouse GC B cells following immunization with SRBCs (A) or VSV (B). The lectin peanut agglutinin (PNA) cells as well as GL-7 (also called “T-and B cell activation markers”) (60) and anti-Fas (CD95) specifically bind to GC B cells, allowing their identification by histology or flow cytometry (Fig. 6).
Fig. 6
Characterization of ex vivo splenic GC B cells in the mouse.
A) is taken from the review by K.L. Wolniak et al. (59). BALB/c mice were immunized i.p. with SRBC or vehicle alone, and spleen cells harvested eight days after immunization. Panels A and C through F represent spleen cells from SRBC-immunized mice. Panel B represents spleen cells from control (BSS injected) mice. Panel A and B: Splenocytes were stained with anti-B220 and PNA. Panel C: splenocytes were stained with anti-B220, anti-CD95 and PNA. The CD95 versus PNA profile is derived from the B220+ gated population. Panel D: Splenocytes were stained with anti-B220 and anti-GL7. Panel E: Splenocytes were stained with anti-B220, anti-GL7 and PNA. The GL7 versus PNA profile is derived from the B220+ gated population.
B) C57BL/6 mice were infected with 2x10⁶ pfu VSV-IND i.v. Six days later spleen cells were stained with anti-GL7, anti-Fas and PNA to detect GC B cells, which are positive for all three markers.

4.3 What decides over extrafollicular versus follicular response?
Although extrafollicular and GC B cell responses have been extensively studied, one obscurity remained: which factors determine the induction of either extrafollicular or GC differentiation in an activated B cell? The decision can potentially be made at four levels (61):
1. During activation, whereby the activating stimulus is determined by the nature of the antigen. Signal strength may be the result of the degree of BCR-cross-linking (62) or affinity (63). 2. Through simultaneous engagement of complement receptors (64-66) or pattern recognition receptors (8) with the BCR. 3. By cytokines or other soluble mediators provided by DCs, T cells, monocytes or stromal cells available at the time point of activation or soon thereafter (67). 4. By contacts with T cells and the consequent formation of immune synapses, engaging several surface receptors on B cells (68) 5. As a consequence of the maturation stage of B cells (39, 69).
Interestingly, it seems that the availability of early specific T help rather promotes a germinal center response and, moreover, inhibits the generation of extrafollicular plasma cells (38, 70).
In our studies with VSV-specific B cells, we observed that following adoptive transfer of B cells which were heterozygous for the tg VH-region did form germinal centers, whereas homozygous donor B cells formed early plasma blasts, but did not enter a GC reaction. However, when co-transferred with Th cells, GCs were initiated both by hetero- and homozygous donor B cells (see "The germinal center response after viral infection: new insights with vesicular stomatitis virus-spefic B and T helper cells, page 94").
These data thus provided evidence that Th cells are decisive for the GC B cell fate. However, additional, yet unidentified (B cell intrinsic?) factors seem to be responsible for the different B cell responses of hetero- and homogenous B cells, since equal (limited) amounts of endogenous Th cells were available in both cases.

5. Chemokines in B cell responses

Chemokines are a large family of cytokines, which have long been recognized for their capacity to attract immune cells. The family of chemokines and the respective receptors and knowledge about their contribution to a cellular organization of immune responses is constantly increasing. More recently, it has been shown that various chemokines also play a role in B cell development, B cell homeostasis and in homing of effector B cells. The "classical" B cell chemokine receptor is CXCR5 since it is expressed on naive B cells and is required for B cell homing to follicles (71, 72). The ligand for CXCR5, CXCL13, is expressed on follicular dendritic cells (FDCs) in B cell follicles and in body cavities, guiding the correct homing of mature B cells (73, 74).

Following activation, B cells up-regulate CCR7. This chemokine receptor seems to prevail over CXCR5 and results in migration of activated B cells towards the T cell zone, where CCR7 ligands CCL19 and CCL21 are expressed (46). This relocation to the T cell zone enables interaction with Th cells, which is required for the generation and maintenance of functional germinal centers. A specialized subset of CXCR5-expressing Th cells, the follicular helper cells, has recently been shown to participate in the GC reaction (75).

Furthermore, chemokines are responsible for the homing of antibody secreting cells (ASCs). Differentiated plasma cells do not migrate towards CXCL13 and CCL19/21 anymore, causing their exit from secondary lymphoid structures into the circulation (76). Homing of circulating plasma cells has been suggested to depend on CXCR4 and CXCL12 (77). Interestingly, the same chemokines are also responsible to retain developing B cells in the bone marrow (78, 79). Table 1, taken from a review by Jason Cyster (77), nicely summarizes the current knowledge about chemokines involved in B cell development and B cell effector functions.
Table 1: Chemokines involved in B cell development and effector functions

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Chemokine(^a)</th>
<th>Chemokine distribution</th>
<th>Receptor expression/ responsive cells(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR7</td>
<td>CCL19 (ELC)</td>
<td>T zone (both), lymphatics and HEV (CCL21), DC (CCL19)</td>
<td>T cells, maturing DCs, B cells</td>
</tr>
<tr>
<td></td>
<td>CCL21 (SLC)(^c)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR9</td>
<td>CCL25 (TECK)</td>
<td>Small intestine, thymus, BM?</td>
<td>DP thymocytes, IEL, memory T, IgA ASCs, early BM B cells</td>
</tr>
<tr>
<td>CCR10</td>
<td>CCL27 (CTACK)</td>
<td>Skin (CCL27); stomach, colon, salivary gland, mammary gland (CCL28)</td>
<td>CLA+ T cells, IgA ASCs</td>
</tr>
<tr>
<td></td>
<td>CCL28(MEC)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCR3</td>
<td>CXCL9 (MIG)</td>
<td>Sites of inflammation, lymphoid tissue; BM?</td>
<td>Effector T cells, NK cells, ASCs, early BM B cells</td>
</tr>
<tr>
<td></td>
<td>CXCL10 (IP10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CXCL11 (ITAC)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCR4</td>
<td>CXCL12 (SDF1)</td>
<td>BM, RP, MCs, near HEV, epithelium, other</td>
<td>pro-B cells, ASCs, B cells, thymocytes, T cells, DCs, other</td>
</tr>
<tr>
<td>CXCR5</td>
<td>CXCL13 (BLC, BCA1)</td>
<td>Lymphoid follicles, body cavities</td>
<td>Mature B cells, B1 cells, helper T cells</td>
</tr>
</tbody>
</table>

\(^a\)Chemokines are shown by their standardized name and, in parentheses, by frequently used common names.

\(^b\)The list of receptor-expressing cell types or chemokine responsive cells is restricted to cells of the immune system and is not meant to be comprehensive. Two CCL21 genes that encode proteins differing by a single amino acid have been defined in BALB/c mice, termed CCL21-ser and CCL21-leu; in some mouse strains there is an additional copy of the CCL21-leu gene. Only a single CCL21 gene has been identified in humans.

ASC, antibody secreting cell; BM, bone marrow; CLA, cutaneous lymphocyte antigen; DC, dendritic cell; DP, double positive; HEV, high endothelial venule; IEL, intraepithelial lymphocyte; MC, medullary cord; RP, red pulp.

From J. Cyster, (77)

6. Peripheral maturation and selection of B cells

6.1 Maturation stages of peripheral B cells

B cells are generated in the bone marrow (BM) and are released into the circulation as immature B cells (80, 81). These early BM emigrants are released into the circulation and further mature to MZ or follicular B cells. Cells with a MZ phenotype are sessile and are found mostly in the spleen whereas follicular B cells circulate between secondary lymphoid organs (82). On their way to maturity in the periphery, BM emigrants pass through at least two transitional stages (83). In vivo \(^3\)H-thymidine and BrdU incorporation studies have revealed that less than 5% of the immature B cell fraction produced in the BM can be recovered in the mature pool. Moreover, it became obvious that newly formed B cells have a
high turnover rate and are short-lived, whereas mature B cells divide less often and live longer (84).

Transitional and mature B cells can be identified by multi-color flow cytometry according to surface marker expression (85, 86). However, the literature is controversial in some points. Table 2 summarizes the classifications generally used to identify peripheral B cell fractions.

Table 2: B cell subsets in the periphery
From Cancro M. et al. (84)

<table>
<thead>
<tr>
<th>Status</th>
<th>Subset</th>
<th>Production rate (cells/d $10^9$)</th>
<th>Cycling</th>
<th>Surface phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transitional</td>
<td>T1</td>
<td>1.5</td>
<td>No</td>
<td>IgM$^+$CD23$^+$B220$^+$AA4.1$^+$</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>No</td>
<td></td>
<td>IgM$^+$CD23$^+$B220$^+$AA4.1$^+$</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>No</td>
<td></td>
<td>IgM$^+$CD23$^+$B220$^+$AA4.1$^+$</td>
</tr>
<tr>
<td>Transitional</td>
<td>T1</td>
<td>ND</td>
<td>No</td>
<td>IgM$^+$CD23$^+$CD21$^+$B220$^+$IgD$^+$</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>ND</td>
<td>Yes</td>
<td>IgM$^+$CD23$^+$CD21$^+$B220$^+$IgD$^+$</td>
</tr>
<tr>
<td>Mature</td>
<td>FO (B2)</td>
<td>0.5</td>
<td>No</td>
<td>IgM$^+$CD23$^+$B220$^+$AA4.1$^+$</td>
</tr>
<tr>
<td></td>
<td>MZ</td>
<td>ND</td>
<td>No (?)</td>
<td>CD9$^+$IgM$^+$IgD$^+$CD23 CD21$^+$</td>
</tr>
<tr>
<td></td>
<td>B1</td>
<td>ND</td>
<td>Yes (?)</td>
<td>IgM$^+$CD43$^+$IgD$^+$CD23$^+$</td>
</tr>
</tbody>
</table>

6.2 Selection of transitional B cells

In vitro BCR cross linking of sorted transitional B cells does not induce proliferation as in mature B cells but triggers apoptosis instead (85, 88). This "immature behavior" of transitional B cells suggested that transitional B cells are targets of peripheral negative selection. Furthermore, studies with HEL-tg B cells in HEL-expressing hosts have demonstrated that self-reactive B cells were negatively selected at the T2 stage (89). These self-reactive cells could be rescued with increased amounts of B cell activation factor (BAFF), in line with the observation that the T2 subset is enlarged in BAFF-tg mice (89, 90). Besides an important role of BAFF, various factors of the BCR signaling cascade and transcription factors have been identified to affect B cell maturation and selection (84, 88).

There is evidence that T2 cells are not only negatively selected, but that positive selection into either the MZ or follicular pool occurs in parallel. Positive selection might be the consequence of BCR-intrinsic mechanisms and of additional stimuli such as triggering of pattern recognition receptors (91). However, this hypothesis is controversial.
6.3 Peripheral B cell subsets in VSV-tg mice

VI10 mice have enlarged MZ and T2 compartments compared to C57BL/6 mice despite normal B cell development in the BM (34). The enlarged MZ may be the result of positive selection of VI10 B cells upon cross-reaction with self-peptides, as suggested in the third manuscript "Shaping of the developing B cell repertoire by maternal antibodies in favor of maternal specificities". VSV-specific B cells as identified by mAb 35.61 resided preferentially in the T2 and MZ compartment, whereas only a minor part of specific cells showed a mature phenotype (Fig. 7)

**Fig. 7**
Phenotype of peripheral B6 and VI10 B cells. Splenocytes of six week old VI10 and B6 mice were analyzed with Abs against the indicated surface markers. M: mature, T: transitional, MZ: marginal zone.
7. **Aim of the thesis**

The humoral immune response in mice following VSV infection has been studied extensively in wild-type mice. However, these studies were mostly restricted to the measurement of antiviral antibodies in neutralization assays and with ELISA. Although VSV-specific B cells could be detected in histology by a sandwich stain involving virus particles (15), specific cells could not be analyzed ex vivo (e.g. for flow cytometry or ELISPOT) due to low frequencies of specific cells in wild-type mice and due to the lack of detecting reagents. H-chain transgenic mice were generated to be able to track VSV-specific B cells following infection and to study the impact of the heavy chain alone on the diversity of anti-viral Abs (92). However, since the transgene for the VSV-specific BCR of the IgM isotype had been inserted randomly, isotype-switched VSV-specific Abs could not be studied. In a new attempt, the VSV-neutralizing H-chain VDH-sequence of mAb VI10 (14) was inserted into the genome of C57BL/6 embryonic stem cells by homologous recombination. A first functional characterization of the VI10 mouse is described in the thesis of Bea Senn and in reference (34, 93).

The aim of this thesis was to address B cell-intrinsic aspects of the VSV-specific response in VI10 mice, involving time-point and requirements of the isotype switch of Abs. To be able to address the impact of selected B cell-specific molecules such as the IFN-α/β receptor or chemokine receptors on the anti-viral response, VI10 mice were crossed to knock-out strains, resulting in VSV-specific B cells which lacked the respective component. The impact of the deleted molecules on specific B cells could then be studied by transferring sorted VI10 B cells into wild-type mice and using anti-idiotypic mAb 35.61 or allotype-specific markers (IgHa versus IgHb) to track VSV-specific B cells.


Introduction


Early Type-I interferon-mediated signals on B cells specifically enhance anti-viral humoral responses in mice

Katja Fink¹, Karl S. Lang¹, Nataly Manjarrez-Orduno¹,², Tobias Junτ³, Beatrice M. Senn¹,⁴, Martin Holdener¹,⁵, Shizuo Akira⁶, Rolf M. Zinkernagel¹ and Hans Hengartner¹

From the ¹Institute of Experimental Immunology, University Hospital Zurich, Schmelzbergstrasse 12, 8091 Zurich, Switzerland, ²Present address: Molecular Biomedicine Dept, CINVESTAV, Ave IPN 2508, Mexico, ³CBR Institute of Biomedical Research, Harvard Medical School, 200 Longwood Ave., 02115 Boston, USA, ⁴Present address: Intercell AG, Campus Vienna Biocenter 6, A-1030 Vienna, ⁵Present address: Immune Regulation Laboratory, Department of Developmental Immunology, La Jolla Institute for Allergy and Immunology, San Diego, CA 92121, 10355 Science Center Drive, USA, ⁶Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita 565-0871, Osaka, Japan

Condensed title: Early IFN-α augments anti-viral B cell responses
Total character count: 42'561
Abbreviations used: VSV, Vesicular stomatitis virus; MZ, marginal zone; pDC, plasmacytoid dendritic cell; TLR, Toll-like receptor; IFNAR⁻, IFN-α receptor deficient

Address correspondence to: Katja Fink, Institute for Experimental Immunology, Schmelzbergstrasse 12, 8091 Zürich. Phone: +41 44 255 37 87; Fax: +41 44 255 20 44; email: katja.fink@usz.ch
Abstract

Type I interferons limit viral spread by inducing antiviral genes in infected target cells and by shaping the adaptive response through induction of additional cytokines. Vesicular stomatitis virus (VSV) efficiently triggers the production of type I interferons in mice, and it is suggested that IFN-α is induced after binding of VSV to TLR7 in infected cells. Our study with virus-specific B cell receptor-transgenic mice demonstrates here that type I interferons directly fuel early humoral immune responses in vivo. VSV-specific B cells that lacked IFN-α/β receptors were considerably impaired in plasma cell formation and in generating anti-viral IgM. At low viral titers, production of IFN-α following VSV infection was independent on TLR7-mediated signals. Interestingly, however, TLR7 ligation in B cells increased the formation of early anti-viral IgM. These findings indicate that IFN-α mediated augmentation of specific B cell responses is a partially TLR7- and virus dose dependent mechanism.

Introduction

IFN-α and -β (Type I IFNs, IFN-I) are key players throughout the course of a host's anti-viral response. They induce immediate anti-viral effects in infected and neighbouring cells, which is critical to limit viral spread (1). In parallel with this instantaneous anti-viral action, IFN-I shape the adaptive immune response by activating dendritic cells and natural killer cells (2-4), by inducing IFN-γ production in T cells, and by providing survival signals during effector and memory T cell formation (5, 6). IFN-β expression can be induced in almost all cell types, whereas production of IFN-α seems to be more restricted to plasmacytoid dendritic cells (pDCs) (7, 8). The receptor for type I interferons, IFN-α/βR, is expressed on most cell types (8) and mediates the induction of an abundant variety of genes via a signalling cascade that includes STAT1, STAT2 and STAT4 phosphorylation (9, 10). Resulting antiviral effects comprise inhibition of protein synthesis, transcriptional control and RNA cleavage (1). The decisive role of IFN-I-mediated signals in vivo following viral infection was convincingly demonstrated with IFN-α/β receptor-knock out mice. These mice are extremely susceptible to infection with acute cytopathic viruses such as vesicular stomatitis virus (VSV) and vaccinia virus (11).
IFN-α augments anti-viral B cell responses

VSV induces high amounts of IFN-α following intravenous infection. Splenic CD11c<sup>lo</sup>, CD11<sup>b</sup><sup>hi</sup>, Gr1<sup>+</sup> pDCs, which enter the spleen via blood stream through the central arteriole (7), have recently been described as main IFN-α producers following intravenous VSV infection (12). The virus is filtered in the marginal zone (MZ) where pDCs may be activated upon endocytosis of the virus. Expression of IFN-α and β following infection with VSV have been shown to be mediated by Toll-like receptor (TLR7) signal, whereby viral RNA binds to TLR7, but not to TLR9 in endosomal compartments of infected cells (13). TLR7 is expressed in significant amounts not only in pDCs, but also in B cells, at least in humans (7, 14). TLR7 triggering may thus not only elicit IFN-I from pDCs, but also from B cells (15), or may have other direct effects on B cell activation and differentiation. Interestingly, it has been shown recently that IFN-α selectively induces TLR7 expression in human B cells, sensitizing them to bind viral ssRNA (16). Additionally, Jiang et al. provide evidence that VSV can cause IFN-I production in macrophages via TLR4 ligation (17). Therefore, TLR7-dependent and -independent pathways may synergize in vivo for an efficient innate and adaptive immune response against VSV.

Given this complexity, not much is known about the spatial and temporal parameters of IFN-I action on lymphocytes in vivo during virus infection. Considering the half life of IFN-I of only few hours (18), proximity of lymphocytes and IFN-I sources may strongly influence the outcome of an immune response. We have analyzed direct effects of type I IFN on specific B cells in spleens of mice after VSV infection. Therefore we used IFN-α/βR-deficient or -competent VSV-specific gene-targeted mice (VHO mice) expressing the rearranged VH-region of a VSV neutralizing antibody (19).

Although TLR7 expression seems to be crucial for the induction of IFN-α following VSV infection in mice (13), it is not clear whether IFN-α induction via TLR7 is restricted to pDCs, and whether additional TLR7-mediated intracellular signals contribute to the anti-viral response independent of the induction of IFN-α. The impact of TLR7 expression on B cells as a potential requirement for efficient activation was analyzed by crossing VI10 mice to TLR7<sup>−/−</sup> mice (20) (VI10xTLR7<sup>−/−</sup>). We found that IFN-α directly acts on B cells to amplify BCR-mediated activation and thereby promotes early antiviral IgM responses. TLR7 ligation by VSV, in turn, promotes antiviral IgM secretion by B cells directly, and at high levels of infection indirectly through its capacity to augment IFN-α production systemically.
Results

IFN-α: a “second” signal to drive specific T cell independent B cell activation

Type I IFNs influence B cell responses either indirectly by activating DCs (3, 21), or directly by binding to the IFN-α/βR expressed on B cells (22, 23). The use of VH/DJH gene targeted (VI10) mice (19) expressing a VSV-neutralizing BCR enabled us to analyze the role of IFN-I on the activation of VSV specific B cells during the course of an infection. First, we tested whether VSV-specific VI10 B cell activation and differentiation could be supported by IFN-α in vitro. We chose IFN-α since it has been shown that IFN-α represents the vast majority of total IFN-I measured following VSV infection in mice (12). Splenic B cells from VI10 (VSV-specific B cells) were sorted with CD19-specific magnetic beads to exclude pDCs, and activated with UV-inactivated VSV, in the presence or absence of IFN-α. IFN-I significantly increased IgM production by VSV-specific B cells (Figure 1A), as analyzed in the supernatant of B cells cultured for five days, a time point when B cells were still viable. Furthermore, cells cultured for 48h were analyzed by flow cytometry to test whether increased IgM titers were due to increased plasma cell formation. As shown in Figure 1B, IFN-α had indeed a boosting effect on the formation of specific, 35.61+ (anti-VI10), CD138+ plasma cells when activated with the specific VSV antigen.

To test whether the IFN-α mediated increase in plasma cell formation and IgM production was a B cell intrinsic mechanism, we crossed VI10 to IFNAR-/- mice (11) and could thereby investigate the impact of IFN-α/β receptor expression on VSV-specific B cells. When specifically activated in vitro in the presence of IFN-α, higher levels of activation markers CD69 and CD86 were detected on specific, 35.61+ VI10 splenic B cells (VI10xIFNAR-/- controls as described in Materials and Methods, abbreviated VI10 controls in the following text) compared to VI10xIFNAR-/- B cells (Figure 2A). Moreover, IFN-α substantially increased plasma cell formation of specifically activated B cells, dependent on IFN-α/β receptor expression (Figure 2B).

In summary, IFN-α supported activation and plasma cell formation of specifically activated B cells in vitro, whereas IFN-α alone did not induce differentiation of B cells to become antibody forming cells.
IFN-α augments anti-viral B cell responses

Figure 1:
Increased specific B cell responses in vitro in the presence of IFN-α.

Spleen B cells from V110 and C57BL/6 mice were incubated with CD19-specific beads, sorted by MACS® or left unsorted and cultured in the presence of 5x10⁶ pfu UV-inactivated VSV, UV-VSV + 100U/ml IFN-α, IFN-α alone or in medium. A) IgM in culture supernatants was measured after five days by ELISA. Bars represent means ± SD of three independent experiments. * p = 0.0057, unpaired t test. B) Plasma cell formation of CD19+ splenocytes cultured for 48 h was analyzed by flow cytometry. Upon gating on viable lymphocytes, percentages of 35.61+CD138+ plasma cells were determined as shown in gated areas. Graphs are representative for one out of five independent experiments, and fold changes are means ± SD of these data.

Figure 2:
Requirement of B cell intrinsic IFN-α/βR expression for IFN-α-mediated boost of specific B cell activation and differentiation.

A) Activation markers CD69 and CD86 were analyzed on splenocytes from V110 and V110xIFNAR+ mice that had been cultured for five or 24 h in the presence of the stimuli described in Figure 1. B) Plasma cell formation of CD19+ sorted splenocytes from V110 and V110xIFNAR+ mice cultured for 48 h. BCR- and IFN-α/βR-mediated signals together resulted in most efficient activation and plasma cell formation. IFN-α alone activated V110 B cells, but did not result in plasma cell formation.
IFN-α/β receptor expression on virus-specific B cells as a requirement for efficient activation

Next, we tested the in vivo relevance of IFN-α/βR expression on VSV-specific B cells following infection. We chose to transfer B220+ VI10 cells into wt recipients to be able to study the humoral immune response with reduced precursor frequencies of VSV-specific B cells. Thirty minutes to one hour following intravenous infection, VI10 cells were strongly activated in the MZ where the virus is filtered by MZ macrophages (24, 25). Activated B cells started to migrate and arrived at the T-B border four to six hours following infection, and extrafollicular plasmablasts started to accumulate 48h following infection. Similar B cell migration and differentiation scenarios have been observed in other studies using transgenic B cells (26-29). Considering the fast kinetics of the anti-viral extrafollicular B cell response, we hypothesized that early induction of IFN-I by VSV would influence the activation of specific B cell in vivo. To address this question, 3x10⁶ B220+ splenocytes from VI10xIFNAR⁺⁺ or VI10 donors were transferred to (C57BL/6xSv129)F1 recipients as described in Material and Methods, followed by infection with 2x10⁸ pfu VSV-IND. In histology of spleen sections, we observed strikingly less specific VI10xIFNAR⁺⁺ B220⁺ plasmablasts compared to the number of VI10 blasts, 48h and 96h following infection (Figure 3A and B). Serum titers of specific antibodies in recipient mice revealed that transferred VI10xIFNAR⁺⁺ B cells produced more than twenty-fold less IgM at day 3 following infection, compared to recipients that had received VI10 B cells (Figure 3C). VI10xIFNAR⁺⁺ B cells were able to switch to IgG, although titers were about ten times lower than with VI10 specific B cells. IgG-specific histology showed that VI10xIFNAR⁺⁺ and VI10 B cells were equally able to switch to IgG (Figure 3D). The serum reduction in IgG could thus be the result of inefficient early activation and expansion, compatible with, but not proving, that primary, early activation of B cells determined the outcome of late antibody titers.

Our in vivo experiments with VSV-specific B cells confirmed the direct impact of IFN-α on B cell activation observed in vitro. In fact, IFN-α/βR expression on VSV-specific B cells was critical for early plasma cell formation following infection.
Figure 3:
Impaired plasma blast formation and antibody production by IFN-α/βR-deficient B cells.

A) and B): 3x10^6 B220+ sorted V10xIFNAR+ or V10 splenocytes were transferred to wildtype recipients. 24 h later, recipient mice were infected with 2x10^6 pfu VSV-IND or left uninfected (naive spleens). Before, 24 h, 48 h and 96 h following infection, spleens were analyzed histologically (representative sections prepared from three independent experiments). The white bar indicates 100 μm. C) VSV-specific IgM and IgG-titers were analyzed in the sera of recipient mice following transfer of 5x10^5 B220+ sorted V10xIFNAR+ or V10 splenocytes. Shown is one of two experiments with similar results with three mice per group, Mean ± SD, p < 0.02 for IgM titers d 3 and for IgG titers d 6 to d 24. D) IgG specific stain of the spleen sections described in A) and B) to show the capacity of V10xIFNAR+ and V10 B cells to switch to IgG. Plasma cell clusters in bridging channels are shown in 2-fold magnifications. The white bar indicates 100 μm.
Poor expansion of IFN-α/βR-deficient specific B cells, but retention of the capacity to differentiate to plasmablasts

To assess whether similar numbers of transferred IFNARxVHO and VI10 cells had homed to splenic B cell follicles at the time point of infection, we analysed naïve spleens of recipient mice 24h after transfer of cells histologically and quantified transferred cells by flow cytometry. Figure 4A shows that even more VI10xIFNAR⁺ cells had accumulated in recipient spleens at the time point of infection, a consequence of higher numbers of 35.61⁺ cells in naïve VI10xIFNAR⁺ donors compared to VI10 donors (25-30% versus 15-20% 35.61⁺ B cells). Following infection, however, transferred VI10xIFNAR⁺ B cells decreased in numbers (Figure 4A, 44h post infection), suggesting that an IFN-α/βR-mediated signal was crucial for efficient B cell activation immediately following infection. Alternatively, VI10xIFNAR⁺ B cells, which bind VSV with high affinity, may be preferentially infected compared to non-specific B cells. Theoretically, cells lacking IFN-α/βR expression may not be protected by IFN-α, and may die due to cytopathic effects of the virus. Surprisingly, after the initial decline in cell numbers in the VI10xIFNAR⁺ B cell population, almost equal numbers of VI10xIFNAR⁻ and VI10 B cells were recovered in recipient spleens 92h following infection. Thus, additional mechanisms, e.g. IFN-γ-mediated signals, seemed to substitute for the action of IFN-α later in the anti-viral response and supported the proliferation and differentiation of IFN-α/βR-deficient B cells. Next, we examined whether a slower proliferation of IFN-α/βR-deficient B cells was the reason for their impaired response. Therefore, we labelled B220⁺ VI10xIFNAR⁺ and VI10 B cells with CFSE before transfer and analyzed the spleens of recipient mice before and 44h after infection by flow cytometry. 44h after infection, IFN-α/βR-deficient cells had expanded poorly compared to IFN-α/βR-competent cells, although no difference in the proliferation rate was observed (Figure 4B). Consequently, less 35.61⁺, B220⁺ plasmablasts and less 35.61⁺, CD138⁺ plasma cells were recovered in mice that had received IFN-α/βR-deficient VI10 B cells (Figure 4C), in line with histology data (Figure 3A and B). Importantly, since only B cells lacked the IFN-α/βR, the observed augmented B cell activation was independent of a potential IFN-α/βR mediated activation of dendritic cells in vivo.

We concluded that IFN-α was required for the efficient expansion of specific B cells following infection with VSV, but that IFN-α-mediated signals did not affect B cell proliferation.
Figure 4

Poor expansion of IFN-α/β-deficient specific B cells despite an unimpaired capacity to differentiate to plasmablasts.

A) Spleens from recipient mice that had received $3 \times 10^6$ B220* sorted V110xIFNAR⁺ or V110 splenocytes were analyzed by flow cytometry to quantify the expansion of specific B cells (35.61⁺) and numbers of specific plasma cells (35.61⁺, CD138⁺). * p = 0.005 (unpaired t test). B) For analysis of the proliferation capacity of V110xIFNAR⁺ versus V110 B cells, sorted B cells were CFSE-labeled before transfer. IFN-α/β-R-deficient and -competent V110 B cells showed similar proliferation profiles, whereas IFN-α/β-R-deficient cells had expanded poorly until 44 h post infection. Graphs are representative for one out of five to six animals per group and time point.

Increased Infection of IFN-α/β-R-deficient cells due to the absence of protective effects of IFN-α

The observation that transferred IFN-α/β-R-deficient B cells decreased in numbers early after infection despite unimpaired proliferation pointed to a higher susceptibility to cytopathic effects of VSV in the absence of IFN-α-mediated protection. First, we tested whether B cells in V110 mice were preferentially infected compared to other potential targets, given the high affinity of the V110 BCR for VSV. Therefore, we infected V110 and C57BL/6 spleen cells in vitro with a recombinant VSV-strain that expresses GFP after productive infection (30). Indeed, we found that VSV-specific B cells amongst V110 spleen cells were main targets of
VSV. In C57BL/6 spleen cell cultures instead, mostly CD11c⁺CD11b⁺ DCs, but very few B cells were infected (Figure 5A).

Next, we used the same in vitro infection strategy to elucidate whether VI10xIFNAR⁺ cells would be infected more easily than VI10 cells (Figure 5B). Flow cytometric analysis of VI10xIFNAR⁺ spleen cell cultures revealed that all cell types analyzed, i.e. B cells, macrophages, pDCs and DCs, were infected more easily in the absence of IFN-α/βR as assessed by GFP expression. This demonstrated the strong effect of IFN-I on inhibition of infection. Interestingly, pDCs expressed high levels of GFP (Figure 5B, pDCs), suggesting that these cells exhibited greatest viral replication. In vivo, cells located in the splenic MZ may be the first cells reached by blood-borne virus and may thus represent major targets for VSV infection and replication. 24h following intravenous infection, a severe reduction in the CD11b⁺CD11c⁺ DC population was observed in 129wt mice compared to naïve 129wt mice, an effect that was even more drastic in IFNAR⁺ mice (Figure 3C). These results suggested that CD11b⁺CD11c⁺ DCs were main targets of VSV in mice. Regarding the role of pDCs as viral hosts and IFN-α producers, we analyzed localization of pDCs in histology at early time points following infection. pDCs were found mainly in the T cell zone before and 45 min after infection (Figure 5D). Six hours following infection, they formed clusters in bridging channels and subsequently populated the MZ at the time when serum IFN-α levels were described to peak (Figure 5D, 6 and 9 h post infection) (12). Hence, following VSV infection, pDCs arrived in the MZ later than VSV-specific B cells, which populated the MZ already in naïve mice (Figure 3A and B). We therefore speculated that pDCs may not be primarily responsible for the early IFN-α required for efficient activation of specific B cells. This point will be further discussed in the next paragraphs in the context of TLR7.

Taken together, virus-specific B cells are easily infected in the presence of a high virus load or in the presence of high frequencies of specific B cells. In vivo, however, DCs and macrophages may be main viral targets due to their MZ localization and exposition to virus.
Figure 5:

*Preferential VSV infection of CD11c*<sup>+</sup>CD11b<sup>+</sup> DCs in wt mice and specific B cells in V10 mice.*

A) Spleen cells from C57BL/6 and V10 mice were infected in vitro with a recombinant GFP-VSV. Cells were infected with an MOI of 3 and cultured for 14 h. Productively infected, GFP-expressing B cells (CD19<sup>+</sup>), CD11c<sup>+</sup>CD11b<sup>+</sup> DCs and CD11b<sup>high</sup> macrophages were then analyzed by flow cytometry. Histograms on the right illustrate the percentage of infected cells identified as GFP-positive cells in the different cell subsets indicated on the left. Graphs on the left were gated on lymphocytes and...
macrophages. For the gating of B cells, VI10 splenocytes are illustrated, showing decreased B cell numbers due to cell loss following infection (normal B cell numbers in VI10 mice are between 20 and 30% of lymphocytes)

B) Equal numbers of spleen cells from VI10xIFNAR' and VI10 mice were infected in vitro with VSV-GFP at an MOI of 10. 14 h following infection, cells were analyzed by flow cytometry, and the percentage of infected, GFP-positive cells in gated cell subsets were analyzed.

C) IFNAR' and 129wt mice were infected with 2x10^6 pfu VSV-IND. 24 h later, spleens were removed and analyzed by flow cytometry. Graphs were gated on lymphocytes and macrophages. Infected animals accumulated CD11b^+ high macrophages in their spleens, whereas the CD11c', CD11b' DC population was smaller in 129wt mice and had disappeared in IFNAR' mice.

D) At different time points following infection with 2x10^6 pfu VSV-IND, spleen sections of (C57BL/6x129SvEv)F1 mice were analyzed histologically with antibodies to B220, CD11c and 120G8 (pDC-specific), revealing that pDCs clustered in bridging channels six hours and arrived in the MZ nine hours following infection. The white bar indicates 100 μm. All experiments were repeated, giving similar results.

TLR7-independent induction of IFN-α at low, but not at high doses of virus

Next we analyzed mechanisms of specific B cell activation by VSV and the nature of the IFN-α-mediated signal required for efficient activation and differentiation. It has been shown recently that VSV can trigger IFN-α production in vivo preferentially through TLR7 ligation (13). Moreover, VSV-mediated B cell activation, as analyzed by CD69 up-regulation following infection in vitro, was dependent on TLR7 expression in the same study. However, it remained unclear whether the observed B cell activation was mediated by TLR7 directly or via IFN-α. We first re-evaluated the induction of IFN-α in TLR7' mice following infection with the standard virus dose of 2x10^6 pfu that was used throughout our experiments. Eight hours following intravenous infection with 2x10^6 pfu VSV-IND, TLR7' and wt littermates showed comparable levels of IFN-α in the serum (Figure 6A), suggesting that VSV did not signal exclusively via TLR7 for induction of IFN-α (17). However, in a situation of high viral load, i.e. after infection with 2x10^8 pfu VSV-IND, TLR7 seemed to be critical for a high production of IFN-α, most probably by pDCs (Figure 6A). Of note, replicating virus was required for the efficient induction of IFN-α, since UV-inactivated VSV-IND did not induce significant levels of IFN-α in both TLR7' and wt mice (Figure 6A).

VSV infection can also induce production of IFN-β, belonging to the group of type I IFNs. Since TLR7-ligation may trigger IFN-β via induction of NF-κB (9, 31), we analyzed the impact of TLR7-expression on the production of IFN-β after infection (Figure 6B). In line with studies
from Barchet et al., levels of IFN-β were generally low after VSV infection (12). Eight and eighteen hours following a high dose of infection, the levels were decreased in the absence of TLR7 expression, indicating that VSV-ligation of TLR7 also affected the IFN-β response (Figure 6B).

Neutralizing antibody titers in the serum of TLR7" mice were weakly, but significantly impaired following infection with 2x10^6 pfu VSV-IND compared to wt-littermates (Figure 6C). This finding implied a B cell intrinsic effect of TLR7 besides its capacity to induce IFN-α.

Thus, following infection with a low virus dose, IFN-α was produced mostly independent of TLR7, in contrast to a TLR7-dependent efficient induction of IFN-α after a high virus dose. In addition, TLR7-ligation supported VSV-induced early antibody formation independent of IFN-α.

![Figure 6:](image)

**Figure 6:**

TLR7-independent induction of high neutralizing titers following VSV infection.

A) Following infection with 2x10^6 pfu VSV-IND, 2x10^8 pfu VSV-IND or 2x10^8 pfu UV-inactivated VSV, IFN-α levels were determined in the sera of TLR7" and wt littermates by ELISA. B) IFN-β levels were measured in the sera of TLR7" and wt littermates by ELISA 8h and 18 h after infection with 2x10^6 pfu or 2x10^8 pfu VSV-IND i.v. . Bars represent means ± SD of three to nine mice per group. *: p = 0.017 (unpaired t test), n.d.: not determined. C) VSV-specific total Ig, and IgG antibody neutralizing titers were analyzed by a VSV-neutralization assay in the sera of mice immunized with 2x10^8 pfu VSV-IND.
Total Igs consisted exclusively of IgM on day 4, whereas on day 7, a large part of total Igs had switched to IgG. Shown are means ± SD of three to six mice per group. *: p = 0.016, unpaired t test.

**Enhanced specific B cell activation of TLR7 expressing B cells**

Expanding the above findings to our transgenic system, we hypothesized that TLR7-expression in VSV-specific B cells was required for their efficient activation and differentiation, and that IFN-α could increase anti-viral responses of both specific TLR7-deficient and –competent B cells. First, CD19+ sorted spleen cells from VI10xTLR7– and VI10xTLR7+ littermates were stimulated in vitro with UV-VSV, UV-VSV + IFN-α or IFN-α alone. LPS and R-848 were used as control stimuli. Figure 7A shows that VHO B cells produced slightly more IgM than VI10xTLR7+ B cells when activated specifically with UV-VSV. Similarly, more plasma cells were formed in cultures with TLR7-competent VI10 compared to VI10xTLR7– B cells when stimulated specifically (Figure 7B). Moreover, IFN-α enhanced IgM production and plasma cell formation from both specifically activated TLR7-deficient and –competent cells. As described recently, IFN-α controlled TLR7-sensitivity of human B cells and expression of TLR and TLR signalling molecules in human macrophages (16, 32). IFN-α may thus contribute to a TLR7-mediated enhancement of specific B cell activation as observed in our in vitro assays. In line with such a scenario, we found a 4.1-fold increase in IgM values for VI10 B cells activated with UV-VSV + IFN-α, compared to a 2.3-fold increase for VI10xTLR7+ B cells. This result hinted to the possibility that the action of IFN-α on B cells was at least partially dependent on TLR7 expression.

Next, we analyzed the requirement of B cell intrinsic TLR7-expression in vivo. Therefore, we transferred VI10xTLR7– or VHO B cells to wt recipients and analyzed VSV-specific serum Ab titers and plasma blast responses in recipient mice (Figure 7C). TLR7-deficient VI10 B cells elicited a weaker early IgM response (Figure 7C, d4 IgM), in line with impaired neutralizing IgM titers in TLR7– mice (Figure 6C). However, similar numbers of TLR7-deficient and –competent VI10 plasma cells were recovered in recipients, at least four days after infection, suggesting an impact of TLR7 exclusively on early B cell activation. Interestingly, VI10 B cells in TLR7+ hosts expanded strongly, implying that TLR7-competent B cells had a decisive proliferative advantage in vivo, leaving behind endogenous TLR7-deficient B cells in the competition for space and survival factors. The potential role of IFN-α in up-regulating TLR7 on B cells and thereby facilitating their proliferation and differentiation needs further investigation on a molecular level to be able to explain the in vitro and in vivo results mechanistically.
IFN-α augments anti-viral B cell responses

To summarize, TLR7-expression on VSV-specific B cells led to an increased production of IgM, independent of IFN-α. However, the boosting effect of IFN-α on specific B cell responses depended at least partially on TLR7 expression. In our transfer system, we observed a comparable B cell expansion and plasma cell formation of TLR7-deficient and – competent specific B cells, but TLR7-ligation in specific B cells triggered induction of higher early IgM levels.
Figure 7:
TLR7 mediated enhancement of specific B cell activation and differentiation through a partially IFN-α dependent mechanism.
Spleen cells from VI10xTLR7−/− and VI10 mice were stimulated in vitro as described in Figure 1. A) IgM was measured in culture supernatants after five days. Bars represent means ± SD from three individual experiments. Fold-changes of cells stimulated with UV-VSV in the presence or absence of IFN-α were calculated from these experiments after subtracting IgM values of cultures incubated with medium alone (means ± SD, p = 0.026 in an unpaired t test). B) Plasma cell formation in cell cultures was analyzed 48h following addition of stimuli.
C) 5x10^5 B220+ sorted spleen cells from VI10x TLR7−/− or VI10 mice were adoptively transferred to wt recipients. 24 h following transfer, mice were infected with 2x10^6 pfu VSV-IND, and 35.61-specific antibody titers in the serum were analyzed at indicated time points by ELISA. D) Total VSV-specific B cell- and plasma cell numbers were determined by flow cytometry in recipient mice following transfer of 3x10^8 B220+ sorted spleen cells and infection of recipients with 2x10^6 pfu VSV-IND. TLR7-deficient and -competent VI10 B cells responded almost equally in wt recipients, whereas TLR7-expressing VI10 B cells expanded strongly in TLR7A recipients compared to wt recipients, suggesting that TLR7-expressing B cells may be more easily activated than endogenous TLR7-deficient B cells and can thus compete for survival and differentiation factors.
Discussion

The present study shows a positive effect of IFN-α and TLR7 triggering on B cell activation and differentiation in a VSV-specific system both in vitro and in vivo. IFN-α/βR-deficient VSV-specific B cells in wt recipient mice were impaired in the early IgM response following infection, as shown by reduced numbers of specific plasma cells in the spleen, and by reduced IgM titers in the serum. IgG titers were similarly decreased, albeit not as strongly as IgM titers. VSV is cleared from the organism within 48 h after infection (33), so that the cause of IFN-α production has already been eliminated when the IgG response begins. We assume that the observed decrease in IgG titers was the result of decreased numbers of IFN-α/βR-deficient compared to IFN-α/βR-competent VSV-specific plasma blasts in recipient mice, since IFN-α/βR-deficient B cells were not impaired in switching to IgG. The action of IFN-α thus seemed to influence early activation and differentiation of specific B cells, yet not the isotype switch. Regarding the transient action of IFN-α, other cytokines are likely more important in regulating the production of isotype-switched Abs. Thus, the presented data suggest a B cell-intrinsic effect of IFN-α in the anti-VSV response. Activation of other cell types such as DCs by IFN-I following infection, which can drive B cell expansion indirectly (3, 21, 34), may synergize with the early direct action of IFN-α on B cells.

Serum levels of IFN-α have been described to peak six to ten hours following infection with VSV (12, 35), when B cells are already activated. What is the reason for this apparent discrepancy in the timing of IFN-α production and B cell activation? We looked for a link between recognition of VSV, IFN-α production by infected cells, and IFN-α-mediated enhancement of B cell activation. Recent data suggested that TLR7 binds VSV-derived ssRNA and induces the production of IFN-α following infection (13). We demonstrated here that systemic induction of IFN-α was TLR7-dependent only after a high dose of virus. Most likely, pDCs were main sources of IFN-α in that situation (36), suggesting that pDCs are only infected at a high viral load. Locally, i.e. in the marginal zone where the virus is filtered, alternative potential IFN-α sources like macrophages and DCs seem to be responsible for the observed supporting effect of IFN-α on early B cell activation. Macrophages may generate significant amounts of early IFN-α, since they capture the virus upon its arrival in the MZ and may consequently be infected easily (24, 25). Recently, Jiang et al. have suggested that VSV also binds to TLR4 on macrophages to induce production of IFN-α (17). Alternatively, binding of viral RNA to TLR7 may induce IFN-α in B cells themselves (15), which could act in an autocrine/paracrine way to support B cell activation. In line with such a scenario, we found a partially TLR7-dependent increase of IgM production in vitro after
specific B cell activation, supporting a relation between the action of IFN-α and TLR7 expression, IFN-α has been shown to increase expression of TLR7 receptors in human B cells (9, 16). Thereby, IFN-α may trigger a feedback amplification loop, further promoting binding of viral RNA and IFN-α production (32, 37, 38).

Independent of IFN-α, TLR7 expression on VSV-specific B cells conferred an advantage for differentiation and IgM production following specific activation. Considering that the transcription factor NF-κB is activated by TLR7 (9, 39), a synergistic effect of TLR7- and BCR-mediated signals seems likely to augment specific B cell activation. Higher titers of VSV-neutralizing antibodies in wt versus TLR7⁻ mice following infection with UV-inactivated VSV not inducing IFN-α (data not shown and Figure 6A) would support this hypothesis. NF-κB may also provide the link between TLR7-signalling and induction of IFN-β. Higher levels of IFN-β in wt versus TLR7⁻ mice after infection could thus be a direct effect of the TLR7-mediated induction of NF-κB, or it may be the indirect result of increased levels of IFN-α, which can induce and amplify the expression of IFN-β (40).

Equal amounts of IgG were produced from both TLR7-deficient and -competent B cells, showing that TLR7-independent mechanisms for B cell activation replaced early IFN-α mediated signals in the course of the infection. Such TLR7-independent mechanisms may comprise IFN-γ, IL-12 and B cell activating factors like BAFF or APRIL (3, 41).

In summary, our results demonstrated a direct action of IFN-α on specific B cell activation and differentiation following viral infection. Additionally, IFN-α may counteract cytopathic effects of the virus in specific B cells. The augmenting effect of IFN-α on B cell activation was partially dependent on TLR7 expression on B cells. In addition, TLR7-ligation directly increased specific B cell responses. The design of conjugate drugs bringing IFN-α in close contact with specific B cells may thus be a valuable strategy. In light of various side effects of systemically administrated IFN-α, such IFN-α targeting may be advantageous for active or passive targeted vaccinations against viruses and for anti-viral treatment.
Acknowledgements

We would like to thank Antje Novotny, Silvia Behnke and Andre Fitsche for preparing cryosections, and Drs. Lars Hangartner and Raphael Zellweger for careful reading of the manuscript and helpful discussions.

This work was supported by the Swiss National Foundation, Grant-Nrs. 3100AO-100068/1 and 3100AO-100779/1.

Material and Methods

Mice

All mice were kept at the Biologische Zentrallabor (BZL) of the University Hospital Zurich under SPF conditions, with the exception of TLR7-KO mice, which were kept in ventilated cages under conventional conditions. C57BL/6 and 129SvEv mice were obtained from the Institute of laboratory animals (Itk) at Irchel University, Zurich. TLR7-KO mice (20) were backcrossed at least four times to C57BL/6 mice. VI10 mice have been described before (19). IFNAR⁻/⁻ mice (11) were on a 129SvEv background. For transfer experiments, splenocytes from VI10xIFNAR⁻/⁻ or VI10xIFNAR⁺/⁺ mice were transferred to (C57BL6x129SvEv)F1 recipients, and splenocytes from VI10xTLR7⁻/⁻ or VI10xTLR7⁺/⁺ littermates were transferred to C57BL6-recipients.

Viruses

Vesicular Stomatitis virus, Indiana strain (VSV-IND, Mudd-Summers isolate) was originally obtained from Prof. D. Kolakofsky (University of Geneva, Switzerland). VSV-GFP was obtained from Prof. G. N. Barber (University of Miami, School of Medicine, Miami, Florida). Viruses were propagated on BHK-21 cells at a MOI of 0.01 and plaqued on Vero cells.

Sorting of cells and cell culture

Splenocytes were sorted with B220-specific magnetic beads (MiltenyiBiotech, Bergisch Gladbach, Germany) for adoptive transfers or with CD19-specific magnetic beads for in vitro B cell cultures. CD19-specific beads were chosen to exclude pDCs. Purity of cells was between 93% and 97%. For proliferation analysis of donor cells, cells were incubated with 1 μM 5(6)-carboxyfluorescein diacetate, succinimidyl-ester (CFSE, Molecular Probes, Eugene, OR) before transfer.
For in vitro activation of B cells, $10^6$ CD19+ cells/ml were cultured in a final volume of 200 μl in the presence of 5x10⁶ pfu/ml UV-inactivated VSV, 100U/ml recombinant IFN-α (HyCult, Uden, The Netherlands), 1 μg/ml R-848 (InvivoGen, San Diego, CA) or 1 μg/ml LPS (Sigma) in RPMI containing 10% FCS, 100 U/ml penicillin, 100μg/ml streptomycin and 5x10⁻⁵ M 2-mercapto-ethanol.

For in vitro infection with VSV-GFP, 3x10⁷ spleen cells/ml or 3x10⁶ CD19+ B cells/ml were infected at an MOI of 3 or 10 at RT for 2h in RPMI without FCS. After addition of FCS to a final concentration of 7%, cells were cultured at 37°C, 5% CO₂ for 14h before flow cytometry analysis.

**Antibodies and flow cytometry**

Anti-idiotypic antibody 35.61 specific for the VSV-neutralizing H- and L-chain variable region of VI10 was described before (19) and labelled with FITC (Sigma-Aldrich) or Cy5 (Amersham Biosciences, Buckinghamshire, England). pDC-specific 120G8 antibody was kindly provided by Schering-Plough, Dardilly, France and labelled with Cy5. Biotinylated horse anti-mouse IgG(H+L) was purchased from Vector Laboratories, Inc., Burlingame, CA. All other antibodies were purchased from BD Pharmingen. For flow cytometry analysis, surface molecules were stained in FACS buffer (PBS, 2% FCS, 1 mM EDTA, 0.1 mg/ml azide) for 30 min at 4°C. For intracellular antibody-staining of plasma cells, splenocytes were fixed and permeabilized with Cytofix/Perm-Solution from BD Pharmingen. Fixed cells were then incubated with antibodies in FACS buffer containing 0.1% saponin for 30 min at 4°C. For acquisition, cells were fixed in FACS buffer/1% formalin and analyzed with a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA) using FlowJo software (TreeStar Inc, Ashland, OR)

**Immunofluorescent histology**

Freshly removed spleens were immersed in Hanks Balanced Salt Solution and snap frozen in liquid nitrogen. Five μm tissue sections were air dried, fixed with acetone for 10 min, and stored at -70°C. After thawing and re-fixation in acetone, cryosections were incubated in PBS for 10min. Sections were blocked with 1 μg/section of the Fc-blocking antibody 2.4G2 for 30 min at 4°C and then incubated with the respective fluorescent antibodies for 1h at 4°C. Streptavidin-conjugated Cy3 (Jackson laboratories, Bar Harbor, ME) was added in a second step. After washing in PBS, nuclei were counterstained with 4',6'-Diamino-2-phenylindolodihydrochloride (DAPI, Sigma Aldrich), and sections were mounted with fluorescence mounting solution (DAKO, Carpinteria, CA).
IFN-α augments anti-viral B cell responses

ELISA
For detection of 35.61-specific antibodies, ELISA plates were coated overnight with 1 μg/ml of 35.61 anti-idiotypic antibody in 0.1 M carbonate buffer, pH 9.3. After blocking with PBS/3% BSA for 2 h at room temperature, plates were incubated with serial three- or four-fold dilutions of mouse sera in PBS, starting at a 1:30 or 1:25 dilution. The starting - and serial dilution giving the ab titer is indicated on the y-axis, whereas \(-\log(X) \times Y = 1/\log(X) \times Y\). After washing, HRPO-coupled goat-anti-mouse IgM (Sigma Aldrich) or AffiniPure rat-anti-mouse IgG(H+L) was added for 1 h.

IgM in culture supernatants was measured on IgM-coated plates. After blocking with PBS/3% BSA for 2 h at room temperature, plates were incubated with serial three-fold dilutions of culture supernatants in PBS (in duplicates), starting at a 1:3 or 1:5 dilution. As an IgM standard, mouse myeloma IgM (Jackson ImmunoResearch) was used. After washing, HRPO-coupled goat-anti-mouse IgM (Sigma Aldrich) was added for 1 h. ELISAs were developed with 0.1 mg/ml 2,2'-azino-di-3-ethylbenzthiazoline sulfonate (ABTS, Roche, Mannheim, Germany) in 0.1 M phosphate buffer (pH 4.0), in the presence of 60% H₂O₂. Plates were read at 405 nm.

IFN-α in sera was determined with the Mouse IFN Alpha ELISA Kit from PBL Biomedical Laboratories, New Brunswick, NJ.

VSV-neutralization assay
Titers of VSV-neutralizing antibodies in sera were determined as described before (42). Sera were diluted in MEM 2% FCS, and the dilution that resulted in 50% reduction of virus plaques was taken as neutralizing titer. For IgG titers, sera were incubated with equal volumes of 0.1 M 2-mercaptoethanol in PBS for 1 h at room temperature before dilution. All mouse sera were heated at 56°C for 30 min for complement inactivation.
52 IFN-α augments anti-viral B cell responses


4. Pogue, S.L., B.T. Preston, J. Stalder, C.R. Bebbington, and P.M. Cardarelli. 2004. The receptor for type I IFNs is highly expressed on peripheral blood B cells and monocytes and mediates a distinct profile of differentiation and activation of these cells. *J Interferon Cytokine Res* 24:131-139.


IFN-α augments anti-viral B cell responses


Extrafollicular plasma cell formation and isotype switch after viral infection is enhanced by CCR7-dependent traffic to the T-B cell zone border

Katja Fink¹, Elke Scandella², Tobias Jun³, Beatrice M. Senn⁴, Nataly Manjarrez Orduno¹,⁵, Reinhold Förster⁶, Rolf M. Zinkernagel, Hans Hengartner¹ and Burkhard Ludewig²

¹Institute of Experimental Immunology, University Hospital Zurich, Schmelzbergstrasse 12, 8091 Zurich, Switzerland, ²Research Department, Kantonal Hospital St. Gallen, 9007 St. Gallen, Switzerland, ³CBR Institute of Biomedical Research, Harvard Medical School, 200 Longwood Ave., 02115 Boston, USA, 4Intercell AG, Campus Vienna Biocenter 6, A-1030 Vienna. ⁵Present address: Molecular Biomedicine Dept, CINVESTAV, Ave IPN 2508, Mexico, ⁶Hannover Medical School, Institute of Immunology, Feodor-Lynen-Str 21, 30625 Hannover, Germany

Protective anti-viral antibody responses depend on proper activation of both B and T cells. Activated B cells up-regulate CCR7 and migrate to the T cell zone, where ligands for CCR7 are expressed. Initial contacts with T cells are made at the B cell follicle-T cell zone interface. In a vesicular stomatitis virus (VSV) specific system, we show here that CCR7-dependent T-B contacts in the early, T cell-independent phase, augmented production of anti-viral IgM. These early chemokine-driven contacts were a prerequisite for efficient B and T cell expansion. VSV-specific germinal centers were formed independent of CCR7 expression on B and T cells, but CCR7-deficient germinal center B cells were unable to maintain contact with T cells at the T-B cell zone. Thus, CCR7 expression on virus-specific B and T cells enables frequent contacts at the T-B border, thereby augmenting extrafollicular plasma cell responses, B and T cell expansion and isotype switch to IgG.
Introduction

Efficient induction of specific immune responses depend on the proper localization of lymphocytes in the micro-environments of secondary lymphoid organs, by enabling frequent and close contacts between effector cells (1-3). In this context, chemokines and chemokine receptor expression have been identified as key players for a coordinated migration of activated lymphocytes in secondary lymphoid organs (4, 5). The influence of chemokine receptors CXCR5 and CCR7 in immune responses has been analyzed in different immunization models. Reif et al. have described that CCR7-expression on B cells mediates their migration to the T cell zone, where CCR7-ligands CCL19 and CCL21 are expressed (6). In their experimental approach, the activation and migration of adoptively transferred CCR7-deficient or competent HEL-tg B cells in wild type spleen was analyzed after immunization with HEL. However, functional consequences of CCR7-ligation in the course of an immune response have not been addressed so far.

Infection with cytopathic viruses require that efficient immune responses are triggered to limit viral spread (7). Different immunological strategies have evolved to optimally meet this task. The first wave of antibodies following immunization is via plasma blast formation in extrafollicular regions (reviewed by I. MacLennan (8)). Antigen enters the spleen through sinuses in the marginal zone (MZ) where it is filtered and captured by MZ macrophages (9, 10). B cells are activated in the MZ and migrate to the T-B border and to the T cell zone, where they arrive as large blasting cells. Later, IgM-producing blasts leave the T cell zone via bridging channels and reside in the red pulp for a short period of time (11-14). The bulk of extrafollicular-derived IgM-producing plasma blasts is short lived (15, 16), whereas few cells eventually survive as long-lived plasma cells in the bone marrow and in the spleen, and as memory cells in the MZ (10, 17, 18). In parallel, germinal centers are formed, which may be required for isotype switch and memory B cell formation, enabling maintenance of high specific antibody titers and efficient defence of secondary infection (19). Both extrafollicular and follicular or germinal center responses depend on chemokine expression, coordinating the meeting of all cell types required for the respective task (6, 20, 21). Vesicular stomatitis virus (VSV) induces both types of B cell responses and is thus optimal to study various aspects of an antibody response. VSV efficiently activates B cells independent of T help, resulting in high IgM titers (22). In addition, the CD4+ T cell-dependent switch of neutralizing Abs to IgG isotypes provides protection by eventually clearing the organism from persisting virus (23, 24).
In the present work we have analyzed the impact of CCR7 expression on the anti-viral B cell response in CCR7\(^{-}\) mice and in an adoptive transfer model with VSV-specific B and T helper cells. For adoptive transfers, B cells from a \(V_{H}DJ_{H}\) gene-targeted mouse expressing the rearranged \(V_{H}\)-region of a VSV-neutralizing Ab (VI10 mice) (25), and T helper cells from T cell receptor-transgenic mice (L7 mice) (24) were used.

We have identified CCR7 as a key player of the thymus-independent anti-VSV response, guiding activated B cells to the T-B border, enabling contacts of B cells with specific T cells in the T cell zone and thereby supporting early extrafollicular antibody responses. Moreover, CCR7-dependent contacts of specific B and T cells were a prerequisite for efficient induction and maintenance of germinal centers and isotype switch in a situation with limited amounts of Ag.

**Results**

**Migration of activated VSV-specific B cells in the spleen is mediated by CCR7**

Immunization triggers migration of specific B and T cells in secondary lymphoid organs, as has been shown in various antigen-specific systems (11, 12, 14, 26). Following immunization with protein antigens, these migration events were shown to depend on the coordinated expression of chemokine receptors CXCR5 and CCR7 (6, 20). We wanted to assess the kinetics and organization of the B cell migration events following an infection with a virus that induces a strong extrafollicular response. To this end, we infected VI10 mice with \(10^9\) pfu VSV-IND and analyzed spleens at different time points after infection by histology. Intravenous infection with VSV induced a massive activation of B cells in the spleen and in lymph nodes (Fig. 1, and data not shown). Within one hour after infection, all VSV-specific B cells, as identified by the anti-idiotypic monoclonal antibody (mAb) 35.61, accumulated in the marginal zone (Fig. 1a, 30 min). Between 6 h and 24 h after infection, these B cells migrated to the T-B zone interface (Fig. 1a, 4 h). Thereafter, most VSV-specific B cells had arrived in the T cell zone as large blasting cells, which were B220-low or -negative (Fig. 1a, 12 h).

Downregulation of B220 on specific cells could be observed in histology because 35.61 (green), B220 (red) double positive cells were yellow whereas 35.61\(^{+},\)B220\(^{-}\) cells were green (Fig. 1a, 12 h – 2 d). 48 hours after infection, large numbers of IgM-producing plasma blasts could be detected in bridging channels and red pulp (Fig. 1a, 48 h). We hypothesized that the observed B cell migration towards the T cell zone, where CCR7-ligands CCL19 and CCL21 are expressed, was dependent on up-regulation of CCR7 (6). Therefore, the migratory
capacity of splenocytes from V110 mice was assessed in an ex vivo migration assay. Six and 12 hours after infection, VSV-specific (35.61*) B cells migrated towards CCL19, whereas non-specific B cells (35.61) and naïve B cells were not responsive to CCL19 (Fig. 1b). In line with the VSV-induced migration of specific B cells to CCL19, CCR7 was up-regulated on specific B cells in infected, but not in naive mice (Fig. 1c).

To summarize, we showed that in the VSV-specific system investigated here, B cells up-regulated CCR7 following infection, suggesting that B cell migration towards the T cell zone was mediated by CCR7.

Figure 1

Migration of activated VSV-specific B cells is mediated by CCR7.

a) Spleens from V110 mice were analyzed by histology at different time points after infection with 10^9 pfu VSV i.v. Spleen sections were stained with the V110-specific mAb 35.61 (green), B220 (red) and CD4 (blue). 35.61*, B220* double-positive specific B cells are yellow, whereas blasting cells have down-regulated B220 and appear as green cells. Sections are representative for one out of at least
three mice per time point, analyzed in different experiments. b) The migratory capacity of VI10 spleen cells towards CCL19 was measured six and 12 hours after infection in an ex vivo migration assay. Total numbers of input and migrated cells were counted, and percentages of 35.61° (specific) and 35.61' (non-specific) B cells were determined by flow cytometry. Shown are means ± SD of triplicates. c) CCR7 expression on B cells from naïve and infected VI10 mice (24 h after infection) was analyzed by flow cytometry. Experiments were repeated, giving similar results.

Impaired anti-viral antibody response in CCR7⁻ mice

In earlier studies with CCR7⁻ mice, the primary immune response to protein antigen was found to be considerably impaired (2). Besides induction of a strong humoral response, VSV also triggers innate mechanisms, which can in turn enhance activation of B and T cells (27). Furthermore, virus may replicate in infected hosts and thereby increase the antigen “dose” in the course of the immune response. In light of these virus-specific activation mechanisms, which may significantly affect the outcome of an immune response, we wanted to analyze the anti-VSV response in CCR7⁻ mice after immunization with VSV. Surprisingly, CCR7⁻ mice were able to generate equal titers of VSV-neutralizing IgM compared to wild type B6 mice at day four after infection with live VSV (Fig. 2a). In contrast, IgG titers were impaired until day 10 after infection, suggesting a defect in the induction of isotype switch in CCR7⁻ mice. At later time points, both CCR7 and B6 mice generated high amounts of VSV neutralizing IgG. Similarly, comparable numbers of IgM - and IgG producing cells were recovered in the spleens of CCR7⁻ and B6 mice (Fig. 2c).

Next, we wanted to compare these findings to a non-infectious situation. Therefore, we immunized CCR7⁻ and B6 mice with VSV glycoprotein (VSV-G). Now, both IgM - and IgG production were markedly impaired in CCR7⁻ mice compared to B6 mice, as shown by decreased VSV-neutralizing titers (Fig. 2b) and lower or even non-detectable numbers of splenic antibody-forming cells in CCR7⁻ mice (Fig. 2d). Evaluating the activation of CD4⁺ T helper cells, we found reduced numbers of IFN-γ producing T cells in CCR7⁻ mice at day 4 following infection with VSV (Fig. 2e). At later time points and in a non-infectious situation, numbers of activated T cells were comparable between CCR7⁻ and B6 mice (Fig. 2e and f). Thus, a comparison of B cell responses in CCR7⁻ and B6 mice revealed that CCR7 expression promoted the induction of isotype switched antibodies following infection with VSV. In turn, CCR7 expression was strictly required for the generation of IgG antibodies in a non-infectious situation, although the activation of CCR7-deficient Th cells was not affected. This finding suggested that insufficient T-B contacts impeded isotype switch to IgG in the latter situation.
Figure 2

Impaired VSV-neutralizing antibody response in CCR7-/- mice at limited amounts of antigen.

CCR7-/- and B6 mice were infected with 2x10^6 pfu VSV-IND i.v. a) or immunized with 5μg VSV-G i.v b), and VSV-neutralizing titers in the sera of mice at the time points indicated. c) and d) Numbers of antibody forming cells AFCs per spleen were analyzed by a VSV-specific ELISPOT assay. e) and f) Numbers of IFN-γ producing CD4+ T cells per spleen were analyzed by an IFN-γ specific ELISPOT assay. Bars represent means of triplicates, and similar results were obtained in two to three independent experiments. nd: not detectable. * : p = 0.02, ** : p = 0.0001 (unpaired t test)
B cell differentiation in follicles in the absence of CCR7 up-regulation on activated B cells

It has been reported that activated B cells are not attracted to the T cell zone in CCR7<sup>−/−</sup> mice and migrated to the red pulp and sinus instead. Moreover, CCR7-negative DCs were unable to migrate to draining LN<sub>s</sub> (2). The consequence of these defects was an impaired adaptive immune response in CCR7<sup>−/−</sup> mice (Figure 2a) and b) and (2)). However, CCR7<sup>−/−</sup> mice show an abnormal lymphoid architecture, which may critically influence the outcome of a humoral response. To address the question whether VSV-specific CCR7-deficient B cells were impaired in the early anti-viral response in a normal lymphoid environment, we have studied the migratory behaviour of VI10xCCR7<sup>−/−</sup> B cells in an adoptive transfer model. Both CCR7-competent and -deficient VSV-specific B cells had homed to B cell follicles 24 h after cell transfer (not shown) and were recovered in equal numbers in recipient spleens (Fig. 3a). Upon immunization with 2x10<sup>6</sup> pfu VSV-IND, B cells from VI10xCCR7<sup>−/−</sup> mice did not migrate to the T cell zone but differentiated to blasts in B cell follicles instead (Fig. 3b). Despite the altered differentiation environment in B cell follicles or at the T-B border, respectively, both VI10xCCR7<sup>−/−</sup> and VI10 formed plasma blasts which accumulated in bridging channels and in the red pulp three days following infection with VSV (see Fig.6).

Next, we addressed whether CCR7-deficiency on specific B cells would affect the production of anti-viral antibodies. Following infection with VSV-IND, comparable amounts of both IgM and IgG antibodies were measured in the sera of recipient mice, independent of CCR7 expression of transferred B cells (Fig. 3c). Despite their differentiation in the B cell follicle environment, CCR7-deficient VSV-specific B cells were apparently able to produce normal amounts of antibodies. However, following immunization with VSV-G, i.e. in a non-infectious situation, CCR7-competent VI10 donor cells produced significantly more IgM and IgG than VI10xCCR7<sup>−/−</sup> donor B cells (Fig. 3d). Similarly, the antibody response in CCR7<sup>−/−</sup> mice was considerably impaired following immunization with VSV-G, as shown in Fig. 2. The results with CCR7<sup>−/−</sup> mice showed that either an intact lymphoid structure or the migration of B cells towards the T cell zone following immunization was required for an efficient antibody response. The decreased titers of CCR7-deficient specific B cells in a wild type environment now demonstrated that the efficiency of an antibody response depended on T-B zone localization of specific B cells in a non-infectious situation with limiting amounts of antigen.
Figure 3

**Impaired antibody response of CCR7-deficient specific B cells in a situation with limited amounts of antigen.**

7x10⁶ sorted B cells from VI10 or VI10xCCR7⁻ mice were transferred into B6-IgHaThy1.1 recipients. a) Numbers of 35.61⁺B220⁺ donor B cells recovered in recipient mice 24 h after adoptive transfer. b) Spleen histology of recipient mice 24 h after infection with 2x10⁶ pfu VSV-IND i.v. c) and d) Serum titers of antibodies of the indicated isotypes were determined by ELISA at different time points following infection with 2x10⁶ pfu VSV-IND or 5 µg VSV-G, respectively. *: p < 0.05 (unpaired t test).

**Reduced extrafollicular plasma cell response by CCR7-deficient VSV-specific B cells**

Although anti-viral antibody titers were unimpaired in infected recipients of VI10xCCR7⁻ B cells (Fig. 3c), we had repeatedly observed in histology that fewer 35.61-bright B cell blasts were formed by VI10xCCR7⁻ compared to VI10 B cells. Considering a CCR7-dependent difference in B cell expansion, we quantified total 35.61⁺ B cells and 35.61⁺ CD138⁺ plasma
cells recovered in recipient spleens by flow cytometry (Fig. 4a) and b). To compensate for an unbalanced precursor frequency of specific B and T cells, we co-transferred VSV-specific sorted T helper (L7) and B cells (VI10), whereby both cell types were either CCR7-deficient or -competent. We found less VI10xCCR7−/− than VI10 B cells when analyzing total 35.61+ cells three and six days following infection (Fig. 4a). Moreover, less 35.61high CD138+ plasma cells were observed in recipients that had received VI10xCCR7−/− compared to those having received VI10 B cells (Fig. 4b). To look into a possible altered B cell proliferation, we CFSE-labeled VI10xCCR7−/− and VI10 B cells before transfer and analyzed the spleens of recipient mice before, 48 h and 96 h after infection. Fig. 4c) demonstrates that the proliferation profiles were similar for VI10xCCR7−/− and VI10 B cells, yet that less 35.61high cells were observed amongst CCR7-deficient donor cells. 35.61high cells from VI10 donors were CD138+ (not shown) and thus represented plasma cells. These data demonstrated that CCR7-deficiency did not affect proliferation, but expansion and differentiation of specific B cells.

Six days after infection, equal numbers of plasma cells were generated by VI10xCCR7−/− and VI10 B cells despite the initial delay of VI10xCCR7−/− cells, suggesting that T-B contacts augmented the very early formation of plasma cells, whereas plasma cell formation at a later time point after infection was independent of CCR7-mediated T-B contacts. The same, albeit more strict, dependence of specific B cell expansion on CCR7 expression was observed following immunization with VSV-G.

We concluded that early extrafollicular plasma cell formation was dependent on CCR7-expression on both specific B- and T cells. CCR7-mediated early contacts of B and T cells at the T-B border thereby seemed to dictate the magnitude of the resulting immune response.
CCR7-mediated B and Th cell contacts after viral infection

**Figure 4**

**CCR7-dependent enhancement of plasma cell formation.**

a) and b) 5x10^5 sorted B220^+ B cells from V110 or V110xCCR7^- mice and 5x10^5 sorted CD4^+ Th cells from L7 or L7xCCR7^- donor mice were transferred into B6-IgHaThy1.1 recipients. 24 h after transfer, recipient mice were immunized with 2x10^6 pfu VSV-IND or 5 μg VSV-G, and total B cell numbers (35.61^+ B220^-) (a) and plasma cell numbers (35.61^+ CD138^-) (b) of donor cells that were recovered three and six days after immunization were determined. c) 3x10^6 B220^+ V110 or V110xCCR7^- spleenocytes were CFSE-labelled and transferred into C57BL/6 recipients. Proliferation profiles of donor cells were determined in recipient spleens before, 48 h and 96 h following infection with VSV.

**T cell expansion following contact with activated B cells**

Having investigated the expansion and generation of an extrafollicular response by CCR7-deficient and -competent B cells, we had found so far that B cells seemed to profit from contacts with specific T cells. In a next step we investigated the T cell side and analyzed T cell activation and expansion after VSV infection, in the absence or presence of CCR7 on specific B and T cells. To address T cell expansion after infection, we co-transferred VSV-specific V110 B cells and Thy1.2^+ L7 T helper cells into Thy1.1^+ recipient mice and infected them with VSV or VSV-G. Fig. 5a) shows that more Thy1.2^+ CD4^+ cells following transfer of CCR7-competent B and T cells were recovered in recipient spleens.
three days after infection with VSV, compared to CCR7-deficient B and T cells. Following
immunization with VSV-G, few donor T cells were recovered after transfer of both CCR7-
competent and - deficient donor cells, and their numbers were comparable (Fig. 5a). The
impaired expansion of CCR7- T helper cells following co-transfer of CCR7-deficient B and T
cells and infection was not due to a defect in T cell activation, since both L7xCCR7- and L7
wild type mice similarly down-regulated CD62L and up-regulated CD44 (Fig. 5b).
We therefore reasoned that B cells contribute to cell expansion. It has been shown before
that activated MZ B cells are efficient antigen presenting cells (APC) (11). We thus
hypothesized that specific B cells activated by VSV in the MZ could act as APCs following
migration to the T-B border and activate specific T helper cells there. To look into a possible
APC-function of specific activated B cells, we infected VI10 mice with VSV, removed the
spleen twelve hours later and sorted CD11c+ dendritic cells (DCs) and B220+ B cells as
potential APCs for a T cell proliferation assay. We chose to dissect spleens twelve hours
after infection since B cells were located at the T-B border at this time point (Fig. 1a). Fig. 5c)
demonstrates that activated VI10 B cells clearly induced T cell proliferation, albeit at lower
levels than CD11c+ DCs.
CCR7-expressing activated B cells thus migrated to the T-B border and had the potential to
efficiently activate specific T helper cells. Our results showed a clear relation between B- and
T cell expansion and CCR7 as mediator of T-B contacts.

Figure 5
Impaired expansion of CCR7-deficient T helper cells due to limited contacts with antigen-presenting B cells. a) Numbers of recovered CD4+ Thy1.2+ donor-derived T helper cells in recipient spleens were determined after adoptive transfer and immunization with 2x10^6 pfu VSV-IND or 5 µg VSV-G. b) Activation markers CD62L and CD44 on donor T cells (gated on CD4+ cells) from either L7 or L7xCCR7+ mice were determined six days after infection of
recipient mice (B6-IgHa-Thy1.1) with VSV. c) The capacity of B cells to induce proliferation of VSV-specific Th cells was determined by $^3$H-thymidine incorporation. As stimulators, L7 Th cells were incubated with irradiated B220$^+$ or CD11c$^+$ sorted splenocytes from naïve VI10 mice or from VI10 mice that had been infected with VSV 12 hours earlier.

**CCR7-independent formation of germinal centers**

So far, we had observed that specific B and T cells interacted at the T-B border, thereby determining the magnitude of T and B cell expansion. These interactions were restricted to a time window between twelve and 24 hours (Fig. 1a and Fig. 3b). We next analyzed spleens histologically after co-transfer of T and B cells at the peak of the extrafollicular response, i.e. three days after infection. Surprisingly, blasting B cells identified as 35.61 bright cells (green in histology) still interacted with specific T cells, despite their advanced differentiation state (Fig. 6a, see selected regions at larger magnification). Apparently due to impaired expansion of CCR7-deficient B and T cells, T-B contacts of VI10xCCR7$^+$ and L7xCCR7$^-$ cells in the bridging channels were scarce (Fig. 6a).

As described above, production of VSV-specific IgG was impaired in the absence of CCR7 expression on B cells, at least after immunization with VSV-G, i.e. in a non-infectious situation. Although isotype switch can occur outside GCs (28), we proposed that formation of GCs by specific B cells may be affected in the absence of CCR7-mediated contacts of B and T cells. Surprisingly, however, GCs were formed with both CCR7-competent and -deficient specific B and T cells. In our transfer model, GC formation with specific cells started at day three following infection, and peak values were observed between day six and eight (data not shown). We quantified GL7$^+$ 35.16$^+$ B cells in recipients at day six following infection with VSV by flow cytometry and observed equal percentages and absolute numbers of GL7$^+$ VI10xCCR7$^+$ and VI10 B cells (data not shown). Following immunization with VSV-G, GC formation by specific cells was very inefficient and started later, between day 5 and 6 following immunization.

We then analyzed the GCs histologically by staining B cell follicles (B220), specific B cells (35.61) and GCs (GL7). As shown in Fig. 6b) and c), less 35.61$^+$ blasting B cells were observed at day six following transfer of CCR7-deficient T and B cells and infection with VSV, compared to CCR7-competent donor cells. B cell blasts at the T-B border, adjacent to GCs, may be the plasma cell output of the respective GCs. The reduction in B cell blasts may explain lower titers of IgG produced by VI10xCCR7$^+$ B cells (co-transferred with specific T cells) at day six after infection, compared to VI10 B cells (Fig. 6d). When VI10xCCR7$^+$ B
cells were transferred alone, however, we did not observe a difference in IgG titers (Fig. 3c), demonstrating the impact of CCR7 expression on both B and T cells.

To summarize, B and T cell contacts in bridging channels persisted beyond the initial interaction at the T-B cell zone border, suggesting that Th cells not only supported B cell activation, but also B cell differentiation. Furthermore, we found that CCR7 expression on T and B cells had no impact on GC formation, but seemed to influence the output of B cell blasts, at least when analyzed in histology.

(a) 
(b) 
(c) 
(d)
Figure 6

*Frequency-dependent persistence of T-B contacts in bridging channels and formation of germinal centers with CCR7-competent versus CCR7-deficient specific B and T cells.*

a) to c) Following transfer of B and T cells and infection with VSV, as described in Figure 4, recipient spleens were analyzed histologically at day three (a)) and at day six post infection (b) and c)). a) and b) VI10 B cells (mAb 35.61): green, L7 Th cells (anti-Thy1.2): blue, B220: red. c) GC cells were stained with anti-GL7 six days post infection. VI10 B cells: green, GL7: blue, B220: red. d) VSV-specific titers produced by donor cells (IgHb-allotype) were measured by ELISA in the sera of recipient mice at day three and six following infection.

Discussion

CCR7 organizes the migration of activated B and T cells towards the T cell zone-B cell zone interphase, fostering the aggregation of B and Th cells and inducing an adaptive immune response. These guided contacts are required in the case of low specific precursor frequencies to ensure activation of sufficient numbers of B and T cells. We have used VSV-specific B and T cells to address the CCR7 dependence of these contacts after infection with VSV. VSV is a cytopathic virus that induces a vigorous IgM response as early as three to four days after infection (29), whereby the onset of high titers of virus-neutralizing antibodies is crucial to protect the host (30). Thus, VSV infection provides a physiologically relevant model to study the organization of initial T-B contacts and to address its functional consequences. In the present study we have shown that CCR7 up-regulation on activated VSV-specific B cells is required for their migration to the T cell zone. T-B cell zone contacts were initiated as soon as B cells arrived at the T-B cell zone border and increased in frequency until 24 hours after infection. In parallel, both T and B cells expanded, suggesting that B and T cells meet at the T-B zone border for direct interaction. A scenario where antigen-presenting DCs activate Th cells, which in turn activate B cells, is therefore unlikely (31). In fact, our data with DTR mice (diphteria toxin inducible DC depleter mice (32)) showed that specific B cells accumulated at the T-B cell zone border independent of the presence of DCs (data not shown).

Following immunization with VSV-G, these guided contacts between B and Th cells were necessary for the induction of virus-neutralizing antibodies. However, following infection with live virus, B cells were able to initiate an extrafollicular response in the absence of CCR7-mediated, organized T-B contacts, suggesting that live virus activates a larger number of specific B cells in the MZ, which increases the likelihood of contacts with Th cells near the T
cell zone or in bridging channels. Thus, initial contacts of activated B cells with naïve Th cells induced Th cell activation and served the expansion of both VSV-specific B and T cells (33-35). Surprisingly, however, also blasting B cells frequently contacted T cells in bridging channels, i.e. on their way leaving the splenic white pulp. This finding suggested a supporting role of T cells in plasma blast maintenance and/or differentiation. Earlier, this role had been attributed mostly to dendritic cells (15). These contacts may also be decisive for the differentiation of a B cell blast to a fully differentiated plasma cell, since we observed an impaired plasma cell generation (identified by CD138 expression and high levels of intracellular 35.61) with CCR7-deficient B and T cells, i.e. in a situation with reduced T-B contacts.

During the GC response, CCR7-expressing B cell blasts accumulated in the T cell zone adjacent to the GC (see Fig. 6c). B cells in the GC response and activated follicular B cells have been shown to express equal levels of CCR7 (21), implying that B cell attraction to the T cell zone also influenced the GC response. It seemed that B cell blasts adjacent to the GC were recent GC emigrants. However, this was a histological observation, and we could not prove the origin of these B cell blasts.

To summarize, comparison of immunizations with live virus or VSV-G showed the importance of CCR7 in coordinating contacts of activated specific B cells with specific T cells. CCR7-expression was a prerequisite for the onset of high anti-viral antibody titers in a non-infectious situation, i.e. at low levels of B cell activation and expansion. Moreover, CCR7 expression on specific B and T cells increased the extrafollicular response after infection with VSV. Histological analysis of specific GCs further revealed that CCR7 also seemed to increase GC output of B cell blasts, possibly by enabling persistent contacts of GC B cells with the T cell zone.

**Material and Methods**

**Mice**

CCR7<sup>−/−</sup> and V110 mice were kept at the Biologische Zentrallabor (BZL) of the University Hospital Zurich under SPF conditions. L7 and L7×CCR7<sup>−/−</sup> mice were kept in ventilated cages under conventional conditions at the Research Department of the Kantosspital St.Gallen. C57BL/6 mice were obtained from the Institute of laboratory animals (Itk) at Irchel University, Zurich. C57BL/6-lgHa-Thy1.1 mice were purchased from The Jackson Laboratories, and kept at Itk, Zurich.
Viruses
Lymphocytic choriomeningitis virus (LCMV), WE strain, was originally obtained from Dr. F. Lehmann-Grube (Heinrich-Pette-Institut, Hamburg, Germany) and propagated as described (36). Vesicular Stomatitis virus, Indiana strain (VSV-IND, Mudd-Summers isolate) was originally obtained from Prof. D. Kolakofsky (University of Geneva, Switzerland). Recombinant VSV-glycoprotein (VSV-G) was obtained from a culture of Spodoptera frugiperda 9 (Sf9) cells after infection with a recombinant baculovirus (37).

VSV-neutralization assay
Titers of VSV-neutralizing antibodies in sera were determined as described before (38). Sera were diluted in MEM 2% FCS, and the dilution that resulted in 50% reduction of virus plaques was taken as neutralizing titer. For IgG titers, sera were incubated with equal volumes of 0.1 M 2-mercaptoethanol in PBS for 1 h at room temperature before dilution. All mouse sera were heated at 56°C for 30 min for complement inactivation.

Sorting of cells
B cells from VI10 and VI10xCCR7<sup>−/−</sup> spleens were sorted with B220-specific beads, and T helper cells from L7 and L7xCCR7<sup>−/−</sup> spleens were sorted with CD4-specific magnetic beads (MiltenyiBiotech, Bergisch Gladbach, Germany) for adoptive transfers. Purity of B220<sup>+</sup> and CD4<sup>+</sup> cells was between 90% and 96%.

Migration assay
For chemotaxis assays, 5x10<sup>5</sup> splenocytes were transferred into the upper chamber of transwell plates with 5μm filters (Corning Costar) containing indicated concentrations of CCL19 (R&D Systems) in the bottom chambers. After 4 hours transmigrated cells were stained with anti-B220 and anti-35.61 antibodies and enumerated by flow cytometry. Transwell assays were performed in duplicate and were repeated with splenocytes from 3 different mice per group.

Proliferation assay
VI10 mice were infected with 5x10<sup>8</sup> pfu VSV-IND and 12 hours later CD11c<sup>+</sup> and B220<sup>+</sup> cells were sorted from splenocytes using MACS-beads (Miltenyi). The amount of VSV-specific 35.61+ B220+ cells was calculated by flow cytometry and indicated numbers of 35.61+ B220+ cells or CD11c+ cells were incubated with 1x10<sup>5</sup> MACS-sorted CD4+ cells from L7 mice for 48 hours. 1 μCi 3H-Thymidine was added to each well during the final 16 hours of
incubation and incorporation was determined in a scintillation counter (Packard). Proliferation assays were performed in duplicate and were repeated with splenocytes from 3 different mice per group.

Flow cytometry
Anti-idiotypic antibody 35.61 specific for the VSV-neutralizing H- and L-chain variable region of V110 was described before (39) and labeled with FITC (Sigma-Aldrich) or Cy5 (Amersham Biosciences, Buckinghamshire, England). All other antibodies were purchased from BD Pharmingen. For flow cytometry analysis, surface molecules were stained in FACS buffer (PBS, 2% FCS, 1 mM EDTA, 0.1 mg/ml azide) for 30 min at 4°C. For intracellular staining of plasma cells, cells were fixed and permeabilized with Cytofix/Perm-Solution from BD Pharmingen. Fixed cells were then incubated with antibodies in FACS buffer containing 0.1% saponin for 30 min at 4°C. For acquisition, cells were fixed in FACS buffer/1% formalin and analyzed with a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA) using FlowJo software (TreeStar Inc, Ashland, OR)

ELISA
For the detection of VSV-specific IgM", IgG2a" or IgG2b", which were derived from adoptively transferred, purified B220+ cells of V110 mice, plates were coated overnight with 5 μg/ml PEG-precipitated VSV-IND particles, blocked with PBS/3%BSA for 2 h at room temperature, washed and incubated with threefold serum dilutions starting at a dilution of 1:30. After washing, biotin-labeled rat-anti-mouse IgG2a" or IgG2b" (BD Pharmingen) were added, followed by Streptavidin-HRPO (Jackson). ELISAs were developed with 0.1 mg/ml 2,2'-azino-di-3-ethylbenzthiazoline sulfonate (ABTS, Roche, Mannheim, Germany) in 0.1 M phosphate buffer (pH 4.0) plus 60% freshly added H₂O₂. OD was measured at 405 nm. The turning point of the sigmoid absorbance curves was determined as ELISA titer.

ELISPOT
To quantify VSV-specific IgM and IgG producing B cells ELISPOT plates were coated overnight with PEG-purified VSV-IND (40 μg/ml). After blocking with RPMI medium containing 10% FCS serial dilutions of splenocytes starting with 5x10⁶ cells were added to the wells in duplicates and incubated for 5 hours at 37°C. Cells were washed off and ASC were detected with alkaline phosphatase conjugated goat anti-mouse IgM and goat anti-mouse IgG antibodies (Jackson) followed by a NBT/BCIP (Applichem) color reaction.
quantify VSV-specific CD4 T cells. Graded numbers of MACS-sorted CD4+ cells were incubated with VSV-loaded bone marrow derived dendritic cells (2x10⁴) in ELISPOT plates coated with anti-IFNγ capture antibody (R&D Systems). After 18 hours cells were washed off and plates were developed with biotinylated anti-IFNγ detection antibody followed by streptavidin conjugated alkaline phosphatase and a NBT/BCIP color reaction.

**Fluorescent histology**

Freshly removed organs were immersed in Hanks Balanced Salt Solution and snap frozen in liquid nitrogen. Five μm tissue sections were air dried, fixed with acetone for 10 min, and stored at -70°C. Cryosections were blocked for 30 min with 1 μg/sample of the Fc-blocking antibody 2.4G2, washed in PBS, and incubated with the respective fluorescent antibodies for 1 h min at 4°C. If necessary, streptavidin-Cy3 was added in a second step. After washing in PBS, nuclei were counterstained with 4',6'-Diamino-2-phenylindoledihydrochloride (DAPI, Sigma), and sections mounted with fluorescence mounting solution (DAKO, Carpinteria, CA). Fluorescence was analysed with a Zeiss Axiophot microscope (Carl Zeiss, Feldbach, Switzerland). Color channels were assembled automatically with the analySIS software (Soft Imaging System, Münster, Germany), and images were processed using Adobe Photoshop, without non-linear operations.


Shaping of the developing B cell repertoire by maternal antibodies in favour of maternal specificities

Katja Fink¹, Nataly Manjarrez-Orduno¹,², Martin Holdener¹,³, Beatrice M. Senn¹,⁴, Hans Hengartner¹, Rolf M. Zinkernagel¹ and Andrew Macpherson¹,⁵

¹Institute of Experimental Immunology, University Hospital Zurich, Schmelzbergstrasse 12, 8091 Zurich, Switzerland, ²Present address: Molecular Biomedicine Dept, CINVESTAV, Ave IPN 2508, Mexico, ³Present address: Immune Regulation Laboratory, Department of Developmental Immunology, La Jolla Institute for Allergy and Immunology, San Diego, CA 92121, 10355 Science Center Drive, USA, ⁴Present address: Intercell AG, Campus Vienna Biocenter 6, A-1030 Vienna, ⁵Present address: Department of Medicine, McMaster University, 1200 Main St. W. Hamilton Ontario L8N 3Z5

Maternal antibodies provide immune protection for newborn mammals. These passively acquired antibodies are supposed to act mostly locally on mucosal surfaces of suckling mice, depending on a continuous supply of antibodies by lactation. This study provides evidence that maternal antibodies also improve immune responses long after weaning. VhDJH-gene-targeted (VI10) mice expressing a B cell receptor (BCR) specific for vesicular stomatitis virus (VSV) produce a high spontaneous titer of VSV-specific antibodies. VI10 offspring with VI10 mothers and nursed by a VI10 mother accumulated more mature specific B cells than VI10 offspring with a wild type mother and nursed by wild type mothers. Strikingly, specific B cells were preferentially selected to the follicular rather than to the MZ compartment in offspring from VI10 mothers. Following infection with VSV, these mice showed a memory-type humoral response. An imprinting, positive effect of maternal antibodies was observed on the anti-viral response up to adulthood.
Introduction

In mouse and man, maternal antibodies of the IgG isotype can cross the placenta and supply the developing fetus with a first immune defence. In mice, maternal IgG uptake continues after birth by uptake through the duodenal neonatal Fc receptor (FcRn) (1, 2). Protective effects of maternal antibodies include steric prevention of pathogen invasion, neutralization of pathogens, and enhanced clearance of pathogens by immune complex formation and activation of complement (reviewed by Van de Perre (3)). The protective effect of passively acquired maternal antibodies is thus prolonged after birth by breast-feeding, whereby maternal antibodies prevent bacterial and viral infections that cannot be cleared by the immature immune system (3).

In mice, FcRn is down-modulated on gut epithelial cells 16 days after birth (2). Thereafter, maternal IgG from milk does not get taken up into the neonatal circulation. In humans, transcytosis of IgG through epithelial cell layers has been demonstrated in vitro and in vivo (4, 5) but the possibility of a small contribution of postnatal maternal antibody uptake is still an open question.

It has been shown in earlier studies that serum immunoglobulins are essential for peripheral selection of B cells and for B cell development in the bone marrow (BM) (6, 7). In newborn mice deficient for the FcRn, i.e. in the absence of maternal antibodies, onset of endogenous IgG titers was delayed, suggesting that maternal antibodies stimulate B cell development (2). More recently, maternal antibodies and their impact on the generation of humoral responses in offspring has been investigated, showing that maternal antibodies seem to be able to optimally prime offspring and thereby increase the Ab response following subsequent infection (reviewed by (8)). However, these studies were carried out with polyclonal BCR repertoires and little is known about the mechanisms how maternal antibodies affect B cell development and selection. Long lasting modulatory effects of maternal antibodies on the immune response of the offspring have been suggested (reviewed by Lemke and Lange (9)), but responsible mechanisms remain unclear.

In the present study, we used V_{H}'D_{J}r-gene-targeted (VI10) mice expressing a BCR specific for vesicular stomatitis virus (VSV) (10) to study the impact of maternal Abs on developing B cells in offspring. VI10 mice produce a high, spontaneous VSV-neutralizing titer with IgM and IgG isotypes. VI10 mothers provided their offspring with VSV-specific (VI10) Abs by transport through the placenta and by lactation. The peripheral development and selection of specific B cells in VI10 offspring, which occurs even in the heterozygous state because of immune exclusion was analysed with a VI10-specific antibody (Ab 35.61). As a negative control, VI10
offspring with a Vi10 father and a B6 mother, thus offspring not receiving specific maternal antibodies yet capable of producing specific B cells, were investigated. Using this experimental setup, significant differences in the peripheral B cell development and selection between offspring with and without supply of specific maternal Abs were observed. Our experiments demonstrated that more specific cells accumulated in the mature, follicular B cell subset in the presence of maternal antibodies, whereas generation of specific B cells with an activated phenotype was suppressed. Strikingly, in offspring receiving VSV-specific maternal Abs, anti-VSV Ab responses following infection were positively influenced up to adulthood.

Results

Suppressing effect of high titers of preformed VSV-specific antibodies on the antiviral humoral response

A suppressive effect of specific antibodies has been described for various antigens. Possibly, the presence of high titers of virus-neutralizing antibodies at the time point of infection leads to the formation of immune complexes as soon as the virus reaches the circulation. As a result, less antigen, or in the case of a viral infection, less active virus, arrives in organized lymphoid structures, leading to a weaker antibody response (11). Additionally, binding of immune complexes to FcyRIIb on B cells is thought to inhibit B cell activation (12, 13). A third hypothesis suggests that the immunogenic structures may be masked by antibodies and can thus not be recognized anymore by B cells (12). To investigate a possible suppressive effect of preformed antibodies in our VSV-specific system, monoclonal VSV-neutralizing antibody Vi10 (mVi10 (14)) was injected to C57BL/6 mice before infection. Formation of neutralizing antibodies was indeed weaker in the presence of preformed VSV-specific antibodies (Fig. 1A). In contrast, VSV-specific memory B cells enhanced the humoral anti-VSV response. When spleen cells from B6 or Vi10 donors, which had been immunized 60 days earlier, were adoptively transferred before infection, a strong neutralizing Ab-response consisting almost exclusively of IgG Abs was induced (Fig. 1A and B). When both memory B cells and high titers of specific Abs were present at the time point of infection, an intermediate response was observed (Fig1A). The requirement of specific preformed antibodies for the suppressive effect of VSV-specific B cells was demonstrated by injecting an irrelevant mAb before infection (Fig 1C).

Thus, a suppressive effect of preformed VSV-neutralizing Abs on both the primary and memory response in adult mice was shown with VSV-specific tg and wild-type memory B cells.
Figure 1

Suppressive effect of preformed specific antibodies on the generation of anti-viral antibodies versus an increasing effect of memory B cells on anti-viral antibody formation.

A) and B) 5x10^7 VI10 or B6 spleen cells from donors that had been infected with VSV 60 days earlier were transferred to B6 recipients. Monoclonal antibody mVI10 anti-VSV was injected right after the cell transfer. 10 days following transfer of cells and/or mAb, recipient mice were infected with 2x10^6pfu VSV-IND, and VSV-neutralizing IgG titers were determined in the sera up to 260 days following cell and/or mAb transfer. C) 6x10^7 VI10 splenocytes were transferred to B6 recipients on day -1, followed by injection of 10μg monoclonal antibody mVI10 anti-VSV or 10μg of an unrelated mAb on day 0. On day 13, recipient mice were infected with 2x10^6pfu VSV-IND, and VSV-neutralizing titers of total IgG were determined in the sera at the indicated time points. Graphs represent data from 2 to 3 mice per group, mean ± SD.

Uptake of specific maternal antibodies by pups

Having found a suppressive effect of VSV-specific antibodies in adult mice, we wanted to address whether exposition of VSV-specific maternal antibodies during B cell development had a similar dampening effect on the immune response of their offspring. First, however, we analyzed the efficiency of specific antibody transmission to offspring. The virus neutralizing capacity of adult VI10 serum was at least 1:320, compared to 1:16 in B6 serum (10). Heterozygous VI10 female mice were bred with B6 male mice (breeding scheme Fig. 2A), and VSV-neutralizing serum titers in offspring were determined at different ages (Fig 2B). As a control, heterozygous VI10 male mice were bred with B6 females (Fig. 2A), and titers in the offspring were analyzed similarly (Fig. 2C).
Measurements of neutralizing Ab titers in tail-typed offspring showed that lactated or freshly weaned mice had antibody titers comparable to those of their mothers (data not shown), demonstrating that VSV-specific antibodies were transmitted normally. After weaning, VSV-specific titers decreased in B6 offspring and were not detected beyond the age of four weeks at a dilution of 1:40 (Fig. 2B), showing a short half-life of maternal antibodies in the circulation of the offspring. VI10 offspring started to produce VSV-specific antibodies soon after birth, as titers above 1:40 were observed at around two weeks of age (Fig. 2C). These data showed normal transmission of VSV-specific Abs from VI10 mothers to their offspring. Furthermore, serum levels of VI10 maternal antibodies decreased progressively in B6 offspring and dropped below a neutralizing titer of 1:40 at the age of four to five weeks.

**Figure 2**

*Normal transmission of VSV-specific maternal antibodies to offspring.*

A) Setup of breeding pairs to analyze transmission of VI10 maternal antibodies to offspring. B) and C) Total and IgG VSV-neutralizing Abs in offspring with a VI10 mother or a VI10 father were analyzed between 16d and 5wk of age. When pups were lactated by a VI10 mother, neutralizing titers decreased in B6 offspring below 1:40 within 4 weeks after birth, whereas VI10 offspring started to produce their own neutralizing Abs, leading to an increase in titers between 3wks and 5wks of age. Offspring was weaned between the age of 20 to 25 days. Data are means ± SD from 5 to 14 mice per group.
Long-term effect of a maternally shaped B cell repertoire on VSV-neutralizing responses in adult mice

Maternal antibodies can potentially influence the immune response of their offspring directly, i.e. protecting passively, or by an imprinting effect on the developing immune system of their offspring. We investigated the impact of maternal VSV-specific Abs on the humoral response in their offspring after infection with VSV, depending on the age after weaning. Therefore, homozygous VI10 mice were crossed with B6 mice as explained in the breeding scheme in Fig. 3A, and VI10 offspring were infected with $10^6$ pfu VSV-IND at four, five and ten weeks after birth. As shown in Fig. 3B, VSV-neutralizing IgG titers in VI10 offspring with VI10 mothers did rise faster than in VI10 offspring with a VI10 father, but the IgG titers that were reached after the initial burst were generally lower in the former group.

VI10 offspring with VI10 mothers generated a weak IgM response, if at all (Fig. 3B, see that total and IgG titers of offspring with VI10 mothers are largely similar), suggesting a suppressing effect of preformed specific antibodies on the IgM response. In turn, maternally derived Abs boosted the induction of VSV-neutralizing IgG antibodies, but led to a more moderate response over time. Strikingly, a difference in responsiveness after infection was still observed in 10 week old adult mice, long after maternal antibodies were lost. Two effects may be responsible for this difference: first, antibody titers in offspring with VI10 mothers were still increased at the age of 10 week compared to VI10 offspring with B6 mothers, exerting a bigger suppressive effect on the anti-viral response. Second, the observed long-lasting effect suggested an imprinting effect of maternal antibodies on the developing B cell repertoire.

Figure 3

Long-term influence of maternal antibodies on anti-viral antibody responses in offspring.

A) Breeding pairs for the analysis of anti-viral responses in offspring with or without transmission of VI10 maternal antibodies.

B) VI10 offspring with a VI10 mother showed faster, but overall weaker VSV-neutralizing IgG responses at the age of 4, 5 and 10 wks compared to VI10 offspring with a VI10 father, most likely as a result of the preimmune maternally derived Abs. In contrast, VI10 offspring with a VI10 father produced higher total Ig titers at early time points, which consisted most likely of IgM. Data are means ± SD from 5 to 7 mice per group, and experiments were repeated with different breeding pairs.
Mature phenotype of specific B cells in suckling mice

Serum antibodies have been suggested to influence B cell development in the bone marrow and selection of B cells in the periphery (6, 7). However, very limited data have been available so far showing a direct effect of maternal antibodies on the developing B cell repertoire and thus on future immune responses in offspring (15). We wanted to elucidate the effect of VSV-specific, maternal antibodies on peripheral maturation and selection of specific B cells in suckling mice. By comparing heterozygous VI10 offspring with or without supply of VSV-specific maternal antibodies, i.e. offspring with a homozygous VHO mother or father as explained in Fig. 3A, a bias to a more mature phenotype of developing B cells was observed in the presence of maternal antibodies. Fig. 4A illustrates that in VI10 offspring with a VI10 father, more B cells had an activated phenotype in a FSC and SSC analysis. When comparing T1 (IgM+HSAmedCD21) and T2 (IgM+HSAmedCD21high) cells in a FSC-SSC gate (Fig. 4A), T2 cells were more granulated and larger than T1 cells, suggesting that T2 cells were actively dividing, whereas T1 cells were in a quiescent stage. Mature and T1 B cells
had the same FSC-SSC phenotype (not shown). Looking into FSC and SSC of specific, 35.61+ splenocytes, it became obvious that the difference between T2 (IgM⁺HSA⁺medCD21⁺high) cells from offspring with or without supply of V110 maternal antibodies was the number of cells and not the differentiation state (Fig 4B). The T1:T2 ratio during peripheral B cell development of VSV-specific B cells was higher in offspring with maternal supply of specific antibodies (Table 1). These data suggested that maternal antibodies suppressed activation of developing specific B cells. Instead, accumulation of B cells with a mature, quiescent phenotype was favored in the presence of maternal antibodies. Between the age of 16 days and 6 weeks, the T1:T2 ratio decreased proportionally with or without supply of specific maternal antibodies, indicating a comparable maturation of specific B cell populations in both groups. In Fig. 4C and D, percentages of mature and transitional cells amongst the specific B cell population were compared in 16 days, 4 week and 6 week old offspring, using two different combinations of antibodies as indicated above the graphs. When absolute numbers per spleen were calculated, similar ratios between the single B cell subsets were obtained (not shown). To address whether this biased development was a specific B cell-intrinsic effect, we also analyzed 35.61⁺, unspecific B cell subsets of the same mice (Fig. 4E). The result of this analysis demonstrated that maternal antibodies influenced the developing B cell repertoire specifically, since unspecific B cells in V110 offspring developed comparably in the presence or absence of maternal antibodies.

These data demonstrated that maternal antibodies specifically suppressed the accumulation of VSV-specific B cells with an activated phenotype and positively influenced differentiation of developing VSV-specific B cells into the mature pool.

Figure 4

Biased maturation of specific B cells in the presence of maternal antibodies.

A) VSV-specific, 35.61⁺ lymphocytes of six weeks old V110 offspring with either a homozygous V110 mother or father and a B6 father and mother, respectively, were localized in a FSC-SSC graph of all splenocytes (upper panels). IgM⁺ cells with a T2 or T1 phenotype as described in D) and E) were localized in a FSC-SSC gate (lower panels). With supply of maternal antibodies, 35.61⁺ were biased to a FSC-low, non-activated phenotype.

B) FSC and SSC of 35.61⁺ T2 B cells from mice with either a V110 mother of a V110 father. Graphs are representative of one out of six animals per group.

C) The distribution of VSV-specific cells in spleens of offspring with either a homozygous V110 mother or father was analyzed at different time points of age. Splenocytes were stained with antibodies
against CD21, IgM, IgD and the VI10 BCR. Specific mature cells were identified as 35.61^+IgD^-IgM^medCD21^low and transitional cells as 35.61^+IgD^-IgM^highCD21^+. Bars represent means ± SD (n=6) of the percentages of mature and transitional B cells amongst the 35.61^+ population.

D) For the discrimination of transitional 1 (T1) and transitional 2 (T2) cells, splenocytes were also stained with antibodies against CD21, IgM, HSA and the VI10 BCR. Mature cells were identified as 35.61^+IgM^-HSA^medCD21^low, T2 cells as 35.61^+IgM^-HSA^highCD21^high and T1 cells as 35.61^+IgM^-HSA^medCD21^low. Bars represent the percentages of mature, T2 and T1 B cells amongst the 35.61^+ population (mean ± SD, n=6).

E) Unspecific, 35.61^- cells were analyzed as described in D). Bars represent the percentages of mature, T2 and T1 B cells amongst the 35.61^- population (mean ± SD, n=6). * : p < 0.03, ** : p < 0.002, *** : p < 0.0005 (unpaired t test)
Table 1
Non-activated, mature phenotype of specific B cells in the presence of maternal antibodies

<table>
<thead>
<tr>
<th>Age of mice</th>
<th>ratio T1:T2</th>
<th>ratio T1:T2</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 days</td>
<td>1.92±0.18</td>
<td>6.33±1.05</td>
</tr>
<tr>
<td>4 weeks</td>
<td>0.75±0.14</td>
<td>1.10±0.54</td>
</tr>
<tr>
<td>6 weeks</td>
<td>0.15±0.04</td>
<td>1.00±0.38</td>
</tr>
</tbody>
</table>

The mean±SD of the T1:T2 ratios of five to six animals per group are shown.

Activated, T2 phenotype of ignorant self-reactive B cells in mice expressing VSV glycoprotein

In different mouse models of autoimmunity, the self-reactive B cell population had a T2 phenotype (16-18). Assuming that VSV-specific B cells were activated by a self-antigen in VI10 mice, the observed increased frequencies of T2 B cells in the absence of maternal antibodies might represent an accumulation of ignorant or anergic B cells. We could test this by crossing VI10 mice to Max mice, which express the VSV-glycoprotein under the H-2Kb promoter (19). The neo-self antigen is thus ubiquitously expressed in a membrane-bound form. Naïve VI10xMax mice expressed very few circulating 35.61-specific B cells compared to VI10 mice due to negative selection in the bone marrow (BM) (0.8–1.5 % 35.61+ B cells in VI10xMax compared to 15-20 % 35.61+ B cells in VI10 mice). Interestingly, the T2 population in VI10xMax mice was increased compared to VI10 mice (Fig. 5A), and all specific peripheral B cells were recovered in the T2 population. This finding suggested that potentially self-reactive B cells, which had escaped negative selection in the BM, acquired a T2 phenotype in the periphery. Of note, mAb 35.61 only recognizes the original VH-VL-combination of mAb VI10, thus the tg V\textsubscript{H}-chain variable region in combination with an endogenous L-chain of the same V\textsubscript{L}-family as in mAb VI10. Sequence analysis of hybridomas generated from spleens of naïve mice had shown that most B cells in VI10 mice expressed the rearranged V\textsubscript{H}-region (data not shown). Considering the huge numbers of cells accumulating in the T2 subset in VI10xMax mice, we hypothesized that other combinations of V\textsubscript{H} and endogenous V\textsubscript{L} chains than the one recognized by mAb 35.61 may also be self-reactive.

To test whether T2 B cells in VI10xMax mice were anergic or tolerant towards live VSV, we infected VI10xMax mice with 2x10\textsuperscript{6} pfu VSV. Strikingly, the T2 population completely disappeared one day after infection, suggesting that T2 B cells were activated and eventually
formed plasma cells to generate anti-viral antibodies (Fig. 5A). V110xMax mice did indeed generate good anti-viral responses as shown in Fig. 5B, although the response was weaker than in V110 mice. FACS analysis in Fig. 5A shows that in V110 mice, the T2 B cell population also seemed to decrease following infection, but the effect was not as drastic as observed in V110xMax mice. Thus, potentially self-reactive V110 B cells in V110xMax mice acquired a T2 phenotype in the periphery. Following infection with live VSV, these cells could be activated, proving that they were not anergic, but tolerant or ignorant. This result suggested that the T2 population that developed in the absence of maternal antibodies in V110 offspring consisted at least partially of ignorant, self-reactive B cells.

**Figure 5**

*Increased levels of VSV-specific T2 B cells in mice expressing VSV glycoprotein as a self-antigen.*

A) and B) Splenocytes from V110xMax and V110 mice were analyzed by flow cytometry before (naïve), and at d 1 and d 2 following infection with 2x10⁶ pfu VSV-IND. Numbers represent percentages of T2 cells in indicated gates. Graphs shown were gated on lymphocytes. Data are representative for at least three mice per time point.

C) VSV-neutralizing titers in sera of V110 and V110xMax mice were measured at indicated time points following infection (mean ± SD, n=3).
Impact of VSV-specific antibodies on follicular versus marginal zone distribution of developing VSV-specific B cells

Marginal zone (MZ) and follicular (FO) B cells of the spleen are specialized B cell subsets, which seem to have evolved to optimize the host's humoral immune response to different antigens. Today's understanding is such that marginal zone B cells are mainly responsible for the generation of early extrafollicular plasma cell responses, whereas follicular B cells are responsible for germinal center formation, isotype switch and affinity maturation (20, 21). However, marginal zone and follicular B cells are able to take over effector functions of the other subset (22, 23). Strikingly, various BCR-transgenic mice have increased numbers of marginal zone B cells, which may be a consequence of the restricted B cell repertoire (21). VI10 mice have indeed higher numbers of MZ B cells as revealed by flow cytometry and histology. MZ B cells in six week old VI10 mice comprise 21.6±2.15% of total B cells, compared to 4.5±0.57% in C57BL6 mice. Considering the differences in the anti-VSV humoral response and in B cell maturation in offspring with either a VI10 mother or a VI10 father, we wondered whether maternal Abs would affect the selection of MZ and FO B cells. Surprisingly, we found that specific B cells in VI10 offspring with VI10 mothers were preferentially guided to the FO area (Fig. 6). The bias was likely more significant in six week old mice since the MZ is only fully developed at three weeks of age. This finding suggested that maternal antibodies significantly influenced peripheral selection of B cells, enabling anti-VSV responses with memory type kinetics, i.e. fast production of IgG following infection. However, a mechanistic explanation for the preferential selection of specific B cells in the presence of maternal antibodies of the same specificity could not be provided with the presented experiments.

Figure 6

Preferential selection of FO B cells in offspring receiving high specific maternal titers.

The distribution of 35.61± B cells amongst MZ and FO compartments was analyzed in spleens of VI10 offspring with either a VI10 mother (filled symbols) or a VI10 father (open symbols) at the age of four and six weeks. MZ and FO B cells were identified as B220+, CD21<sup>high</sup>, CD23<sup>low</sup> and B220<sup>+</sup>, CD21<sup>low</sup>, CD23<sup>+</sup> cells, respectively. Offspring that received specific maternal antibodies via placenta and lactation (e.g. offspring with a VI10 mother) showed a preferential selection of specific B cells to the FO compartment. * p<0.002, ** p=0.04 (unpaired t test).
Contribution of FcR expression to a directed B cell development in offspring

MZ versus FO selection of B cells has been suggested to depend on self antigens (24-28). Alternatively, a difference in BCR signal strength has been proposed to decide over the destiny of a B cell emigrating from the bone marrow (29). The high specific titers of VI10 offspring with VI10 mothers may influence peripheral B cell development and selection by: i) masking potential self antigens or ii) by binding of specific Abs to FcγR-expressing cells, thereby multiplying the amount of self-antigen, or by inhibiting BCR derived signals in specific B cells by FcγRllb expressed on B cells (30). To test the influence of VI10 antibodies bound to the low affinity FcγRlll on maturation and selection of VI10 B cells, VI10 mice were crossed with FcRγ chain" mice. FcγRlll is expressed on macrophages, neutrophils and mast cells and is thus widely distributed in the organism (31). VI10xFcRγ" females or males were crossed with FcRγ" males or females, respectively. We could then analyze the developing B cell repertoire in VI10xFcRγ" offspring with or without supply of specific maternal antibodies by their mothers. VI10xFcRγ" mice have the same pre-immune neutralizing titer as VI10 mice (data not shown), a prerequisite for a direct comparison with VI10 mice. Fig. 7A shows the improved maturation of specific B cells by maternal antibodies in offspring lacking FcγRlll expression, as in FcγRlll-competent VI10 mice. Similarly, a bias to FO B cell accumulation in the presence of specific maternal antibodies also seemed to occur in these mice, at least at the age of four weeks (Fig. 7B).

These results suggested that systemic binding of maternal antibodies to FcγRlll did not influence B cell development and selection in suckling mice. However, more data and studies involving additional FcγRs are required for final conclusions,
Figure 7

No effect of maternal IgG-binding to FcgRI and III in offspring on the developing immune system.

A) Spleens from FcR γ chain-deficient V110 offspring with either a FcR γ'xV110 mother or a FcR γ'xV110 father were analyzed as described in Figure 4. Bars represent means ± SD.

B) The distribution of specific MZ and FO B cells was analyzed in four and six weeks old FcR γ chain-deficient V110 offspring as described for Figure 3.

Discussion

The importance of maternal antibodies for the immune defense of newborns has long been recognized. However, suppressive effects of maternal antibodies impairing vaccination efficiencies in children have also raised doubts about the benefit of lactation in developed countries with high hygiene standards. Most studies addressing the benefit of milk-derived Abs have been performed in the context of atopy and allergy (32). Recent studies suggest that maternal antibodies increase the risk of developing autoimmune diseases and allergies in susceptible offspring, whereas others attribute a beneficial effect to maternal antibodies in this context (15, 33, 34). Furthermore, it has been observed that children whose mothers had been vaccinated during pregnancy with Haemophilus influenzae type b (HiB) generated higher anti-HiB titers following vaccination (35-37). Taken together, there is evidence that maternal antibodies influence immune responses in offspring beyond the limited half-life of antibodies. It is conceivable that the immune repertoire of mothers shape the evolving repertoire in offspring, providing a survival advantage and adaptation to environmental antigens during evolution (11).
In the present study we have investigated the effect of VSV-specific maternal antibodies in V_{n, DJ_{n}}-gene targeted mice (VI10). VI10 mice with VI10 mothers showed an initial boost of VSV-neutralizing IgG antibodies within five days following infection, compared to VI10 offspring with B6 mothers and a VI10 father which had a slower onset of IgG antibodies, yet a better IgM response. This difference in the anti-viral response may be the consequence of the preferential selection of FO versus MZ B cells in offspring with VI10 mothers. This B cell distribution seemed thus optimal for the strong onset of an isotype-switched virus-neutralizing response. Different mechanisms leading to the observed biased selection in dependence of Ab concentrations during B cell ontogeny may be considered. Many studies have investigated the dependence of MZ versus FO selection on self antigen (25-27, 38-40). Assuming that high titers of VSV-specific antigens masked cross-reactive self- or environmental antigens present in the gut flora, our data suggested that MZ selection depended on a positive signal on B cells. A scenario where maternal antibodies bound to FcγRs increase the amount of presented antigen did not seem to hold true since VI10xFcγR- mice showed the same FO/MZ distribution and B cell maturation as VI10 mice. In VI10 offspring with B6 mothers, more B cells had an activated phenotype typical for T2 and MZ B cells. Interestingly, the activated phenotype of specific cells did not result in a faster onset of IgM antibodies, as would have been expected at least for MZ B cells (41, 42). One possible explanation may be that a part of these cells was tolerized. Indeed, a T2 phenotype has been attributed to autoimmune B cells in different studies (16-18), where T2 B cells were normally or negatively selected in a healthy environment (18). VI10 mice do not show signs of autoimmune diseases, suggesting that potentially autoreactive cells are tolerized. The capacity of maternal antibodies to prevent development of activated, potentially self-reactive B cells in our VSV-specific experimental setup may be adaptable to other antigens. Maternal antibodies would thus mask self- or non-self antigen in their offspring according to the mother's repertoire.

In summary, our data showed that maternal antibodies specifically shaped the antibody repertoire and peripheral B cell development in their offspring. The observed selection of B cells with a mature phenotype and suppression of B cells with an activated phenotype may have two effects: first, development of potentially self-reactive T2 and MZ B cells and thus the onset of autoimmune diseases may be debilitated, and second, the mature B cell repertoire may enable memory-type and thus optimized immune responses following primary viral infections.
Material and Methods

Mice
All mice were kept at the Biologische Zentrallabor (BZL) of the University Hospital Zurich under SPF conditions. C57BL/6 mice were obtained from the Institute of laboratory animals (Itk) at Irchel University, Zurich. VI10 mice (10) and Max mice (19) have been described before. FcgR− mice (B6;129P2-Fcer1gtm1Rav/J) were originally purchased from Taconic, Germantown, NY, USA.

Virus and VSV neutralization assay
Vesicular Stomatitis virus, Indiana strain (VSV-IND, Mudd-Summers isolate) was originally obtained from Prof. D. Kolakofsky (University of Geneva, Switzerland). Viruses were propagated on BHK-21 cells at a MOI of 0.01 and plaqued on Vero cells. Titers of VSV-neutralizing antibodies in sera were determined as described before (43). Sera were diluted in MEM 2% FCS, and the dilution that resulted in 50% reduction of virus plaques was taken as neutralizing titer. For IgG titers, sera were incubated with equal volumes of 0.1 M 2-mercaptoethanol in PBS for 1 h at room temperature before dilution. All mouse sera were heated at 56°C for 30 min for complement inactivation.

Antibodies and flow cytometry
Anti-idiotypic antibody 35.61 specific for the VSV-neutralizing H- and L-chain variable region of VI10 was described before (10) and labelled with FITC (Sigma-Aldrich) or Cy5 (Amersham Biosciences, Buckinghamshire, England). Hybridoma 7G6 specific for CD21/35 was a kind gift of John F. Kearney. The antibody was biotinylated or labelled with FITC. All other antibodies were purchased from BD Pharmingen. For flow cytometry analysis, surface molecules were stained in FACS buffer (PBS, 2% FCS, 1 mM EDTA, 0.1 mg/ml azide) for 30 min at 4°C. For acquisition, cells were fixed in FACS buffer/1% formalin and analyzed with a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA) using FlowJo software (TreeStar Inc, Ashland, OR)

Adoptive transfer of cells and antibodies
For cell transfer, spleens of donor mice were smashed using metal grids to prepare single cell suspensions. Cells were counted by excluding dead cells with trypan blue, and injected into the tail vein of recipient mice in 200μl Balanced Salt Solution. Concentrations of monoclonal Ab VI10 or an LCMV-neutralizing mAb (IIIIC4.1) (10) as a control Ab were determined by measuring the OD, and Abs were injected into the tail vein in 200μl PBS.


The germinal center response after viral infection: new insights from vesicular stomatitis virus-specific B and T helper cells

Katja Fink, Nataly Manjarrez-Orduno, Beatrice M. Senn, Martin Holdener, Rolf M. Zinkernagel and Hans Hengartner.

Germinal centers (GCs) are still mysterious regarding their role in thymus independent responses, in affinity maturation, isotype switching and memory cell formation. These events can take place outside GCs, which raises the question: what triggers the development of GCs? We have analyzed GC formation after infection with vesicular stomatitis virus (VSV) in a transfer model with VSV-specific B and T helper cells. Following infection, GCs were built early and disappeared three or four days after their onset when T cell help was the restrictive factor. Even with adequate T cell help, GCs with specific cells were relatively short lived. Activated B cells can form extrafollicular IgM producing plasma blasts, become memory cells or differentiate to isotype switched plasma cells. At which time points B cells decide to follow one or the other path and whether the destination can still be changed once B cells have taken one route is still unclear. We have investigated the role of T cell help in this differentiation process by analyzing the kinetics of VSV-specific GC formation quantitatively and by measuring virus-neutralizing antibody formation. We show here that specific interaction of T helper and B cells suppressed extrafollicular plasma blast formation, whereas GC formation was promoted. In turn, the transient nature of VSV-specific GCs was not affected by T cell help, suggesting a B cell intrinsic mechanism deciding over GC maintenance and disappearance.
**Introduction**

When B cells are activated and migrate to the T-B cell border of secondary lymphoid organs, they interact with T helper (Th) cells, proliferate and differentiate into plasma blasts, or they join follicular dendritic cells (FDCs) in the B cell follicle, becoming GC founder cells (reviewed by (1, 2)). Initially, a germinal center response is characterized by an extensive proliferation phase of B cells, called centroblasts in this stage. Centroblasts form dense cell aggregates, which can be identified histologically as dark zones. Following proliferation, non-dividing centrocytes move to the light zone for selection. Centrocytes are either positively selected by antigen presented on FDCs, or they will die by apoptosis in the absence of an antigenic stimulus. Positively selected B cells will eventually mutate and further divide in the dark zone for a next round of selection (1, 3, 4).

Original studies on the GC reaction have mostly used haptens as immunizing agents and the established models have only partially been extended to thymus independent (TI) antigens (5-8). TI antigens are of special interest since they both trigger a potent extrafollicular response and initiate the formation of germinal centers. TI antigens represent thus a good model situation to elucidate which factors determine the fate of an activated B cell. Generally, interaction of B cells with Ag-primed Th cells has been suggested to drive GC formation (9-11). Following strong B cell activation by pathogens, vigorous extrafollicular responses are induced independent of T cell help, although an impact of (specific) Th cells cannot be excluded (12-14). These observations demonstrate a role for the activating antigen, whereas the impact of Th cells on B cell differentiation at this early time point of a B cell response is unclear.

Once a GC is established, it is unclear why it disappears again. The existence of long-lived GCs has been shown following viral infection (15), whereas protein antigens elicited transient GCs (16), suggesting a role of the immunizing antigen.

We have addressed the impact of B cell intrinsic factors and of T cell help on GC formation in an adoptive transfer model with VSV-specific B and Th cells. B cells were derived from VL10 mice expressing the rearranged V_{H}DJ_{H}-region of a VSV-neutralizing Ab. As Th cell donors, we used TCR-transgenic mice with Th cells specific for a VSV glycoprotein epitope presented on I-A\(^b\) (17). Furthermore, we compared hetero- and homozygous VL10 donor B cells since we had found earlier that homozygous cells were activated earlier than heterozygous cells, probably due to different expression levels of specific BCRs on the cell surface.
We found that specific B cells, when co-transferred with specific T cell help, efficiently led to the onset of specific GCs, whereas extrafollicular plasma cell formation was delayed. When B cells were transferred alone, most cells differentiated early into extrafollicular plasma cells after VSV infection. Homozygous VI10 B cells elicited high early IgM titers, but less IgG compared to heterozygous VI10 B cells. These data suggested that B-Th cell interaction plus B cell intrinsic mechanisms decided over extrafollicular versus follicular response. GCs formed with specific cells were always transient. Viral infection alone did thus not guarantee long-lived germinal centers, at least not with VSV-specific cells used in our experiment. We discuss the formation and performance of GCs in a viral system extends established models for the GC reaction.

Results

No germinal center formation with high affine VSV-specific B cells present at high frequencies

In VI10 mice, 15-20% of all B cells are VSV-neutralizing (18). These animals can thus generate huge titers of VSV-neutralizing antibodies following infection with a high dose of VSV. We have observed that VI10 mice form immense numbers of extrafollicular foci in the spleen, peaking at day 2 or 3 following infection. These early extrafollicular plasma cells produced mostly IgM, as revealed by flow cytometry and histology (data not shown). Preimmune neutralizing titers in VI10 mice instead consisted of both IgM and IgG isotype, and an increase of IgM and IgG was measured in the sera of infected mice (Fig. 1a). We wondered whether class switch would occur in germinal centers or in extrafollicular foci in these mice. To address this question both histologically and with flow cytometry, we infected VI10 mice with 10⁹ pfu VSV-IND and removed the spleen at day 5 following infection. Fig. 1b) demonstrates that GC structures, as identified with marker GL7, were present in spleens of VI10 mice, but that they seemed to be formed exclusively by endogenous, 35.61' B cells. This result showed that VSV-specific B cells in VI10 mice did not enter germinal center reactions and formed exclusively extrafollicular responses instead.
VSV-specific germinal center formation

Figure 1

Absence of specific germinal center formation in VI10 mice.
A) VI10 and C57BL/6 mice were infected with $2 \times 10^8$ pfu VSV-IND i.v., and the neutralizing capacity of total serum antibodies or IgG antibodies was analyzed at the indicated time points. Symbols are means ± SD, n=3 to 4 mice per group. B) Spleens of VI10 mice infected with $2 \times 10^8$ pfu or $2 \times 10^9$ pfu VSV-IND i.v. 5 days earlier were analyzed in histology with antibodies against B220 (red), 35.61 (anti-VL10) (green) and with the anti-GC B cell marker GL7 (blue). The same sections are shown with and without GL7 stain to demonstrate that GCs were derived exclusively from endogenous cells. Sections are representative for one mouse out of at least four independent experiments.

Requirement of T cell help for germinal center formation

In VI10 mice, there seemed to be a mechanism that efficiently triggered VI10 cells to enter the extrafollicular path, or a mechanism that prevented the formation of germinal centers. It is known that the interaction of B cells with Th cells is required for GC formation (10, 11, 19, 20). We therefore hypothesized that an imbalance between specific B cell frequencies and specific T cell help would prevent efficient germinal center formation in VI10 mice. To lower specific B cell frequencies, we adoptively transferred VI10 splenocytes into wild type recipients and analyzed germinal center formation at day 9 following infection. Flow cytometry analysis showed that a small population of transferred specific B cells was GL7+ (Fig. 2a). Moreover, when specific T cell help was co-transferred in the form of splenocytes from L7 mice, the population of VSV-specific cells that had entered a GC response was significantly larger (Fig. 2b).

We concluded that specific T cell help was a prerequisite for the germinal center response of VSV-specific B cells, since GC responses increased in parallel with availability of specific T cell help.
Impact of T help on the time course of germinal center formation versus extrafollicular response

In an earlier study with quasi-monoclonal (QM) mice expressing a high affinity NP-specific BCR, it was shown that GCs were formed following immunization with the TI antigen NP-ficoll in the absence of T help. However, these T cell independent GCs were short-lived (6). Although T cell help seemed to be required or at least promoted the formation of GCs with VSV-specific B cells, we wondered whether specific T cell help would affect the duration of a GC response. We therefore transferred VMO B cells with or without co-transfer of L7 Th cells into wild type recipients and analyzed serum antibody titers and splenic germinal center formation at different time points following infection.

IgM and switched VSV-neutralizing titers in the serum were higher in the presence of T cell help, showing that T cell help supported both early extrafollicular formation of IgM and the subsequent formation of IgG (Fig. 3a). However, we could not tell whether IgG was produced from GC emigrants or from isotype switched extrafollicular blasts. To address this question, we quantified GL7+ 35.61+ cells in recipient mice in the presence or absence of specific T cell help. Analysis of total recovered B cells in recipient mice showed that specific B cells expanded stronger until day 6 in the presence of specific Th cells and remained in the spleen at high numbers until day 8, whereas specific B cells without co-transfer of specific Th cells had collapsed at day 8 following infection (Fig. 3b). Fig. 3c shows that also more specific GC B cells were recovered at day 6 following infection in the presence of T cell help, whereas less GC B cells were detected at the same time point without T cell help.
When analyzing CD138+ cells for their intracellular expression of IgM, we revealed that T cell help extended IgM production by CD138+ plasma cells, since only with co-transfer of L7 cells IgM+ plasma cells could be detected at day 6 following infection, whereas the IgM plasma cell response of VI10 B cells transferred without L7 T cell help collapsed after day 4 (Fig 3D, 35.61+ IgM plasma cells). Moreover, co-transfer of T cell help induced the generation of switched (i.e. IgG) plasma cells at day 6 following infection, whereas hardly any switched plasma were recovered in the absence of L7 cells (Fig. 3d, 35.61+ IgG plasma cells).

These experiments demonstrated that early T-B cell interaction promoted GC formation following viral infection, whereas most VSV-specific B cells differentiated into early IgM-producing plasma cells in a situation with restricted specific T cell help. Formation of switched VSV-specific plasma cells was dependent on specific Th cells, providing indirect evidence that isotype switch occurred in early VSV-induced GCs.

![Graph A: VSV-neutralizing titers](image)

![Graph B: Total VSV-specific B cells](image)

![Graph C: VSV-specific GC B cells](image)

![Graph D: 35.61+ IgM and IgG plasma cells](image)
Figure 3

*Th cell-mediated increase in GC formation and isotype switch.*

3 x 10⁶ B220* sorted V110 splenocytes were adoptively transferred into B6 recipients with or without co-transfer of 1.5 x 10⁶ CD4* sorted L7 splenocytes. 24 h after cell-transfer, recipient mice were infected with 10⁶ pfu VSV-IND. A) VSV-neutralizing titers in the sera of recipient mice were measured at different time points after infection. None: no cells transferred. B) Total 35.61+ cell numbers recovered in recipient mice with or without co-transfer of L7 Th cells were determined at the indicated time points following infection. C) Additionally, a time course of specific GC formation was evaluated in the same mice by quantifying 35.61+ GL7+ lymphocytes. Each symbol represents one mouse. D) Percentages of intracellular 35.61+, CD138*, IgM* (IgM plasma cells) or IgM* (IgG plasma cells) were analyzed by flow cytometry at the indicated time points after infection (mean ± SD, n=3). All experiments were repeated, giving similar results.

Efficient germinal center formation with specific B cells expressing reduced BCR density

So far we had found that T cell help was crucial for the extent of GC formation and the generation of isotype switched Abs. We thus assumed that the low availability of T help in V110 mice would prevent formation of GCs. However, when we crossed V110 mice to gene-targeted KL25 mice expressing the rearranged VµDJκ-region of an LCMV-neutralizing Ab (18), we found: a) that B cells in V110xKL25 mice had dual BCR specificities, and b) that B cells formed specific GCs following infection with VSV (Fig. 4a). This result was surprising since the frequency of 35.61+, VSV-specific cells was comparable between V110 and KL25xV110 mice (Fig. 4b). Consequently, endogenous T cell help in KL25xV110 mice should be insufficient for GC formation of specific B cells as in V110 mice. We hypothesized that a B cell intrinsic effect accounted for the observed difference in GC formation of V110 and V110xKL25 B cells. Since B cells in V110xKL25 mice expressed both BCR specificities, it seemed reasonable that the BCR density on B cells with both specificities would be lower than on single-tg B cells. We analyzed the mean fluorescence of B cells from V110 and V110xKL25 cells stained with Ab 35.61 and found in fact that the density of the VSV-specific BCR was lower on V110xKL25 B cells compared to V110 B cells (Fig. 4c). A lower BCR density might affect the cross-linkage of BCRs by the antigen, resulting in a weaker BCR-mediated signal (21, 22). BCR density has also been found to affect B cell development in the bone marrow and in the periphery, which may influence B cell responses indirectly (23, 24).
VSV-specific germinal center formation

B cell intrinsic mechanisms deciding over extrafollicular or follicular response

The results with VI10xKL25 mice had suggested a mechanism for GC fate of specific B cells that seemed to be independent of T help. We wanted to investigate this point more closely and compared the responses of B cells from heterozygous and homozygous VI10 mice. Homozygous VI10 mice (VI10+/+) had higher numbers of VSV-specific B cells and expressed higher pre-immune titers of VSV-neutralizing antibodies compared to heterozygous VI10 mice (VI10+/−). Following infection, B cells in homozygous VI10 mice up-regulated activation markers CD86 earlier than B cells in heterozygous VI10 mice, showing a more efficient activation of homozygous B cells compared to heterozygous B cells (Fig 5a). Differences in
BCR densities were indeed not consistent between hetero- and homozygous mice, and we observed some variability within and between hetero- and homozygous groups.

Next, we compared the response of B cells from heterozygous and homozygous V110 mice following transfer to wild type recipients. Strikingly, hardly any V110+/+ B cells could be recovered at day 4 following infection in recipient mice, whereas V110+/ B cells had expanded and had also formed GCs (Fig. 5b). When measuring VSV-neutralizing titers in the sera of recipient mice, we observed that IgG titers were at least 10-fold lower in recipients of V110+/+ donor cells, whereas IgM titers were comparable between recipients of homozygous or heterozygous donor cells. The lower IgG titer was in line with strongly reduced GC formation of V110+/+ B cells, suggesting that isotype switch was supported in early GCs following viral infection (Fig. 5c). Spleen histology of recipients of V110+/+ or V110+/ B cells at day 2 following infection showed that the majority of V110+/+ B cells had differentiated to plasma blasts, whereas most V110+/ B cells showed a naïve phenotype (not shown). When L7 CD4+ T cells were co-transferred with V110+/+ or V110+/ B cells, B cells from both donors formed transient GCs, peaking at day 7 and disappearing until day 11 following infection. This result suggested that V110+/+ B cells strictly required T cell help for GC formation, whereas V110+/ B cells when adoptively transferred alone formed GCs, albeit with lower efficiency than in the presence of specific T cell help.

We thus revealed that B cell intrinsic mechanisms decided over the destiny of activated B cells to take either the extrafollicular or follicular route. In addition, experiments with co-transfers of B and Th cells showed that T cell help dominated over B cell intrinsic factors in driving the onset of a GC response.
Figure 5

Different B cell responses of heterozygous and homozygous VSV-specific BCR-tg B cells.

A) Expression of activation marker CD86 was analyzed on VI10+/− versus VI10+/+ B cells at days 1 and 2 following infection of tg mice with 10⁹ pfu VSV-IND. Shaded areas are the expression levels of naïve B cells. B) C) B cells from VI10+/− or VI10+/+ mice were adoptively transferred into B6 recipients as described in Figure 2. Graphs represent VSV-specific B cells (total specific B cells in the upper graph and specific GC B cells in the lower graph) recovered in recipients at the indicated time points following infection.
Discussion

What drives a B cell to become a GC founder cell and to generate a GC has remained an immunological mystery. A role of Th cells has been implicated, but cannot explain GC formation in the absence of T cells (6). Furthermore, at which stage B cells switch from IgM to other isotypes is still an open question. Following viral infection, IgG Abs appear rapidly, implying that the isotype switch may occur independent of GC formation in an early phase of the immune response.

In the present study we have analyzed GC formation of VSV-specific B cells in an adoptive transfer model following infection with VSV. We found that most B cells entered the extrafollicular path when specific T cell help was restricted, i.e. when only endogenous Th cells were available. The functional consequence was early production of neutralizing IgM, which may prevent cytolytic effects of systemically spreading virus and viral persistence (25). Most specific B cells were exhausted in the early extrafollicular response, and only few cells acquired a GC phenotype. In contrast, when VSV-specific Th cells were co-transferred with specific B cells, extrafollicular plasma cell formation was strongly reduced, whereas GCs were induced efficiently. This result showed that interaction of B and Th cells affected the outcome of an early humoral response following viral infection. On first sight, B cell exhaustion in the absence of co-transferred T cell help may make little sense since this strategy is potentially dangerous for the host if the virus is not cleared with the first wave of Abs. We suggest that the high affinity of the VSV-specific BCR in VI10 mice (26) was decisive for the observed strong activation of B cells (27). Ab VI10 contains hypermutations, but germline Abs against VSV are already very high affine (28), likely the result of evolutionary adaptation of the immune system to harmful pathogens, which need to be cleared early to assure survival of the host. In our viral system and in contrast to immunization with haptens, we have to consider immediate unspecific B cell stimulating mechanisms such as triggering of pattern recognition receptors (29) and induction of inflammatory cytokines (30) in addition to BCR-mediated activation. Viral activation of immune cells and the degree of viral replication may indeed influence B cell activation and isotype switching (31-33).

Interestingly, Th cells suppressed specific extrafollicular plasma cell differentiation of specific B cells and induced early formation of IgG (Fig. 3a). GCs were already formed at day 4 following infection, implying that isotype switch occurred in these early GCs. In contrast, extrafollicular plasma cells induced in the absence of transferred T help produced mostly IgM, as we had observed by flow cytometry and in histology when staining for IgM (Fig. 3D
and data not shown). Although isotype switch can occur outside germinal centers (7), this did not seem to happen when enough specific T cell help was available.

Our results with VI10xKL25 mice suggested that B cell intrinsic factors critically influenced the outcome of a B cell response. We suppose that BCR density and a subsequent altered BCR-mediated signal accounted for the different responses of VI10 and VI10xKL25 mice. Low BCR density of VI10xKL25 B cells thus seemed to provide a signal for the GC path, whereas high BCR density on VI10 B cells efficiently induced formation of extrafollicular plasma cells. In addition, our data with VI10++ and VI10+/- cells pointed to a B cell-intrinsic mechanism influencing the GC response. However, a proposed difference in B cell activation needs to be addressed on a molecular level to allow this conclusion.

In summary, we find that at least three factors contributed to the decision whether virus-activated B cells were destined to the extrafollicular- or GC response: a) The availability of specific T cell help, b) The precursor frequency of specific B cells, and c) the efficiency of B cell activation. When B cells were activated early by a strong stimulus, B cells exclusively entered the extrafollicular response, whereas a more moderate B cell activation also led to the formation of GCs. When T cell help was not limiting and/or when the precursor frequency of B cells was low, efficient GC formation was induced. Moreover, even strongly activated B cells can be directed into a GC reaction in the presence of specific T cell help.

**Material and Methods**

**Mice**

Homo- and heterozygous VI10 mice (18) and L7 mice (17) were kept at the Biologische Zentrallabor (BZL) of the University Hospital Zurich under SPF conditions. C57BL/6 mice were obtained from the Institute of laboratory animals (Itk) at Irchel University, Zurich.

**Virus**

Vesicular Stomatitis virus, Indiana strain (VSV-IND, Mudd-Summers isolate) was originally obtained from Prof. D. Kolakofsky (University of Geneva, Switzerland).

**VSV-neutrlization assay**

Titers of VSV-neutralizing antibodies in sera were determined as described before (15). Sera were diluted in MEM 2% FCS, and the dilution that resulted in 50% reduction of virus plaques
was taken as neutralizing titer. For IgG titers, sera were incubated with equal volumes of 0.1 M 2-mercaptoethanol in PBS for 1 h at room temperature before dilution. All mouse sera were heated at 56°C for 30 min for complement inactivation.

**Sorting of cells**

B cells from V110 spleens were sorted with B220-specific beads, and T helper cells from L7 spleens were sorted with CD4-specific magnetic beads (MiltenyiBiotech, Bergisch Gladbach, Germany) for adoptive transfers. Before sorting, erythrocytes were lysed with 1.66% NH₄Cl. Purity of B220⁺ and CD4⁺ cells was between 90% and 96%.

**Flow cytometry**

Anti-idiotypic antibody 35.61 specific for the VSV-neutralizing H- and L-chain variable region of VI10 and anti-idiotypic antibody IIIC4.1 specific for the LCMV-neutralizing H-chain variable rebion of KL25 were described before (18) and labeled with FITC (Sigma-Aldrich) or Cy5 (Amersham Biosciences, Buckinghamshire, England). All other antibodies were purchased from BD Pharmingen. For flow cytometry analysis, surface molecules were stained in FACS buffer (PBS, 2% FCS, 1 mM EDTA, 0.1 mg/ml azide) for 30 min at 4°C. For intracellular staining of plasma cells, cells were fixed and permeabilized with Cytofix/Perm-Solution from BD Pharmingen. Fixed cells were then incubated with antibodies in FACS buffer containing 0.1% saponin for 30 min at 4°C. For acquisition, cells were fixed in FACS buffer/1% formalin and analyzed with a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA) using FlowJo software (TreeStar Inc, Ashland, OR)

**Fluorescent histology**

Freshly removed organs were immersed in Hanks Balanced Salt Solution and snap frozen in liquid nitrogen. Five μm tissue sections were air dried, fixed with acetone for 10 min, and stored at -70°C. Cryosections were blocked for 30 min with 1 μg/sample of the Fc-blocking antibody 2.4G2, washed in PBS, and incubated with the respective fluorescent antibodies for 1 h min at 4°C. If necessary, streptavidin-Cy3 was added in a second step. After washing in PBS, nuclei were counterstained with 4',6'-Diamino-2-phenylindolehydrochloride (DAPI, Sigma), and sections mounted with fluorescence mounting solution (DAKO, Carpinteria, CA). Fluorescence was analysed with a Zeiss Axiophot microscope (Carl Zeiss, Feldbach, Switzerland). Color channels were assembled automatically with the analySIS software (Soft Imaging System, Münster, Germany), and images were processed using Adobe Photoshop, without non-linear operations.


Discussion

VSV as polyclonal B cell activator

When C57BL/6 mice are infected with VSV, a large proportion of B cells up-regulates B cell activation marker CD69 and down-regulates CD62L few hours after infection. Furthermore, CD21/35 (complement receptor 1/2) and CD23 (FceR) are down-regulated. This activation cannot be Ag-specific since the activated population is too large to be VSV-specific. When C57BL/6 splenocytes are infected in vitro with VSV, or when they are incubated with UV-VSV in the presence of IFN-α, splenic B cells produce low amounts of IgM, suggesting that some cells differentiate into plasma cells following binding of VSV (see Fig. 1a, page 35). One explanation for this observation would be that VSV-specific B cells in the C57BL/6 repertoire are activated and expand. It has been shown that C57BL/6 produce a considerable pre-immune VSV-neutralizing titer (1). Moreover, VSV-specific B cells can be identified in histology of spleens from wild type mice already four days following infection (2). However, the frequency of VSV-specific B cells at day four after infection is too small to be detected in flow cytometry with biotinylated VSV or with VSV and a secondary anti-VSV Ab. In summary, VSV activates far more cells than can be VSV-specific. The recent finding that VSV can bind to TLR4 on macrophages and specifically induce production of IFN-α and other cytokines is worth mentioning in this context (3). TLR4 is well known as a ligand for the polyclonal B cell stimulator lipopolysaccharide (LPS). In the study of Jiang and Co-workers, binding of VSV to TLR4 has indeed only been shown with macrophages, which required a CD14-mediated co-signal to start cytokine production. CD14 is expressed on macrophages, but not on B cells. Nevertheless, it may be worthwhile to analyze the activation of TLR4⁺ B cells upon binding of VSV.

This aspect of polyclonal activation observed after VSV infection may provide an explanation for differences in the course of the humoral response when comparing VSV with haptens or artificial TI antigens such as ficoll as immunizing agents. Nevertheless, it is the unspecific induction of inflammatory cytokines that makes VSV infection attractive as a physiologically relevant model for viral infections.
B cell selection in VI10 mice

Marginal zone B cells

VI10 mice show up to 50% reduced B cell numbers compared to wt mice (4). Analysis of the BM did not reveal a block at a specific developmental stage, although total B cell numbers were already reduced in the BM. The CD19^{int}B220^{int} immature B cell fraction was slightly increased, in contrast to a reduced mature fraction, suggesting that negative selection of developing B cells in the BM is substantial in VI10 mice.

Marked differences between B cell repertoires of VI10 and wild type mice become manifest in the periphery. The most striking observation is the enlarged MZ B cell population in VI10 spleens. Despite some variability in the distribution of specific, 35.61^{+} B cells between the MZ and follicular compartment, we observed a bias of specific cells to accumulate in the MZ (about 45% in the MZ versus 35% in the follicles). Enlarged MZs have been observed in many BCR-tg or -knock in lines (reviewed in (5)). Martin and Kearney suggest that the increase in MZ B cells is a means to compensate for a restricted B cell repertoire. According to their opinion, the MZ B cell pool as a first line of defense for blood-borne Ags is specifically increased to combat harmful invaders early and efficiently (5). Our data from heterozygous VI10 offspring with a homozygous VI10 mother and a B6 father showed a bias to follicular B cell development in the presence of maternal VI10 Abs (see Fig.6, page 87). We hypothesized that VSV-specific maternal Abs masked cross-reactive self-Ags in the offspring, and that MZ B cell development was basically determined by positive selection of developing B cells on self-Ags. Several recent studies addressing the question of peripheral B cell selection argued in the same line (6-10).

The fast and vigorous extrafollicular plasma cell formation of VI10 B cell suggested that MZ B cells were responsible for this type of response (11, 12).

In spleen histology the MZ seemed to be emptied 12 hours following infection. Both in VI10 and wild type mice, white pulps appeared contracted after 24 hours, indicating that the MZ B cells were main responders following intravenous infection with VSV. This makes sense when considering the location of MZ B cells. VSV arriving via arterial blood is emptied into the marginal sinus, and MZ cells thus first see the Ag (13). However, following their arrival in the MZ, also follicular B cells participate in the early extrafollicular response (see Fig.1, page 58)
Transitional B cells

In addition to an increase in the MZ population, we also found enlarged transitional B cell populations in V110 mice. 35.61* B cells were particularly enriched in the CD21\textsuperscript{high} IgM\textsuperscript{high} HSA\textsuperscript{med} transitional T2 population (Fig. 4, page 84). We have not analyzed yet whether there is a specific block at the T2 stage, preventing transition of specific B cells from the T2 to the mature stage. Moreover, we can not exclude that the T2 population V110 mice as described in the manuscript “Shaping of the developing B cell repertoire by maternal antibodies in favor of maternal specificities” contains MZ B cells, although MZ B cells have been described to be HSA\textsuperscript{low} (14, 15). The activated phenotype of the cells we assign T2 B cells in our experiments is indeed generally attributed to MZ cells (12). In V110xMax mice, where the T2 population was “erased” at day one following infection, we observed a partial recovery of this population already two days after infection, showing very efficient homeostatic reconstitution of this pool (Fig. 5, page 86). It is unlikely that “sessile”, slowly proliferating MZ B cells (8, 13) account for this fast recovery.

Considering the disappearance of the T2 population following infection, we wondered whether these cells would mature (16) and differentiate into plasma cell, or whether T2 cells would directly differentiate into plasma cells. Preliminary results from BrdU incorporation experiments performed one and two days after infection did not support maturation of activated T2 cells. In light of the fast kinetic of plasma cell formation in V110 mice, it is also possible that T2 cells do not expand upon activation, but differentiate directly into blasts instead. This scenario has not been described before in the literature and may demonstrate an additional pathway of B cell responses.

Plasma cell formation of VSV-specific B cells

As outlined in the introduction, the functional relation between plasma blasts and plasma cells is unclear. During our studies with VSV-specific B cells we also brought up the question whether fully differentiated plasma cells necessarily go through a plasma blast stage. Transfer of CFSE-labelled sorted V110 B cells into recipients allowed the analysis of proliferation of specific cells following infection. At day two after infection, when both plasma blasts (B220\textsuperscript{CD138}) and plasma cells (B220\textsuperscript{CD138+}) were present, the proliferation profile of these cell populations was analyzed. We found that plasma cells had divided only once or twice, whereas plasma blasts showed a more variable distribution with most cells having divided more than two times. Moreover, intracellular staining with 35.61 showed that plasma cells contained more Ig, indicating that plasma cells are “high producers” of secretory IgM.
This finding does indeed not exclude that proliferating blasts differentiate into plasma cells later in the course of the response. At day four after infection, new CFSE-negative plasma cells could be detected, possibly representing the end product of blasting cells observed at day two.

We hypothesize that two waves of plasma cells are produced in mice following infection with VSV: the first wave may derive directly from MZ or T2 B cells without expansion, and a second wave of plasma cells, which requires B cell proliferation, may derive from follicular B cells. So far, IgM plasma cells could be identified only at day four after infection, suggesting that isotype switched Igs are only produced by the second wave of plasma cells.

**General discussion and outlook**

This thesis addresses various aspects of the anti-viral humoral immune response following infection with VSV. To this end, gene-targeted BCR-tg VI10 mice were studied, aiming at contributing to a characterization of this mouse strain which had only recently been established (4). Despite the advantages of this VSV-specific strain, there are limitations of studying B cell responses in VI10 mice. The un-physiologically high frequency of specific B cells and the high pre-immune titer in VI10 mice require high viral doses (10⁶ pfu VSV-IND) to trigger a response comparable to the wild-type situation. Immune complex (IC) formation must be immense in these mice after infection. Even without infection, ICs may be formed with self-antigens, as suggested in the manuscript “Shaping of the developing B cell repertoire by maternal antibodies in favor of maternal specificities”. IC with VSV can indeed increase a secondary response (17). However, GC formation has been shown to occur independent of IC formation on follicular dendritic cells (FDCs) (18). ICs can indeed also be deposited on macrophages, monocytes or B cells themselves, exerting an enhancing effect on the B cell response independent of FDCs and GC formation. More experimental data will be required to clarify this point.

To circumvent high precursor frequencies and pre-immune titer, B220⁺ sorted VSV-specific B cells were transferred into wt recipient. In the project “Early Type-I interferon-mediated signals on B cells specifically enhance anti-viral humoral responses in mice”, transfer of IFN-α/βR⁺×VI10 B cells allowed us to study the effect of IFN-α directly on B cells, i.e. independent of Th cells and DCs. Thereby we have revealed an enhancing effect of IFN-α on B cell activation, differentiation to plasma blasts and antibody production. An activating and pro-survival effect of IFN-α on BCR-activated B cells has been shown earlier on a molecular level (19). Complementary to these data, we reported the functional consequence of IFN-α,
acting on specifically activated B cells in vitro and in vivo. The VSV-specific system that we used links the effect of IFN-α induction by the virus and its direct action on specific B cells in vivo, resulting in enhanced B cell responsiveness and protection from VSV infection.

In the study “Extrafollicular plasma cell formation and isotype switch after viral infection is enhanced by CCR7-dependent traffic to the T-B cell zone border”, we have addressed the impact of CCR7 expression on B and Th cells on the anti-viral response. Therefore we co-transferred CCR7-deficient Vi10 specific B cells and CCR7-deficient specific L7 Th cells into wt recipients. Three important observations were made: first, CCR7-mediated contacts of B and Th cells at the T-B border and in the T cell zone following viral activation significantly supported B and Th cell expansion. Second, CCR7-mediated T-B contacts and subsequent Th and B cell proliferation were required for efficient Ab production in the case of low amounts of antigen, i.e. following immunization with VSV-G, whereas Ab titers were not impaired after infection with live virus. This result showed that VSV induces a B cell response independent of T cell help, as has been shown before (20), and that the induction of IgG in not impaired at high B cell frequencies, which may enable contacts with Th cells independent of CCR7. The efficient Tl B cell activation may not only depend on the amount of antigens, but is likely the result of innate mechanisms triggered by VSV. Third, B cells could potentially act as APCs and induce proliferation of specific Th cells as soon as 12 h after infection, and T-B contacts at the T-B border were independent of DCs (data not shown). The “three cell contact model” involving B cells, T cells and DCs (21) was thus not valid for induction of early extrafollicular B cell responses. B cells can be activated without DC-induced T cell help, and it seems to be the B cells that support Th cell expansion rather than vice versa in that situation (22). For efficient activation of B cells, BCR-independent second signals are indeed required. Such unspecific signals are induced after viral infection (23, 24).

Binding of VSV to pattern recognition receptor TLR7 and the consequent induction of the anti-viral cytokine IFN-α has been studied in the first manuscript, showing that TLR7 is only one component of a redundant system, ensuring efficient triggering of the innate immune response by VSV (25).

Availability of several knock-out strains lacking components of the innate B cell activation system provide excellent experimental tools to dissect unspecific viral effects from BCR-mediated B cell activation in the future. This knowledge is required to reconsider and improve vaccination strategies and anti-viral treatments.
Discussion


## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>(4-hydroxy-3-nitrophenyl)acetyl NP</td>
<td>Antibody</td>
</tr>
<tr>
<td>Ab</td>
<td>Antigen</td>
</tr>
<tr>
<td>Ag</td>
<td>A proliferation-inducing ligand</td>
</tr>
<tr>
<td>APRIL</td>
<td>B cell activating factor</td>
</tr>
<tr>
<td>BAFF</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BCR</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BM</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DC</td>
<td>Follicular Dendritic cell</td>
</tr>
<tr>
<td>FDC</td>
<td>Immune complex</td>
</tr>
<tr>
<td>IC</td>
<td>Interferon</td>
</tr>
<tr>
<td>IFN</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>Ig</td>
<td>Lymph node</td>
</tr>
<tr>
<td>LN</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MHC</td>
<td>Marginal zone</td>
</tr>
<tr>
<td>MZ</td>
<td>p-azophenylarsonate Ars</td>
</tr>
<tr>
<td>phosphorylcholine</td>
<td>PC</td>
</tr>
<tr>
<td>Sheep red blood cells</td>
<td>SRBC</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Tg</td>
<td>Transgene, transgenic</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TI</td>
<td>Thymus independent</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>VSV</td>
<td>Vesicular Stomatitis Virus</td>
</tr>
</tbody>
</table>
Bibliography


*both authors contributed equally to this work

In revision:

Acknowledgements

First of all I would like to thank Hans and Rolf for their support and for the good working and social atmosphere in the ExpImm. I appreciated the independence I was given to realize my ideas and to choose the research fields I was interested in.

When I started my thesis, it was Bea who introduced me into the immunology field, and she did that in a great way. I would like to thank her for everything she did for me.

Then I would like to thank my PhD- and lab-fellow Rafi for sharing scientific and other sorrow. I really liked to work with you, Rafi. Who knows, perhaps we will meet again somewhere in the world for a new collaboration?

During my thesis, all members of the ExpImm contributed to a very nice atmosphere, and I would like to thank all of them. In particular, I would like to thank the G53 lab members for many enjoyable and entertaining days (Lars und Tili...), and my PhD fellows Vero and Philipp for being a good team.

I thank Martin and Nataly for the excellent work they did during their time in our lab. They have both contributed a lot to my projects.

A very big thankyou goes to Jacqueline for being a wonderful friend, for listening to my problems and for all the nice girl talk. Without her, life in the lab would have been much colder.

For the CCR7 project I had a very nice collaboration with Elke and Burkhard, and I want to thank them for their support and their interest. I always enjoyed being in St.Gallen, and I hope that we can finish our project successfully.

I would like to thank all my friends and colleagues outside the lab. They were an important change to the scientific side. I am thankful that they always reminded me that immunology is probably not the most important thing in the world and that they helped me to see my work in a different light. Thank you all for being so patient with me.

Finally I would like to thank my parents and my sisters Petra and Bettina. It is invaluable to have you.
Curriculum vitae

Name: Katja Fink

Work address: Institute of Experimental Immunology
University Hospital Zurich
Schmelzbergstrasse 12
8091 Zurich
Switzerland
Telephone: +41 44 255 37 87
Fax: +41 44 255 44 20
e-mail: katja.fink@usz.ch

Home address: Dorfstrasse 31
9535 Wilen

Nationality: Swiss
Date of birth: 6.3.1975
Citizenship: Mosnang SG and Aadorf TG

Education
1982 – 1988 Primarschule Bichelsee
1988 – 1991 Sekundarschule Balterswil-Bichelsee
1996 – 2001 Graduate Studies in Biochemistry, University of Zurich
degree dipl.biochem.
Thesis title: "Characterization of Calsyntenin-3: Generation of antibodies
against Calsyntenin-3 and in situ hybridization" under the supervision of
Gustav Hintsch and Prof. P. Sonderegger, Institute of Biochemistry, University
Zurich
2001 – 2005 PhD student at the Institute of Experimental Immunology, University
Hospital Zurich under the supervision of Prof. Hans Hengartner and Prof. Rolf
M. Zinkernagel