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Mechanisms of Establishment and Maintenance of RNA Virus Persistence in Primary Lymphocytes: a Dissertation

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MECHANISMS OF ESTABLISHMENT AND MAINTENANCE OF RNA VIRUS PERSISTENCE IN PRIMARY LYMPHOCYTES

A Dissertation Presented

By

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Abstract

RNA virus persistence in lymphocytes has been studied extensively *in vitro*, but the influence of lymphocyte homeostatic mechanisms and antiviral immunity on persistence has not been well studied in an *in vivo* system. It is demonstrated here that vesicular stomatitis virus (VSV), a negative-strand RNA virus, is maintained in B lymphocytes *in vivo* despite the existence of homeostatic mechanisms that drive the cells to proliferate under conditions of B cell deficiency and a strong antibody response to the virus. It is also shown that antiviral antibodies inhibit VSV reactivation from persistently infected primary B cells *in vitro*. A model is proposed for virus persistence *in vivo* in which B cell homeostatic signals drive virus expression in some infected cells, resulting in an antibody response, which maintains virus persistence in B cells.

In the course of conducting experiments to define the homeostatic signals that might act on persistently infected B cells *in vivo*, it was found that a fraction of small, resting splenic B cells proliferates after adoptive transfer into B cell deficient hosts (sublethally irradiated, *xid*, or SCID). This process, termed homeostatic proliferation, is driven by B cell deficiency since proliferation is limited in B cell sufficient hosts. This reveals the existence of a mechanism by which B cells sense their own numbers. The proliferation is unique in that the replicating cells do not upregulate cell surface markers, such as CD25 and B7-2, associated with antigen or mitogen induced proliferation. They do, however, show transient increases in other activation markers (CD69, CD71), demonstrating the action of an inductive signal. Homeostatic proliferation is a property of both mature and immature B cells, but in competition experiments, only mature B cells

inhibit proliferation. *xid* B cells express a defective form of Bruton's tyrosine kinase (Btk); as a result, these cells proliferate poorly in response to stimulation through a number of cell surface receptors including the BCR, IL-5R, IL-10R, the toll-like receptor RP-105, and CD38. Homeostatic proliferation is severely reduced in *xid* B cells; thus, this process is regulated by a Btk-dependent inductive signal, which is counterbalanced by an inhibitory signal provided by mature B cells. B cell homeostatic proliferation does not rely on transcription factors (c-rel and p50) critical for conventional proliferation induced by antigen or mitogen (c-rel), or for peripheral B cell survival (p50), suggesting that multiple signals drive this process and that survival and proliferation signals are not identical.

VSV persists in small, resting primary B cells for several weeks *in vitro*, and virus replication is restricted at multiple levels depending on the activation state of the cells. After adoptive transfer of infected B cells into B cell deficient (*xid*) recipients, viral RNA, but not infectious particles, can be detected by RT-PCR in recipient spleens for at least 72 days. RT-PCR analysis of FACS sorted donor cells stained with CFSE reveals that viral RNA is maintained in transferred B cells but can also found in recipient cells. Infected B cells can undergo homeostatic proliferation and an antibody response is generated to the virus, suggesting that homeostatic signals induce virus expression in some transferred cells. Virus persistence is maintained despite an active immune response to the virus. In fact, persistence may be maintained by antiviral antibody since *in vitro* treatment of infected primary B cells with anti-VSV antibody inhibits virus reactivation at multiple levels (transcription, protein synthesis, assembly/release of

infectious particles). This inhibition is reversible upon antibody removal, demonstrating that functional virus is maintained in antibody treated cells. Antibody specific for a single viral protein (VSV G) is sufficient since inhibition is mediated by monoclonal antibodies specific for a VSV G; neutralizing activity is not required because inhibition occurs with non-neutralizing monoclonal antibodies to VSV G. It is proposed that antibody binding to VSV G on infected B cells generates inhibitory signal(s) that suppress signaling pathways required for virus replication in B cells. Finally, a model of RNA virus persistence in B cells is proposed in which lymphocyte homeostatic signals promote virus expression, leading to the production of antiviral antibodies, which suppress virus replication inside infected B cells and help to maintain persistence.

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Introduction

Establishment of persistent infections is a property of many RNA and DNA viruses. Persistent infections are generally characterized by the maintenance of viral genomes in a non-infectious form (latent infection) or continuous production of virus through non-cytolytic replication in host cells (chronic) (1). Herpes simplex virus (HSV) is a double-stranded DNA virus that productively infects epithelial cells but establishes latent infection of neurons; viral DNA is maintained, but no infectious virus is produced and the only viral transcripts seen are the latency-associated transcripts (LATs) (1). Infections of this kind are associated with periodic reactivation of infectious virus from latently infected cells, resulting in disease. This example illustrates two strategies by which viruses establish persistence; diminished viral gene expression in certain cell types and infection of immunologically privileged tissues such as neurons (2). Chronic infections, on the other hand, are associated with the continuous shedding of low levels of virus over the lifetime of the host. Lymphocytic choriomeningitis virus (LCMV) is a single-stranded RNA virus containing two genome segments (3). Infection of neonatal mice results in a chronic infection in which persistence is favored by a non-lytic replication strategy as well as suppression of the immune response to the virus (2). In some cases chronic production of viral antigens results in the formation of immune complexes which can be deposited in sites such as the renal glomeruli, resulting in disease (3). These two examples clearly show that multiple mechanisms can lead to persistent infection. Elucidating these mechanisms is important for two reasons. First,

persistently infected individuals are natural reservoirs for virus and can disseminate the infection to others in the population. Second, persistent viral infections may also cause recurrent or chronic disease in the host. Understanding how these infections occur may lead to the development strategies to interfere with the establishment or maintenance of persistence or to induce virus clearing from the host.

Despite the absence of overt cytopathic effects in persistently infected cells, there is strong evidence that such infections can result in alterations of cellular functions and, in some cases, disease. In the C3H/ST strain of mice, LCMV persistent infection is associated with retarded growth as the result of non-cytolytic virus replication in growth hormone producing cells in the anterior pituitary (4, 5). This reduction in growth hormone production is the result of specific inhibition of growth hormone transcription by the viral protein NP (2). Borna disease virus (BDV) is a non-cytolytic, negative-strand RNA virus which causes a persistent infection of the CNS in neonatally infected mice (6). Such infections are associated with a number of behavioral changes and developmental abnormalities of the CNS including impaired learning abilities, loss of spatial discrimination, and diminished taste (6-8). Taken together, these studies demonstrate the potential for viruses that persist to subtly affect cellular function and cause disease.

A common property of many viruses causing persistent infections is the ability to infect lymphocytes, sometimes resulting in altered lymphocyte function leading to immunodeficiency (9). Infections with measles virus, a negative-strand RNA virus have long been associated with immunosuppression, and experimental infection of human

lymphocytes with this virus inhibits lymphocyte proliferation, NK cell activity and secretion of immunoglobulin without affecting cell viability (9, 10). Suppression of B cell proliferation and antibody secretion is a direct effect of measles virus infection of B cells, and may be the result of measles nucleocapsid protein binding to Fc receptors on B cells (11, 12). Measles virus infection of T cells does not affect cytokine secretion, but does inhibit T cell proliferation, by the impairment of IL-2 receptor signaling mediated through Akt kinase (9, 13). There is evidence that interactions with the measles virus glycoproteins F and H on infected cells inhibits proliferation in uninfected cells, providing a mechanism by which a small number of infected lymphocytes could mediate general immunosuppression (14). Thus, measles virus can alter the function of infected and uninfected lymphocytes without killing the cells. Another example of immunosuppression associated with virus infection of lymphocytes is HIV, which predominantly infects $CD4^+$ T cells, but can also infect dendritic cells and macrophages (15). HIV infection gives rise first to functional defects in $CD4^+$ T cells (reduced proliferation in response to antigen or mitogen) which is followed by a depletion of these cells over time; some of the proposed mechanisms for $CD4^+$ T cell depletion are direct cell killing by HIV, formation of syncytia with uninfected cells, immune clearance of infected cells, and disruption of T cell homeostasis (16). Thus, immunodeficiency associated with infection of lymphocytes with measles virus, HIV, and many other viruses is well documented and of great medical importance.

It has been demonstrated that a number of RNA viruses, including poliovirus, measles, influenza A, vesicular stomatitis virus (VSV), and lymphocytic choriomeningitis

virus (LCMV) can infect lymphocytes (9). The ability of RNA viruses, especially highly cytopathic ones such as VSV, to persist in lymphocytes *in vivo* and the mechanisms involved have not been established. VSV is an ideal virus for these types of studies because its replication and persistence (in lymphocytes and other cell types) have been well studied *in vitro*. VSV is a negative-strand RNA virus that causes a disease of great economic importance in cattle and horses (17). The replication of VSV has been reviewed elsewhere (17) and will be briefly summarized here. VSV encodes five proteins (N, P, M, G, and L) which are found in the virion plus two proteins derived from a second reading frame within the P mRNA that are only found in infected cells (18, 19). Attachment occurs by binding of the viral glycoprotein (G) to phosphatidylserine on target cells and virus enters cells by receptor mediated endocytosis; subsequently, the entire viral replicative cycle occurs in the cytoplasm. Transcription and genome replication are carried out by the RNA-dependent RNA polymerase (a complex of L and P proteins) carried in the virion. The template for transcription is the genomic RNA complexed with nucleoprotein (N); transcription requires phosphorylation of P and is downregulated by matrix (M) protein. The switch from transcription to replication of the viral genome requires de novo synthesis of viral N and P proteins. The N, P, and L proteins then form ribonucleoprotein (RNP) cores, which associate with G protein at the plasma membrane through interactions with M protein, followed by budding and release of infectious particles.

VSV is generally considered to be highly cytolytic; however, persistence of the virus, defined as a low level of virus replication in the absence of cytopathic effect, has

been demonstrated in cell lines such as baby hamster kidney cells and mouse L cells. In these *in vitro* systems, restriction of virus replication and inhibition of cytopathic effects occur by three mechanisms; generation of defective interfering (DI) particles, generation of viral mutants, and the induction of interferon. DI particles carry genomes containing significant deletions such that they only replicate in cells co-infected with wild-type virus; furthermore, they interfere with replication of wild-type virus by efficiently competing for viral replicative proteins (20). Persistence of VSV in cell lines can be established by co-infection of cells with wild-type virus and DI particles; this limits wild-type virus replication and minimizes cytopathology (21, 22). Long term persistence of VSV in cell culture also results in the appearance of temperature sensitive (ts) viral mutants that may be important in maintaining persistence; such mutants can establish persistence without DI particles, demonstrating that persistence does not absolutely require DI particles (23, 24). These ts mutants have a small plaque phenotype, and the mutations generally fall within the polymerase gene (L) or matrix protein gene (M) resulting in less efficient virus replication and the inability to carry out host shut-off functions (24-27).

Host cell factors may also be involved in maintaining persistent infections with VSV *in vitro*. Interferons are a class of cytokines known to induce an antiviral state in responsive cells by restricting virus transcription, translation, and assembly of virus particles (28). Pretreatment of mouse L cells with interferon followed by infection with VSV leads to a persistent infection characterized by chronic low level virus production with limited cytopathic effect (25). Small plaque mutants and DI particles are also

generated in these cultures, suggesting virus replication is restricted by a combination of interferon, viral mutants, and DI particles (25). This idea is reinforced by the finding that some DI particles and ts mutants generated during persistent infections *in vitro* are strong inducers of interferon (29, 30). VSV in persistently infected cells seems to be subject to constant selective pressures since genome sequences change constantly over time; this suggests that persistence in these *in vitro* models is a result of continuous evolution of the virus which favors the generation of less cytopathic variants (31). Thus, in cell culture models, VSV replication and cytopathic effects can be controlled by a combination of viral and host cell factors resulting in cultures that can be persistently infected for years.

The studies described above have examined VSV persistence in cell lines that are normally fully permissive for virus replication. Persistence can also be established and maintained in cell types that are nonpermissive for virus replication. A number of viruses, including VSV, can infect resting lymphocytes, but virus replication is not supported; antigen or mitogen stimulated lymphocytes, on the other hand, support full virus replication (32-36). Thus, lymphocytes can act as conditionally permissive host cells for viruses. Studies in this laboratory have shown that VSV can infect small, resting primary splenic B cells, but no viral proteins or infectious particles are produced and only low levels of viral transcripts are detected (35, 36). All stages of virus replication can be induced by treatment of infected cells with B cell specific activators, and different B cell activation pathways support different levels of virus replication (35, 36). For example, treatment of infected B cells with IL-4 enhances viral mRNA levels, but viral proteins

and infectious particles are not produced; treatment with PMA enhances viral protein production while stimulation with anti-Ig+IL-4 makes B cells fully permissive for virus replication, resulting in production of infectious virus (36). Signals for B cell proliferation and differentiation are not required for virus replication because some activator combinations such as PMA+IL-4 make B cells fully permissive for virus replication without inducing cell proliferation or antibody secretion (36). Thus, virus replication can be regulated at many levels in B cells, and the ability to produce infectious virus identifies a stage of B cell activation not apparent by conventional assays such as [^{3}H]TdR incorporation or antibody secretion. More recent studies have demonstrated that VSV can be maintained in culture for weeks in small resting B cells from bcl-2 transgenic mice (B/bcl-2 cells) with no viral proteins or infectious virus produced; virus can be reactivated from these cells at any time after the initiation of persistent infection by treatment with B cell activators (M. Schmidt, manuscript in preparation). This demonstrates that an RNA genome can be maintained in the absence of continuous virus replication; thus, RNA virus persistence can be maintained by mechanisms other than the generation of DI particles and virus mutants. During *in vivo* persistent infections, the ideal host cell should have two properties: first, it should maintain virus without expressing viral proteins so as to evade the host immune response; second, it should have the ability to periodically produce infectious virus to maintain persistence within the host and allow for the dissemination of virus to other hosts. Given the properties of VSV infection of primary B cells *in vitro,* it is proposed here that resting B cells harbor RNA viruses such as VSV during persistent infections *in vivo*. It is also

proposed that signals involved in the maintenance of B cells (homeostatic signals) induce virus expression in these cells while immune mechanisms suppress virus replication and maintain persistence. In order to better understand how VSV infected B cells might be maintained *in vivo*, it is important to understand how lymphocytes are maintained and the role immune responses play in persistent infections.

Lymphocyte homeostasis refers to the maintenance of a relatively constant number of lymphocytes in the periphery throughout life. It reflects a balance between the production of cells in the primary lymphoid organs and the loss or expansion of these cells in the periphery. This process is regulated at many levels by signals through the antigen receptor and other molecules on the surface of lymphocytes. In the thymus, signals through the T cell receptor (TCR) mediate positive and negative selection, a process by which T cells are selected for their ability to recognize non-self peptides in the context of self-MHC (37). In addition to selection in the bone marrow, B cells are also subject to a selection step in the periphery during the transition from immature to mature B cells, which involves signals through the B cell receptor (BCR) (38). These selection steps regulate the number of lymphocytes that are able to leave the primary lymphoid organs and enter mature lymphocyte pools. Once lymphocytes become part of the long-lived recirculating pool, survival of both B and T cells is an active process requiring signals induced through lymphocyte interaction with environmental ligands that control the expression of anti-apoptotic molecules (39, 40). These signals are delivered to lymphocytes through their antigen receptors (41-43), and to B cells by other molecules including CD40L, IL-4, and BAFF/Blys (44-46). Similarly, survival of naive T cells is

influenced by additional factors; primarily IL-7 and other γ_c cytokines as well as B7 costimulation and TGF-β (47-50).

Lymphocyte numbers in the periphery are also affected by the proliferation of mature lymphocytes. Under lymphopenic conditions, naive T (51) and B cells (this dissertation) can proliferate without signs of overt activation or terminal differentiation; this process has been termed homeostatic proliferation. T cell homeostatic proliferation requires interactions between the TCR and peptide-MHC complexes as well as other signals (52-58); in B cells this process is Btk dependent (this dissertation). Activation of specific lymphocytes by antigen and co-stimulatory signals, resulting in expansion and differentiation to effector function, also occurs during the course of normal immune responses. Thus, a variety of mechanisms acting on lymphocytes *in vivo* alter their physiology and possibly their ability to support virus replication.

One example that illustrates the interplay between lymphocyte homeostasis and virus persistence in these cells is Epstein-Barr virus (EBV). EBV is a member of the herpesvirus family that establishes a latent infection of resting memory B cells in human hosts (59-61). A model for EBV persistence in B cells *in vivo* is emerging which proposes that the virus manipulates normal B cell activation and homeostatic signals to establish and maintain persistence. While EBV infects all B cell subsets *in vivo*, the pattern of viral gene expression varies depending on the differentiation state of the cell. Naive B cells express the "growth program" which induces proliferation of these cells; tonsillar memory and germinal center cells, on the other hand, express a more restricted set of genes (EBNA1, LMP1, and LMP2); peripheral resting memory B cells express

only LMP2 (62). By the current model, EBV initially infects naive B cells and expresses a pattern of genes that promote proliferation and differentiation to memory cells thereby establishing a persistent infection in these cells (62). Infected memory B cells may be maintained by the action of LMP2a (63) through which survival signals are transmitted (64). LMP2a also blocks BCR signaling by entering lipid rafts, and this may interfere with signals that would otherwise lead to B cell activation and reactivation of EBV from latency (65, 66). Thus, EBV persistence may be maintained in resting B cells *in vivo* by virally encoded proteins which promote survival of infected B cells and interfere with the normal homeostatic or activation signals generated through the BCR. EBV persistence is also controlled by the host immune response to the virus; it is proposed that productively infected (activated) B cells are killed by CTL while latently infected resting memory B cells are poor CTL targets (59, 60). This idea is supported by the fact that immunosuppression results in increased shedding of virus and increased incidence of EBV-positive lymphomas (59). Thus, a combination of homeostatic controls imposed on infected B cells by viral proteins and the adaptive immune response to the virus ensures the maintenance of latently infected B cells. EBV is an example of a complex DNA virus that encodes proteins which modify lymphocyte function to establish and maintain virus persistence in B cells. Simple RNA viruses such as VSV, on the other hand, do not encode such regulatory proteins; they likely take advantage of the normal physiology of lymphocytes and the immune responses of the host to establish and maintain persistence in lymphocytes.

The immune response to viral infections has two potential outcomes: clearance of virus from the host after acute infection or persistence; many RNA viruses establish and maintain persistent infections by evasion or suppression of the host immune response (67). Given the high mutation rate of RNA viruses (68), it is not surprising that these viruses generate mutants *in vivo* that escape recognition by neutralizing antibody or CTL, resulting in persistence (69, 70). RNA viruses can also establish persistence by inducing immunosuppression in the host. General immunosuppression after LCMV infection in mice is mediated by immune destruction of cells important for the immune response such as antigen presenting cells and $CD4^+$ T cells (71). LCMV can suppress the immune response to itself by exhaustion of antiviral CTL (72); it has also been proposed that LCMV promotes persistence in part by infecting B cells producing virus-specific neutralizing antibodies, resulting in their elimination by CTL and suppression of the neutralizing antibody response (73). Thus, RNA viruses have evolved a number of ways in which to evade or interfere with host immune responses in order to establish persistence.

There is also evidence that immune mechanisms are actively involved in the maintenance of virus persistence. Reactivation from latency by members of the herpesvirus family such as cytomegalovirus (CMV) is associated with immunosuppression; murine CMV is reactivated from latently infected mice after depletion of $CD4^+$, $CD8^+$, and NK cells (74). Furthermore, $CD8^+$ T cells restrict expression of herpes simplex virus type 1 (HSV-1) late proteins and infectious virus in explanted, latently infected trigeminal ganglia without destruction of the cells (75).

These studies demonstrate that cellular immune mechanisms can control virus replication while sparing infected cells and thus have the potential to support viral persistence.

Persistent viral infections are often maintained despite the presence of virus specific antibodies; studies by several groups suggest that antiviral antibody can facilitate a persistent infection by stripping viral antigens from the surface of infected cells or by restricting virus replication inside infected cells (2). Treatment of measles virus infected HeLa cells with measles immune serum results in removal of measles antigens from the cell surface and renders the cells resistant to complement mediated lysis (76). Interestingly, treatment of infected cell lines with measles antiserum or monoclonal antibodies to measles HA protein results in downregulation of both externally and internally expressed viral proteins (77-81). Antibody mediated inhibition of measles virus mRNA synthesis has been observed in a rat model of measles encephalitis and in persistently infected neuroblastoma cells (82, 83). Antiviral antibodies have also been shown to inhibit replication in neuronal cells infected with reovirus, Sindbis virus, and herpes simplex virus (84-86). This form of immune inhibition is not restricted to cells of neural origin since it has been observed in rubella virus infected human PBMC or lymphoblastoid cell lines and measles virus infected macrophage cell lines (81, 87). These studies demonstrate the ability of antiviral antibodies to act directly on cells infected with RNA viruses (measles virus, Sindbis virus, reovirus, rubella virus) and suppress replication at the levels of transcription, protein synthesis, and infectious virus production without killing the cells. Thus, antibody dependent restriction of virus

replication may play a role in the establishment or maintenance of RNA virus persistence *in vivo*.

In this dissertation, a model for studying the mechanisms by which an RNA virus (VSV) persists in B lymphocytes *in vivo* is described. Persistence of VSV in primary B lymphocytes *in vitro* has two important characteristics which can be extended to explain the behavior of the virus *in vivo*. First, the virus can be maintained for extended periods of time in resting B cells without production of viral proteins or infectious particles (M. Schmidt, manuscript in preparation); this implies that *in vivo*, the virus could evade the immune response by infecting resting B cells. Second, reactivation of infectious virus is always induced when resting B cells are driven to proliferate, but proliferation is not absolutely required for production of infectious particles (36). Thus, virus expression (the production of viral proteins or infectious particles) in B cells could be induced *in vivo* by signals that maintain B cells (survival), signals that promote proliferation (homeostatic expansion), or signals that promote proliferation and differentiation to effector function (response to specific antigen). This is important because it provides an opportunity for the virus to exit resting lymphocytes to maintain the pool of persistently infected cells and to potentially disseminate infection to other hosts. The host immune response to the virus also impacts persistence *in vivo*. In response to VSV infection, mice generate an early T-independent neutralizing IgM response followed on day 7 by a T-dependent neutralizing IgG response (88). While $CD8^+$ T cells are generated in response to VSV infection, they are not required for a protective immune response to the virus (89, 90). Infection is lethal in the absence of an antibody response, and passively

administered neutralizing antibodies are sufficient for protection (90-94). Type I (α/β), but not type II (γ) interferons are also essential in controlling virus replication before the onset of the antibody response (95, 96). Thus, VSV infection in mice is controlled by a combination of type I interferons and neutralizing antibody, suggesting that these immune mechanisms might play a role in the establishment or maintenance of a persistent infection *in vivo*.

Based on this background information, the studies described here had four goals: 1) to demonstrate that B cells persistently infected with an RNA virus (VSV) could be maintained *in vivo* for an extended time; 2) to better understand the mechanisms by which normal peripheral B cell homeostasis is maintained; 3) to determine if homeostatic signals can induce virus expression in B cells *in vivo*; 4) to determine the role of antiviral antibody in persistence of this virus in B cells. The experiments in Chapter III show that naive peripheral B cells are capable of undergoing proliferation in B cell deficient hosts (termed homeostatic proliferation) without adopting an effector phenotype; since B cells that proliferate *in vitro* can support full VSV replication, this provides a mechanism by which virus might be reactivated from persistently infected B cells *in vivo*. In Chapter IV it is shown that VSV can be maintained in infected B cells *in vivo* in spite of these homeostatic signals and an antibody response to the virus. Furthermore, evidence is presented that homeostatic signals acting on some infected B cells induce virus expression, resulting in an antibody response to the virus. Finally, it is shown that antiviral antibody can inhibit the reactivation of virus from persistently infected B cells *in vitro*, suggesting a mechanism whereby antiviral antibodies play a role in maintaining

RNA virus persistence in lymphocytes *in vivo*. These results lead to a model of *in vivo* persistence in which viral RNA is maintained in resting B cells; homeostatic signals drive production of virus or viral proteins in some infected cells which results in an immune response to the virus. Antiviral antibody, in turn, inhibits reactivation of virus leading to the maintenance of persistently infected B cells. By this model, reactivation of an RNA virus from resting lymphocytes can occur when antiviral antibody titers decay over time and under conditions of immunodeficiency, when mechanisms such as homeostatic proliferation are induced to restore lymphocyte numbers.

Chapter II

Materials and Methods

Mice

CBA/N x A.By female (normal), C57BL/6, CBA/Ca-*bcl-2* transgenic, or CBA/N-*bcl-2* transgenic (age 8-24 weeks) were used as sources of spleen cells and B cells. CBA/N x A.By female (normal) or male (*xid*), CBA/Ca (normal), CBA/N (*xid*), C57BL/6, or C57BL/6.scid mice (age 12-16 weeks) were used as recipients. In some experiments, recipients were irradiated using a ^{137}Cs source (Gammacell, Toronto, Canada) 24-48 hours before adoptive transfer. All mice were bred and maintained in the animal facilities at the University of Massachusetts Medical School using stock mice obtained from the National Cancer Institute (Frederick, MD) or Jackson Laboratories (Bar Harbor, ME). Mice expressing the human Ig-bcl-2 transgene, originally obtained from Dr. S. Korsmeyer (Washington University, St. Louis, MO) (97), were produced as previously described (98). C57BL/6.scid mice were generously provided by Drs. Dale Greiner and Eva Tsuda, University of Massachusetts Medical School or purchased from Jackson Laboratories (Bar Harbor, ME). $CD40^{-/-}$ mice were generously provided by Drs. Dale Greiner and Nancy Phillips, University of Massachusetts Medical School. IL-$4^{-/-}$, IL-$6^{-/-}$, and $p50^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME). c-$rel^{-/-}$ and B6.*xid* mice were generously provided by Drs. Ranjen Sen and Joan Press, Brandeis University. All animal care and procedures were carried out in accordance with the Animal Welfare Act.

Virus stocks

VSV-Indiana (Salt Lake City subtype) was obtained from Dr. Trudy Morrison (University of Massachusetts Medical School, Worcester, MA), and stocks were prepared as previously described (35). B cells were infected with VSV at a multiplicity of infection of 10-20. VSV ΔG-HA was generously provided by Dr. Jack Rose, Yale University (99).

Cell preparation: B cell homeostasis experiments

Total spleen or lymph node (excluding mesenteric) cell suspensions were prepared by gently pressing lymphoid organs between glass slides in BSS-BSA (0.3% w/v) supplemented with 100 μg/ml streptomycin, 10 μg/ml penicillin, and 10 μg/ml gentamycin. B cell enriched spleen cell populations were prepared by treating pooled spleen cell suspensions with anti-Thy 1.2 mAb (clone J1J10) and mouse-adsorbed rabbit complement as previously described (100). Small resting cells (either B cells, total spleen cells or lymph node cells) were prepared by fractionation on a Percoll step gradient (50, 65, 75%) prepared in HBSS. Purified B cells analyzed by flow cytometry were 85-95% $B220^+$.

B lymphocyte preparation: Virus persistence experiments

Small B lymphocytes were prepared and cultured as previously described (35). B cell enriched spleen cell populations were prepared as described above. For *in vitro* persistence experiments, small resting B cells were prepared by fractionation on a step gradient of Percoll (50, 65, 75%) prepared in HBSS. Purified B cells were 85-95% $B220^+$. For *in vivo* persistence experiments, small resting B cells were purified by

counter-current centrifugal elutriation, had a mean cell diameter of 6 μm as determined by Coulter counter analysis, and constituted 20-30% of input B cells.

Preparation of immature B cells

Spleen cell populations enriched for immature B cells were produced in auto-reconstituting, sublethally irradiated donors as described by Allman and Cancro (132). Briefly, spleen cells were taken from CBA/Ca or CBA/NxA.By normal donors irradiated 12-13 days previously with 550 rads of whole body irradiation. B cells were prepared by anti-Thy1.2 and complement treatment followed by depletion of dead cells and erythrocytes on 50:60% Percoll gradients and harvesting cells at the 50:60% interface. Immature B cells prepared by this procedure were greater than 95% $B220^{+}$, and uniformly CD24 $(HSA)^{hi}$, p130-140 (493 and AA4.1)$^{+}$, IgM^{hi}, IgD^{lo}.

B lymphocyte culture and activation

B cells were cultured at 5-10 x 10^6 cells/ml in RPMI-1640 supplemented with 10% FCS (Gibco), 2 mM glutamine, 50 μM 2-mercaptoethanol, 100 μg/ml streptomycin, 10 μg/ml penicillin, 10 μg/ml gentamycin, and MEM nonessential amino acids. The following B cell activators were used: $F(ab')_2$ fragments of affinity purified rabbit anti-mouse $F(ab')_2$ at 10μg/ml and recombinant IL-4 at 100 U/ml; LPS (*Escherichia coli* O55:B5 Westphal, Difco); PMA (Sigma) and ionomycin (Sigma) were dissolved in DMSO at 1 mg/ml and used at 10 ng/ml and 1 μg/ml respectively. Proliferation of activated B cells was assayed by [^{3}H]TdR (Amersham) incorporation. Triplicate samples of $2x10^5$ cells/well in 96-well microtiter plates were pulsed with 1μCi/well [^{3}H]TdR for 6 to 8 hours. Plates were

harvested with a LKB (Wallac, Inc., Gaithersburg, MD) cell harvester and counted on a LKB Betaplate reader.

CFSE staining

Small resting lymphocytes or immature B cells were stained with the vital dye CFSE (5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester) (Molecular Probes (Eugene, OR) as previously described (101). Briefly, cells were washed and resuspended in serum-free PBS or Hanks' Balanced Salt Solution (HBSS) at $50x10^6$ cells/ml and mixed with an equal volume of 2μM CFSE in PBS (1μm CFSE final concentration). The cells were then incubated at 37° C for 10 minutes with gentle shaking, and washed in HBSS-BSA (0.3% w/v) twice. In virus persistence experiments, B cells were stained prior to infection.

Adoptive transfer

Cells were washed twice in BSS-BSA (0.3% w/v), once in PBS, resuspended in PBS at the appropriate concentration. Cells (0.5 ml) were injected via the lateral tail vein. In some virus persistence experiments, extracellular virus was neutralized before adoptive transfer by treating infected B cells with a 1:1000 dilution of VSV immune serum at 37° C for 20 minutes. Serum was produced by immunization of CBA/NxA.By female mice with $2x10^6$ PFU of VSV-SLC.

FACS analysis: B cell homeostasis experiments

Single cell suspensions made from the spleens of individual adoptive transfer recipients were treated with Gey's solution to remove red blood cells and resuspended in ice-cold FACS buffer (PBS, 3%FCS, 0.02% sodium azide). Cells were treated with anti-FcR

antibody (2.4G2) and stained with APC, PE, or Tricolor conjugated anti-B220 (RA3-6B2), PE conjugated anti-I-A^k (14V.18), anti-B7-1 (RMMP-2), anti-B7-2 (RMMP-1), anti-CD25 (PC61.5.3), anti-CD71 (RI7217.1.4), PE or APC conjugated anti-Thy1.2 (CT-TH1) (Caltag); PE conjugated anti-IgD (11-26) or anti-CD69 (H1.2F3) (Southern Biotechnology Associates); PE conjugated anti-HSA (M1/69) (Pharmingen). Biotinylated goat anti-mouse IgM (FisherBiotech) or biotinylated anti-CD38 (clone 92) (PharMingen) were detected with streptavidin-Tricolor (Caltag) or streptavidin-Cychrome (PharMingen). The p130-140 differentiation antigen of immature B cells was detected using a mixture of 493 antibody (102) produced from a hybridoma generously provided by Drs. A.G. Rolink and F. Melchers, Basel Institute for Immunology, and AA4.1 antibody (103) generously provided by Dr. R. Gerstein, University of Massachusetts Medical School. Biotinylated antibodies to 493 or AA4.1 were detected with streptavidin-Tricolor (Caltag) or streptavidin-Cychrome (PharMingen). After staining, cells were washed with FACS buffer and fixed with 2% paraformaldehyde in PBS before analysis on either a FACSCalibur or FACSVantage machine. In all experiments, 100,000-150,000 events were collected. Dead cells were excluded based on forward and side scatter and data analyzed using FlowJo (Tree Star, Inc.).

FACS sorting: Virus persistence experiments

Separation of transferred infected $CFSE^+$ B cells was performed by sorting on a FACS Vantage (Becton-Dickinson). $CFSE^+$ (donor) and $CFSE^-$ (recipient) cell populations were collected and were 60-88% and >99% pure, respectively. The efficiency of donor cell transfer was assessed by staining total spleen cell populations with APC conjugated B220

(Caltag) and determining the percentage of $CFSE^+$ $B220^+$ cells using FlowJo software (Tree Star, Inc.)

In vivo BrdU labeling and analysis

Adoptively transferred cells were labeled with BrdU *in vivo* by administering 1 mg of BrdU in PBS i.p. immediately after adoptive transfer, and then feeding the recipients BrdU continuously in their drinking water (1 mg/ml) for one or two weeks. BrdU containing drinking water was shielded from light and changed every three days. Single cell suspensions were made from the spleens of individual recipients and stained for incorporated BrdU using the procedure of Lentz, et al. (104) which was modified by using unlabeled anti-BrdU (Becton Dickinson) as the primary antibody and PE conjugated rat anti-mouse IgG_1 (Becton Dickinson) as the detecting antibody.

ELISA

Detection of VSV specific antibodies was performed essentially as described previously (94). Briefly, 96 well polyvinyl chloride plates (Falcon) were coated with 5-10 mg of protein from VSV-SLC infected CHO cells in carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C. Coated plates were blocked with PBS-1% BSA for one hour at room temperature. Serial three-fold serum dilutions were made in PBS-1% BSA, added to the blocked plates, and incubated at room temperature for 2 hours. VSV specific antibodies were detected by incubating the plates for1h at room temperature with biotinylated rat anti-mouse k (Zymed), goat anti-mouse IgM (Fisher), or goat anti-mouse IgG1 (Fisher) at a 1:2000 dilution followed by streptavadin-HRP (Zymed) at a 1:4000 dilution for 1h at room temperature. The substrate TMB (Sigma) was prepared as described by the

manufacturer then added to each well (50-100 ml) and the plates incubated for 10-15 minutes at room temperature. O.D. was determined at 370nm on a plate reader (Molecular Devices), and ELISA titers calculated by determining the serum dilution which gave the half-maximal O.D.

Antibodies: Virus persistence experiments

Normal mouse serum (NMS) was obtained from a pool of unimmunized 129, CBA/NxA/J and CBA/NxA.By mice maintained under SPF conditions (18 ml total volume). Anti-VSV serum was obtained from CBA/NxA.By female mice immunized with $2x10^6$ PFU of VSV-SLC, boosted on day 21, and bled out on day 28; this serum was combined at 1:1 with the pooled NMS to increase the total volume to 15ml. Sera from normal or VSV immune mice were filter sterilized before storage at -80°C. Purified antibodies or antibody fragments were prepared from NMS or anti-VSV serum. Briefly, immunoglobulin was initially fractionated using a 50% ammounium sulfate cut, the precipitate dialyzed against 50mM Tris pH 8.0, 50mM NaCl, and loaded on a DEAE column equilibrated in the same buffer. Immunoglobulin was eluted using a 50mM Tris pH 8.0, 50mM-500mM continuous NaCl gradient, concentrated, then further purified on a protein A column. $F(ab')_2$ and Fab fragments were made from purified anti-VSV Ig by digestion with pepsin and papain respectively. Purified intact Ig, $F(ab')_2$ and Fab fragments were further purified on a G-100 sizing column followed by a protein A column to remove any intact immunoglobulin or contaminating Fc fragments. Anti-VSV $F(ab')_2$ and Fab fragments showed no evidence of intact immunoglobulin when run on a 10% SDS-PAGE gel and stained with Comaissie blue. In some experiments, Fab

fragments were spun in a Beckman airfuge before use to eliminate aggregates. Ascites fluids containing the following monoclonal antibodies were generously provided by Dr. Leo LeFrancois, University on Connecticut Health Science Center: 1E9 (Indiana specific neutralizing anti-VSV G); 8E11F2 (Indiana specific non-neutralizing anti-VSV G); 5E4D8 (Indiana/New Jersey cross-reactive, non-neutralizing anti-VSV G); 10G4 (anti-VSV N). All monoclonal antibodies are IgG2a, κ (105, 106). In some experiments, ascites fluids prepared in our laboratory from the hybridomas 1E9F9 or 8E11C9 (generously provided by Dr. LeFrancois) were used.

Antibody inhibition of virus reactivation from B cells *in vitro*

Small resting splenic B cells from bcl-2 transgenic mice were infected with VSV-SLC at moi=10, washed 2-3 times, then placed in culture at a density 5-10x10^6 cells per ml with anti-VSV antibody or control antibody (NMS, NMS Ig, or control ascites). Half of the media in each culture was replaced every 3-4 days with media containing fresh antibody. After 7-9 days, cells were washed three times with complete RPMI, counted, and placed into culture with or without the following B cell activators: anti-Ig+IL-4, LPS, or PMA+ionomycin. Separate cultures were set up to monitor virus production (0.5x10^6 cells/ml), RNA (5x10^6 cells/ml), and protein (1x10^6 cells/ml). Virus production cultures were frozen at -20°C, while cells from RNA and protein cultures were counted, resuspended in RLT buffer (Qiagen) or cell lysis buffer (36) respectively, and stored at -80°C.

Assay for infectious virus

For *in vivo* persistence experiments, tissues were homogenized in PBS and subjected to one freeze-thaw cycle. The homogenate was then vortexed, spun briefly to pellet debris, and a 10-fold dilution of the supernatant assayed for plaque formation. For *in vitro* persistence experiments, serial 10-fold dilutions were assayed. Supernatants (200 ml) were placed on CHO monolayers in 60mm dishes, incubated at room temperature for 30 minutes, and the monolayers overlayed with a 1:1 mixture of 2X RPMI and 2% agar. Plaques were counted after incubation of the monolayers at 37°C for two days.

Protein analysis

Viral protein synthesis was analyzed by continuous labeling with [^{35}S]methionine (Amersham, SJ-1515) for 3-4 hours, 18-24 hours after activation of infected B cells as previously described (36). Viral proteins were immunoprecipitated using a high titer rabbit anti-VSV serum specific for VSV G, N, and P proteins as previously described (36). VSV proteins were analyzed by running $1x10^6$ cell equivalents of protein on 10% SDS-PAGE gels and visualized by autoradiography or on a Molecular Imager (BioRad).

RNA extraction

Total RNA was prepared from cells or tissues using the Qiagen RNeasy Total RNA kit as described by the manufacturer.

Northern blot analysis

Total RNA (4μg or RNA from equivalent cell numbers) was separated on 1% formaldehyde-agarose gels and transferred to Hybond N^+ membranes for analysis. Filters were prehybridized at 42°C overnight in 50% deionized formamide, 1X

Denhardt's, 1% SDS, 5X SSC, 10mM EDTA, 50 mM $NaPO_4$ (pH6.7), 0.2 mg/ml yeast tRNA, and 0.2 mg/ml denatured salmon sperm DNA. DNA probes for VSV M, G, and L were made from restriction fragments by random primer labeling as previously described (36). Filters were hybridized with a mixture of the VSV M, G, and L probes overnight at 42°C in prehybridization buffer. Filters were washed at least twice with 0.1X SSC and 0.1% SDS for 45 min at 50°C before exposure to film or a phosphorimager screen.

RT-PCR

Analysis of RNA was also performed by RT-PCR. Contaminating DNA was removed from RNA samples essentially as described previously (107). Briefly, 2 μg of RNA was resuspended in 50 μl of a reaction mix containing 1x Promega M-MLV Reverse Transcriptase Buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT), 0.5 mM of each dNTP, 20 U RNasin (Promega), and 1 U DNase (Promega). Reactions were incubated at 37°C for 30 min and then 75°C for 5 min. A mix of 0.5 μg of random primers (Promega) or 0.25 μg of oligo dT primers (Promega) and 100 U M-MLV Reverse Transcriptase (Promega) was then added, and the reactions incubated at 23°C for 10 min, 37°C for 1 h, and 65°C for 10 min. In some cases, antisense primers specific for VSV G or VSV M (see below) were used instead of random primers or oligo dT. One-tenth of each RT reaction was subjected to PCR amplification. The primers used for PCR were as follows: VSV M external sense primer 5'-ATGAAGAGGACACTAGCATGG-3', VSV M external antisense primer 5'-TCTGAAGTGCTCTGGTACATT-3' (397 bp product); VSV M internal sense primer 5'-CGAGCGCTCCAATTGACAAAT-3', VSV M internal antisense primer 5'-

TACCGCTGGAGTGGCCTTTAG-3' (260 bp product); actin sense primer 5'-ATGGATGACGATATCGCT-3', actin antisense primer 5'-ATGAGGTAGTCTGTCAGGT (571 bp product). PCR reactions were in 45 μl volumes: 1x Sigma Taq polymerase buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.001% gelatin), 2.5 mM (actin primers) or 4 mM (VSV M primers) $MgCl_2$, 6.25 pmol of each primer, and 0.2 mM of each dNTP. Samples were heated at 95°C for 5 min, quick chilled on ice, and 2.5 U of Taq polymerase (Sigma) added to each. Amplification was performed in an MJ Research MiniCycler for 35 cycles: denaturation 93°C 30 sec, annealing 55°C 30 sec, extension 72°C 30 sec, final extension 72°C 10 min. Ten microliters of each reaction were run in 2% agarose (NuSieve) gels in TAE buffer. Products were visualized by ethidium bromide staining.

Chapter III

B Lymphocytes Undergo Homeostatic Proliferation In B Cell Deficient Recipients

Introduction

Poorly understood homeostatic mechanisms maintain constant lymphocyte numbers in naive animals. Homeostasis is an ongoing process as lymphogenesis in primary lymphoid organs continuously provides naive lymphocytes to the periphery, and although slowed by aging (108, 109), lymphocyte production continues for the life of the individual. Understanding the mechanism of normal peripheral homeostasis will contribute new insights into disease processes, since autoimmunity, cancer, and immunodeficiency are all associated with dysregulation of lymphocyte numbers. Central to the notion of homeostatic regulation in the periphery are lymphocyte-intrinsic sensing and response mechanisms that detect variation in the size of the total lymphocyte pool and make compensatory adjustments to lymphocyte numbers through feedback regulation. This sensing mechanism likely functions in a unique local microenvironment and is lymphocyte subpopulation specific since B and T cell numbers are independently regulated (110).

Naive lymphocyte numbers are controlled at the level of lymphogenesis and by mechanisms regulating cell proliferation and survival in the periphery. Survival of both B and T cells is an active process requiring signals induced through lymphocyte interaction with environmental ligands that control the expression of anti-apoptotic

molecules (39, 40, 111). These signals are delivered to lymphocytes through their antigen receptors (41-43), and to B cells by other molecules including CD40L, IL-4, and BAFF/Blys (44-46). Similarly, survival of naive T cells depends on additional factors; primarily IL-7 and other γ_c cytokines as well as B7 costimulation and TGF-β (47-50). Competition between individual lymphocytes for ligands that promote survival is a major regulatory control on lymphocyte numbers (100, 110, 112).

Peripheral lymphocyte numbers are also maintained by homeostatic proliferation; cell division induced in lymphopenic hosts in the absence of overt antigenic stimulation. Adoptive transfer of CD4 or CD8 T cells into T cell deficient hosts results in expansion of the donor T cells, and, like survival, requires interactions between the TCR and peptide-MHC complexes as well as other signals (52-58). The similarity in inductive signaling has led to the proposition that homeostatic proliferation and survival are part of a stimulatory continuum, with the intensity of signal being titrated by the number of competing lymphocytes. In this model, when T cell numbers are reduced, access to the ligand(s) promoting survival is increased and proliferation is induced (113). A recent report showing homeostatic proliferation requires $p56^{lck}$ while survival does not, however, indicates that the interrelationship between these two processes is complex (114).

The mechanisms that maintain the naïve peripheral B cell pool are still poorly understood. While it is known that signals through the BCR affect mature B cell survival *in vivo* (41), it remains to be determined whether other regulatory mechanisms are active. Indeed, it seems reasonable to hypothesize that B and T cells use similar mechanisms to

maintain peripheral homeostasis. In this report, we present direct evidence that both immature and mature B cells proliferate in response to a B cell deficiency. This replication is under homeostatic control since neither population proliferates extensively in B cell sufficient hosts. Unlike antigen or mitogen induced stimulation, B cells that proliferate display a naïve antigenic phenotype, and do not progress to effector functions. Homeostatic proliferation requires Bruton's tyrosine kinase (Btk) a cytoplasmic kinase critical for B cell proliferation, upregulation of anti-apoptotic molecules, and maturation (115). We also find that two members of the Rel/NF-κB family of transcription factors, c-Rel and p50, are dispensable for homeostatic proliferation. The requirement for c-Rel in BCR and mitogen induced proliferation (116) illustrates the distinction between homeostatic and antigen/mitogen induced replication. Since p50 is required for B cell survival *in vivo* (116), but not homeostatic proliferation, the signals promoting these processes are distinct. These data suggest that homeostatic proliferation is an important component of both B and T cell peripheral maintenance.

Results

Splenic B cells divide after adoptive transfer into B cell deficient, but not B cell sufficient recipients

xid mice carry a mutation in Bruton's tyrosine kinase (Btk) which impairs full B cell development, resulting in a marked reduction of mature B cells in the periphery (117, 118). This selective B cell deficiency provides the opportunity to assess the ability of transferred normal B cells to sense and respond to a deficit in the mature B cell compartment without having to manipulate the system with ionizing radiation, which

could induce pro-proliferative factors. Small resting normal splenic B cells were stained with CFSE (101) to allow tracking and determination of cell divisions, and adoptively transferred into unirradiated normal or *xid* recipients. $CFSE^+$ B cells were readily detected in the spleens of *xid* recipients early after transfer (day 2), but little cell division had occurred (Figure 1A). By day 14, however, a significant proportion (27%) of the $CFSE^+$ $B220^+$ B cells in *xid* recipients had divided, with three divisions being readily distinguished (Figure 1A). Our analysis likely underestimates the extent of cell division because cells that have divided more than three times will appear to be in the CFSE negative gate and excluded from the analysis (Fig 1A). Homeostatic proliferation of the transferred B cells was dependent on B cell deficiency since a significantly smaller proportion (7.4%, $p<0.001$) of the $CFSE^+$ B cells divided in normal recipients by day 14 (Figure 1A and 1C). Analysis of replication at early time points showed that proliferation was ongoing over the 14 day assay period (Figure 2 and data not shown).

In some experiments the fluorescence intensity of cells in the main $CFSE^+$ peak (presumably undivided cells) decreased between day 2 and day 14 (Figure 1A). This could indicate that all the cells in this peak had divided or the loss of dye by the normal turnover of intracellular proteins to which CFSE is covalently bound (119). To distinguish between these two possibilities, BrdU was administered continuously for 14 days to *xid* recipients of CFSE labeled normal B cells. Thereafter, spleens were harvested, cells permeabilized, stained for BrdU, and analyzed by FACS. Of the cells in the brightest $CFSE^+$ fraction, only 5% of the cells incorporated BrdU (Figure 1B), whereas 98% of the cells in peaks with lower CFSE intensity did so. These results

clearly show that the most intense CFSE peak consisted of undivided cells while the $CFSE^+$ cells with lower fluorescence intensity had divided.

Homeostatic proliferation was also evident in normal irradiated (20.1±1.0%) or B6.scid (33.4±2.0%) recipients, indicating that *xid* (23.4±0.8%) recipients were not unique (Figure 1C). Furthermore, proliferation was apparently T-independent because SCID mice contain very few mature T cells. The T independence of B cell homeostatic proliferation was further confirmed by the finding that B cells from $CD4^{-/-}$ donors replicate as well in irradiated $CD4^{-/-}$ recipients as they do in irradiated normal recipients (see below). We conclude from these experiments that splenic B cells undergo homeostatic proliferation in a T cell independent manner when transferred into a B cell deficient environment.

Figure 1. Normal splenic B cells proliferate in B deficient, but not B sufficient recipients

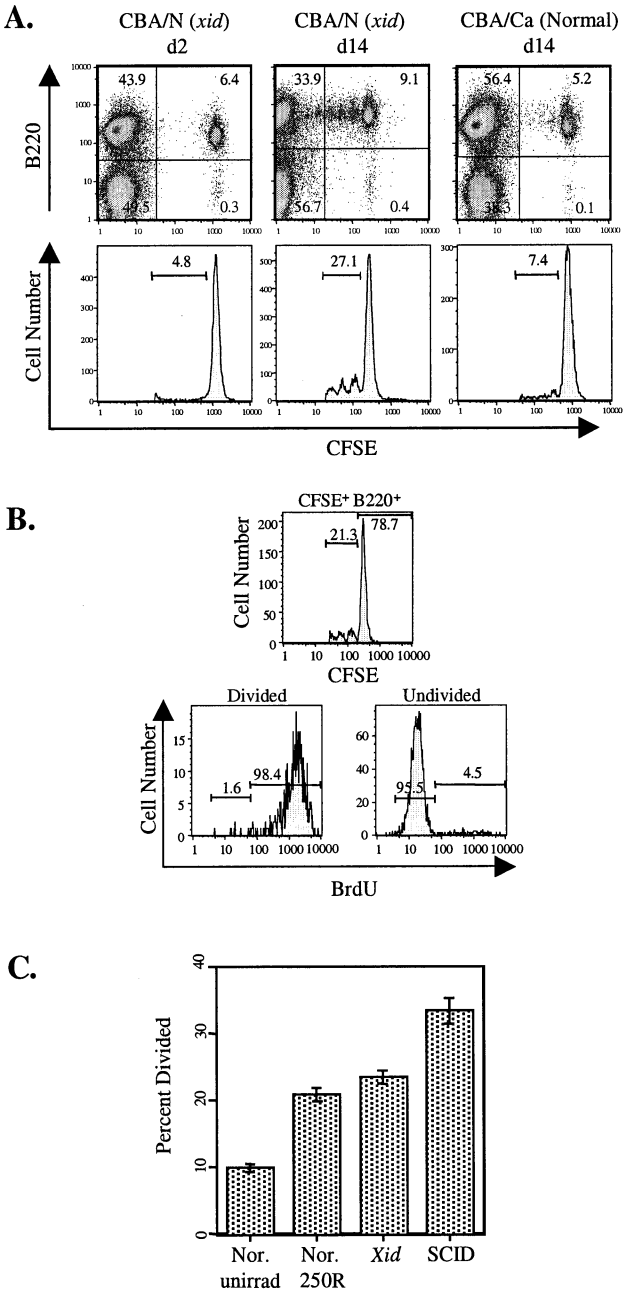


Figure 1. Normal splenic B cells proliferate in B deficient, but not B sufficient recipients. **A.** $40x10^6$ CFSE labeled small resting splenic B cells from normal donors (CBA/Ca or CBA/NxA.By females) were transferred into *xid* recipients (CBA/N or CBA/NxA.By male). Spleen cells from individual recipients on day 2 or day 14 were analyzed by flow cytometry. The gate drawn in each histogram shows the percentage of $B220^+CFSE^+$ cells that have divided. **B.** $20x10^6$ CFSE labeled small resting splenic B cells from normal (CBA/NxA.By female) donors were transferred into *xid* (CBA/NxA.By male) recipients. Recipients were fed BrdU continuously in their drinking water for 14 days before sacrificing. Spleen cells were analyzed for BrdU incorporation on day 14 as described in Materials and Methods. Results are representative of two independent experiments. **C.** 20-$40x10^6$ CFSE labeled small resting splenic B cells from normal donors (CBA/NxA.By female, or C57BL/6) were transferred into syngeneic recipients; unirradiated normal (CBA/NxA.By female or C57BL/6, n=9); sublethally irradiated (250R) normal (CBA/NxA.By female or C57BL/6, n=21); unirradiated *xid* (CBA/NxA.By male, n=15); or unirradiated C57BL/6.SCID (n=6). The percent of $B220^+$ $CFSE^+$ spleen cells that have divided by day 10-14 is shown ±SE. Results are from two to seven independent experiments.

B cells that divide after adoptive transfer maintain a resting phenotype

Antigen or mitogen stimulation changes the cell surface antigen phenotype of both B and T cells. Homeostatic proliferation in T cells differs from antigen induced replication in that memory, but not other activation markers, are upregulated (54, 56, 58, 120-122). Accordingly, we examined a collection of cell surface markers known to change their expression pattern on antigen/mitogen activated B cells: IgD and CD38, which decrease on mitogen activated cells (123, 124); IgM, which is lost on B cells undergoing class switching (125); CD25 (IL-2Rα chain), CD43, syndecan, CD69, CD71 (transferrin receptor), MHC class II, B7-1 and B7-2 which are all upregulated (126, 127). CD69 is routinely used as an early lymphocyte activation marker, whereas CD43 and syndecan are a late activation markers expressed on antibody secreting, but not naive B cells (128, 129).

We analyzed surface marker expression on the replicating and non-replicating fractions of small resting normal B cells transferred into *xid* recipients before proliferation had occurred (day 2), at the beginning of proliferation (day 4), and at later times (day 9) after transfer. B cells analyzed less than 24 hours after transfer served as the unstimulated baseline whereas B cells stimulated *in vitro* with anti-Ig+IL-4 were used as activated controls. In anti-Ig+IL-4 activated cells, all of the markers selected for analysis underwent the expected increases or decreases (IgD) in expression by 2 days in culture (Figure 2 and data not shown). For transferred B cells, although division was negligible on day 2, there was a pronounced increase in CD69 expression on most of the cells. Neither cell size nor other cell surface markers were affected at this time point. By

day 4, B cells which had divided demonstrated increased forward scatter, a slight decrease in IgD, and expressed higher levels of CD71, whereas CD69 had returned to a baseline level and other markers were unaffected. In the non-dividing fraction, CD69 had also returned to a baseline level, and there was no noteworthy change in any of the other markers analyzed. By day 9, divided cells were slightly larger by forward scatter and all other markers had returned to baseline levels. The expression of other activation markers, including MHC class II, B7-1, IgM and CD38, as well as syndecan, a marker for differentiation to effector function, was unchanged at all times analyzed on both divided and undivided cells (data not shown).

These data suggest that homeostatic B cell proliferation is distinct from antigen or mitogen driven proliferation in that the cells undergoing replication maintain a resting phenotype. The transient increase in CD69 expression suggested that a substantial fraction of transferred B cells received some inductive signal upon transfer, whereas changes in forward scatter, CD71, and IgD expression on divided cells may reflect continued signaling in these cells. This signaling, however, is not sufficient to induce differentiation to effector function.

Figure 2. B cells undergoing homeostatic proliferation maintain a resting phenotype

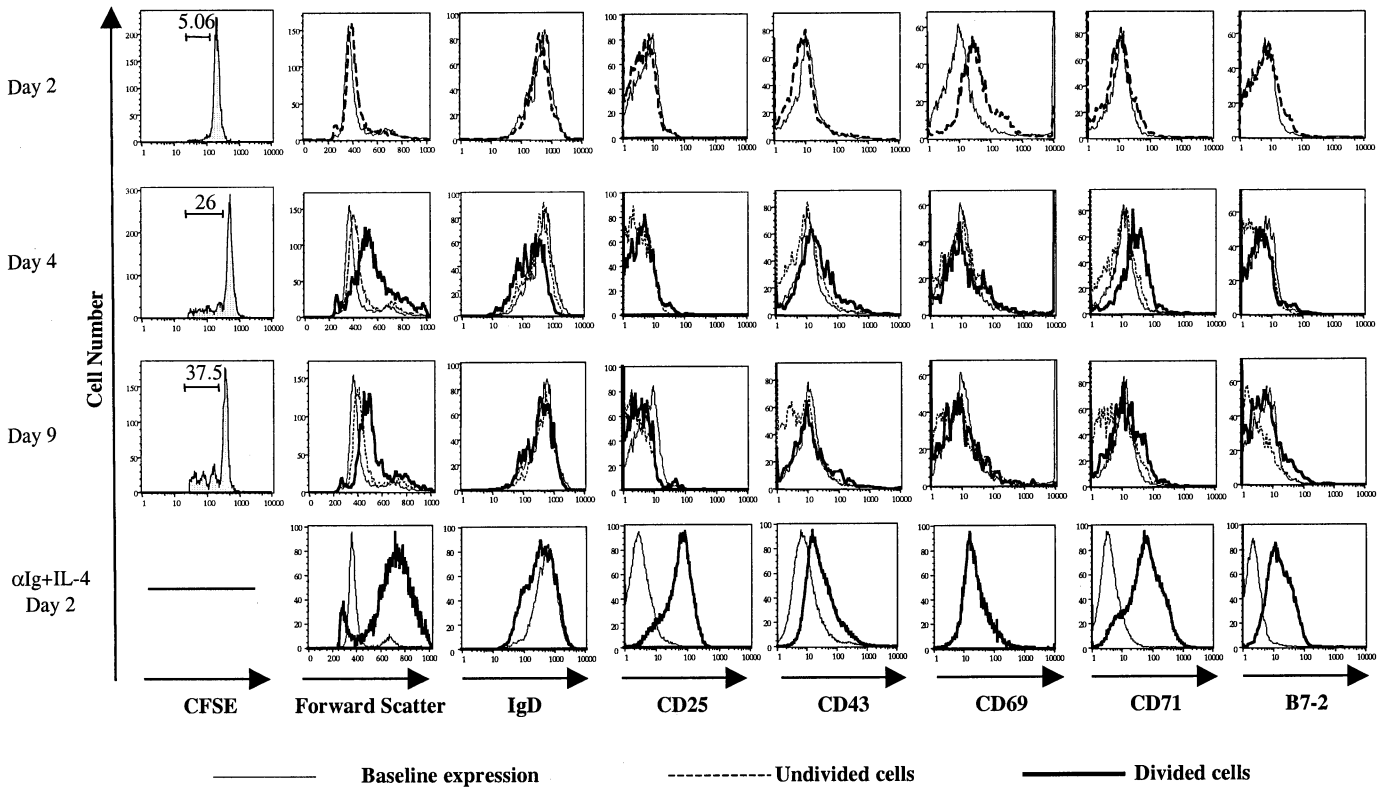


Figure 2. B cells undergoing homeostatic proliferation maintain a resting phenotype. $20x10^6$ CFSE labeled small resting splenic B cells from normal donors (CBA/NxA.By female) were transferred into *xid* recipients (CBA/NxA.By male). Recipients were sacrificed on day 2, day 4, or day 9 after adoptive transfer and spleen cells were analyzed as described in Materials and Methods. Results are representative of four mice for each time point from two independent experiments. Histograms show CFSE fluorescence intensity or expression of the indicated cell surface markers on $CFSE^+ B220^+$ donor cells. The marker expression and forward scatter of $CFSE^+ B220^+$ donor cells on day 1 after transfer was used as a baseline for comparing marker expression at later time points. The forward scatter and surface marker expression of purified small resting splenic B cells stimulated with $F(ab')_2$ fragments of affinity purified rabbit anti-mouse $F(ab')_2$ (10 μg/ml) plus rIL-4 (100 U/ml) for two days is shown for comparison.

Mature and immature B cells undergo homeostatic proliferation

Our data clearly show that resting splenic B cells undergo homeostatic proliferation. Splenic B cells are a heterogeneous assortment of developmentally distinct subpopulations. In order to unambiguously demonstrate that mature B cells underwent homeostatic proliferation, we used lymph nodes as a source of donor cells, since immature and marginal zone B cells are absent and B-1 cells are extremely rare (130, 131). A typical experiment is shown in Figure 3A; donor B cells ($B220^+$) from the spleens or lymph nodes of normal mice divided after adoptive transfer into *xid* recipients, 24% and 18% respectively, while donor T cells ($Thy1.2^+$) from spleens or lymph nodes did not, 7% and 5% respectively. Overall, the extent of cell division was similar in lymph node B cells compared to those purified from the spleen (Figure 3B; 19.3±0.5% versus 21.4±0.6%). The fact that T cells from the same innoculum did not divide extensively in these T cell sufficient recipients confirmed that the expansion was lymphocyte subpopulation specific. We conclude from these data that mature B cells are capable of homeostatic proliferation in B cell deficient recipients.

To assess the capacity of immature B cells to undergo homeostatic proliferation, we isolated a highly enriched population of immature donor B cells from the spleens of mice auto-reconstituting after sublethal whole body irradiation (132). In these mice all peripheral B cell populations are depleted by irradiation; two weeks thereafter, the spleens consist almost exclusively of newly emergent immature B cells (>95% $pB130\text{-}140^+$ and HSA^{hi}) (102, 132). Purified, CFSE labeled immature B cells replicated as extensively as mature B cells when transferred into *xid* recipients (36.2±2.9%, Figure 3C

versus 21.4±0.6%, Figure 3B). As was found for mature B cells (Figure 1), immature B cells failed to replicate when transferred to normal unirradiated recipients (5.7±2.2%, Figure 3C). Taken together, these data demonstrate that both mature and immature B cells are capable of homeostatic proliferation in hosts deficient in mature B cells.

Figure 3. Homeostatic proliferation is a property of mature and immature B cells

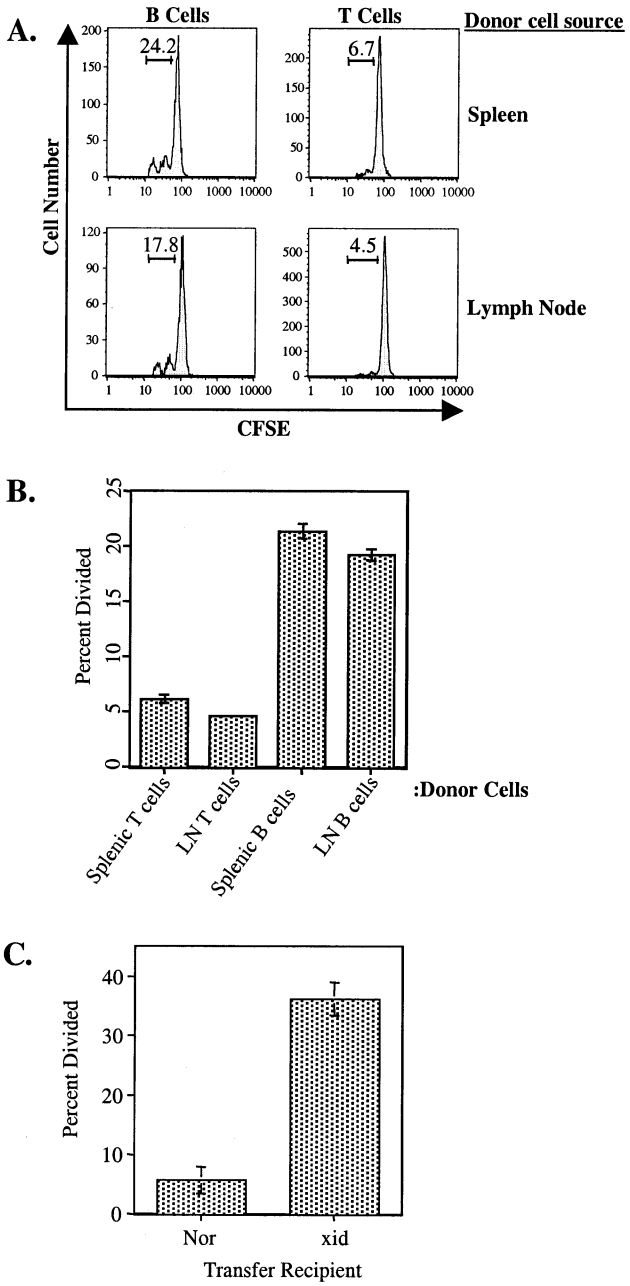


Figure 3. Homeostatic proliferation is a property of mature and immature B cells. **A.** 20-40x10^6 CFSE labeled small total spleen or lymph node cells from normal donors (CBA/NxA.By female) were transferred into *xid* recipients (CBA/NxA.By male) and recipient spleen cells analyzed on day 14. Representative histograms demonstrating the extent of cell division in $CFSE^+$ donor B cells ($B220^+$) and T cells ($Thy1.2^+$) are shown. **B.** Comparison of the extent of cell division in the indicated donor cell populations after adoptive transfer into *xid* recipients. Results were compiled from 10 recipients in each group from three independent experiments and are shown as percent divided ± SE. **C.** Pure populations of transitional B cells were prepared from normal donors (CBA/NxA.By female) as described in Materials and Methods. 25x10^6 cells were adoptively transferred into either unirradiated normal (CBA/NxA.By female) or *xid* (CBA/NxA.By male) recipients and analyzed on day 10 after transfer.

Mature, but not immature, B cells mediate feedback inhibition of homeostatic proliferation

Homeostatic proliferation is inhibited under conditions of lymphocyte sufficiency; induced experimentally by transferring either high doses of homologous cells or using normal recipients. Indeed, the extent of T cell homeostatic proliferation is inversely related to the dose of transferred cells (121). Accordingly, we adoptively transferred increasing numbers of CFSE-labeled purified splenic B cells to *xid* recipients to demonstrate that susceptibility to competitive inhibition was also a property of peripheral B cells (Figure 4A). There was clearly an inverse correlation between B cell dose and the degree of homeostatic replication, with the least proliferation occurring at the highest cell dose. These differences were highly significant ($p=0.03$ to <0.001), and demonstrate that proliferation is limited by competition with other B cells. Likewise, inhibition was demonstrated by transferring increasing numbers of unlabeled normal splenic B mixed with a constant dose of normal CFSE-labeled B cells (Figure 4B). In contrast, competing *xid* B cells had no inhibitory effect (Figure 4B). Whether this reflects competition between B cells for environmental ligands that drive proliferation or direct B cell:B cell interactions that suppress proliferation is unknown.

We find that immature B cells replicate upon transfer to *xid* recipients, despite the fact that *xid* mice have normal numbers of immature and marginal zone B cells. This suggests that all B cells may not be subject to the same negative regulation or the susceptibility to inhibition is developmentally acquired. The finding that immature B cells transferred into normal recipients show significantly reduced replication, however,

suggests that they are subject to regulation, but not by the homologous B cell subpopulation. To determine if homeostatic proliferation of immature B cells could be autoregulated in a subpopulation specific manner, graded doses of immature B cells were transferred into *xid* recipients. In striking contrast to the behavior of mature B cells, homeostatic proliferation of immature B cells was equivalent at all cell doses transferred (10, 30, and 90 million cells, Figure 4C). These data indicate that mature B cells play a central role in peripheral homeostasis by regulating their own homeostatic proliferation and that of their immediate precursors.

Figure 4. Mature, but not immature B cells inhibit homeostatic proliferation

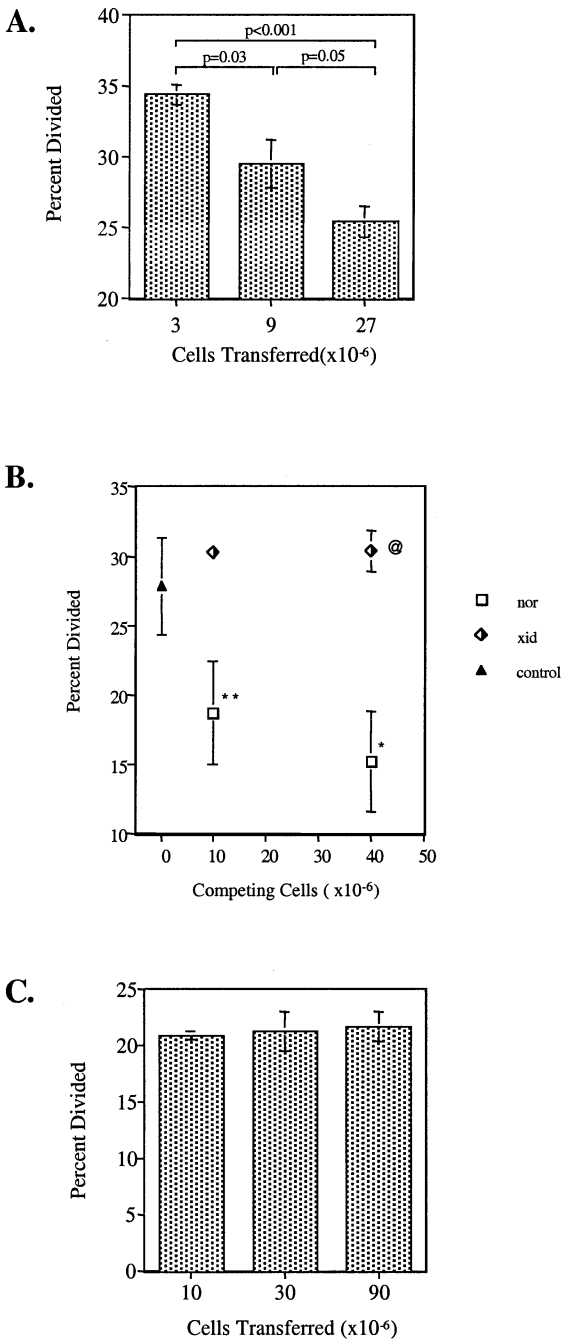


Figure 4. Mature, but not immature B cells inhibit homeostatic proliferation. **A.** The indicated numbers of CFSE labeled small resting splenic B cells (mature B cells) from normal donors were transferred into *xid* recipients and recipient spleen cells analyzed on day 12. The percent of $B220^+ CFSE^+$ cells that had divided ± SE is shown. Gates were set to only look at one cell division to avoid the inclusion of background $CFSE^-$ host cells at the lower cell doses. Results of two independent experiments were averaged, n=9-10 mice per cell dose. **B.** $15x10^6$ CFSE labeled small resting splenic B cells from normal donors were mixed with the indicated numbers of unstained normal or *xid* splenic B cells, transferred into unirradiated *xid* recipients, and analyzed on day 13 after transfer. @p>0.3; *p<0.04; **p<0.01. **C.** The indicated numbers of purified immature B cells were transferred into unirradiated *xid* recipients and analyzed on day 10.

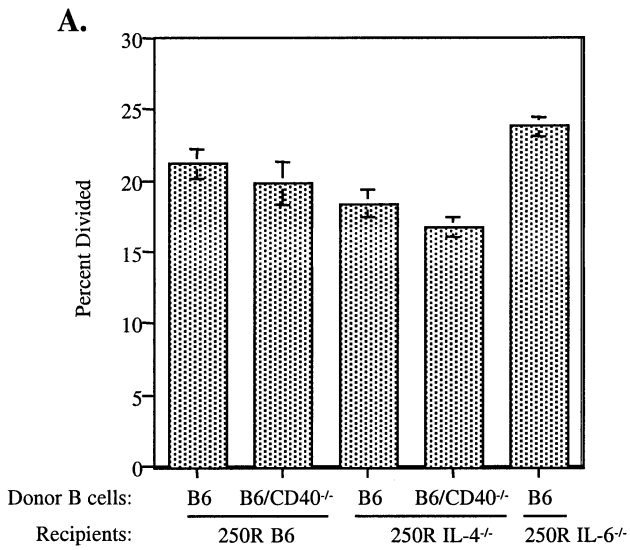
B cell homeostatic proliferation does not require signals from CD4$^+$ T cells

CD40 is expressed on naive mature B cells, and signaling through this receptor *in vitro* induces B cell proliferation; the ligand for CD40 (CD40L) is expressed on activated CD4$^+$ T cells (133). IL-4 and IL-6 are cytokines that are secreted by activated T cells; IL-4 promotes B cell proliferation in combination with other signals (such as anti-immunoglobulin) while IL-6 promotes B cell hybridoma growth and the differentiation of antigen stimulated primary B cells (134, 135). Since signals through CD40 or signals generated by IL-4 or IL-6 have the potential to promote B cell proliferation or differentiation, we wanted to determine if these signals are involved in driving homeostatic proliferation. Accordingly, CFSE labeled small resting B cells from wild-type (B6) or CD40$^{-/-}$ mice were transferred into irradiated wild-type or IL-4$^{-/-}$ recipients; wild-type B cells were also transferred into irradiated IL-6$^{-/-}$ recipients. Thus, the mutant donor B cells lacked CD40 expression (136), and the mutant recipients were unable to produce IL-4 or IL-6 (137, 138). Wild-type and CD40$^{-/-}$ B cells proliferated to the same extent after transfer into irradiated B6 mice (Figure 5A, 21.1±1% vs. 19.7±1.5%). Similarly, wild-type B cells replicated at similar levels in irradiated B6 (21.1±1%) , IL-4$^{-/-}$ (18.3±1%), or IL-6$^{-/-}$ (23.8±0.7%) recipients (Figure 5A). Compared to wild-type B cells transferred into irradiated B6 recipients, however, proliferation was slightly reduced upon transfer of CD40$^{-/-}$ B cells into IL-4$^{-/-}$ recipients (Figure 5A, 21.2±1% vs. 16.7±0.7%), suggesting that a combination of CD40 and IL-4 signals might play some role in this expansion. This combination of signals was not absolutely required, however, because the proliferation of CD40$^{-/-}$ B cells in IL-4$^{-/-}$ recipients was higher than that seen upon

transfer of wild-type B cells into unirradiated normal recipients (Figure 1, 10±0.6%). This demonstrated that there was no absolute requirement for CD40, IL-4, or IL-6 in B cell homeostatic expansion.

Since CD40L, IL-4, and IL-6 are expressed on or secreted by activated $CD4^+$ T cells (133-135), these data suggested that $CD4^+$ T cells were not involved in driving B cell homeostatic proliferation. To test this idea directly, B cells from $CD4^{-/-}$ mice, which lack $CD4^+$ T cells (139), were transferred into irradiated wild-type (B6) or $CD4^{-/-}$ recipients. The extent of homeostatic proliferation was not diminished in the absence of $CD4^+$ T cells, demonstrating that these cells are not required for proliferation (Figure 5B, B6-->irrad B6, 22.7±0.7% vs. $CD4^{-/-}$-->irrad $CD4^{-/-}$, 23.8±0.2%). Taken together, these data demonstrate that the inductive signal(s) driving B cell homeostatic expansion are not delivered by $CD4^+$ T cells.

Figure 5. B cell homeostatic proliferation does not require signals from CD4$^+$ T cells



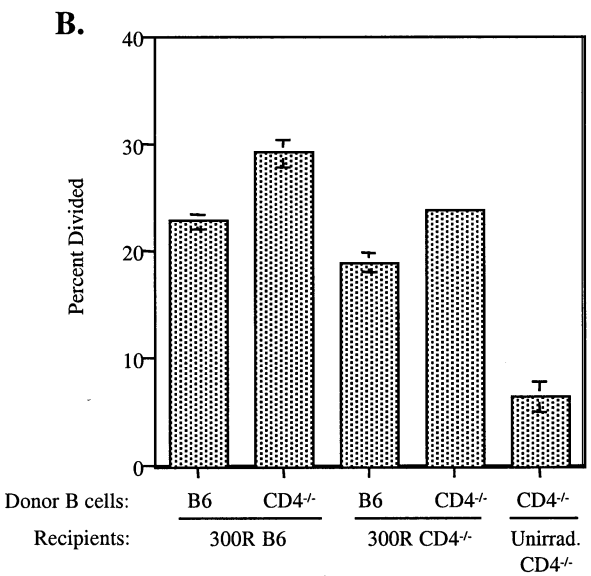


Figure 5. B cell homeostatic proliferation does not require signals from $CD4^+$ T cells. **A.** 20-25x10^6 CFSE labeled small splenic B cells from wild-type (C57BL/6) or $CD40^{-/-}$ donors were transferred into irradiated (250R) wild-type(C57BL/6), IL-$4^{-/-}$, or IL-$6^{-/-}$ recipients and analyzed on day 14 as described in Materials and Methods. The mean percent divided ± SE in each donor cell population, gated on $CFSE^+B220^+$ cells. Results are from one to two experiments for each donor cell group. IL-$6^{-/-}$ recipients, n=3; all others n=6-15. **B.** 20x10^6 CFSE labeled small splenic B cells from wild-type (C57BL/6) or $CD4^{-/-}$ mice were transferred into irradiated (300R) wild-type or $CD4^{-/-}$ recipients and analyzed on day 13 as described in Materials and Methods. $CD4^{-/-}$ B cells were also transferred into unirradiated $CD4^{-/-}$ mice. The mean percent divided ± SE in each donor cell population, gated on $CFSE^+B220^+$ cells; n=4 per group.

Btk is involved in signaling pathways for B cell homeostatic proliferation

B cells are capable of homeostatic expansion in a B cell deficient environment, but the inductive signals are unknown. The critical role of the BCR in B cell survival suggests that it may also play a role in homeostatic proliferation. Similarly, it has been established that TCR signals are critical for survival and homeostatic proliferation (42, 43, 53-57, 140). *xid* mice have a defect in Btk, a Tec family tyrosine kinase that is involved in numerous signaling pathways in B cells (115). As a result, *xid* B cells proliferate poorly in response to stimulation through a number of cell surface receptors including the BCR, IL-5R, IL-10R, the toll-like receptor RP-105, and CD38 (141-145).

The poor proliferation seen *in vitro* following stimulation through these receptors led us to hypothesize that the signaling defects in *xid* B cells might render them less responsive to the *in vivo* signals that drive homeostatic expansion. We examined this possibility by comparing the replicative ability of normal and *xid* B cells after transfer into *xid* recipients. We found that splenic *xid* B cells underwent significantly less homeostatic proliferation than normal B cells (Figure 6A; 12.2±1.1% versus 24.3±1.1%, $p<0.001$). To exclude poor survival of *xid* donor cells (146, 147) as a basis for this effect, we transferred splenic B cells from *xid/bcl-2* transgenic mice into *xid* recipients. B cells from *xid/bcl-2* transgenic mice are enriched for mature IgM^{lo} IgD^{hi} B cells, have enhanced survival *in vitro*, but remain refractory to stimulation with Btk dependent activators, including anti-Ig (146). As with non-transgenic cells, *xid/bcl-2* transgenic B cells showed significantly reduced homeostatic proliferation compared to normal *bcl-2* transgenic B cells (Figure 6A; 10.9±0.3% versus 23.7±0.7%, $p<0.001$), demonstrating

that the lack of proliferation was not due to reduced donor cell survival. Splenic populations from *xid* mice have a higher proportion of immature B cells than normal and it is possible that the most developmentally mature *xid* B cell is capable of homeostatic proliferation. To look directly at the behavior of these cells, we used lymph node B cells as the donor population in transfer experiments. As was found with spleen cells, lymph node *xid* B cells have a significant defect in homeostatic proliferation compared to normal lymph node B cells (Figure 6A; 11.9±0.9% versus 19.3±0.5%, $p<0.001$). These results are consistent with the data from *xid/bcl-2* transgenic donors, which have increased numbers of mature splenic B cells relative to non-transgenic *xid* mice.

We noted that homeostatic proliferation was not completely suppressed by the Btk mutation in the strains tested. This could be due to the fact that the severity of the Btk mutation is effected by genetic background (148). To test this possibility, we transferred B6 or B6.*xid* congenic B cells into B6.scid recipients. Under these experimental conditions, reduced homeostatic proliferation of *xid* B cells was again observed (Figure 6B; *xid*,5.5±0.2% versus normal, 33.4±1.4%, $p<0.001$), showing that restricted *xid* replication is not an unexpected consequence of transferring into an *xid* environment. It is also noteworthy that replication of normal cells is highest in SCID recipients (see Figures 1, 6, and 7) and that the inhibition of replication of B6.*xid* B cells is more prominent. Taken together, these data demonstrate that Btk-mediated signaling is important for B cell homeostatic proliferation.

Figure 6. Reduced homeostatic proliferation of *xid* B cells

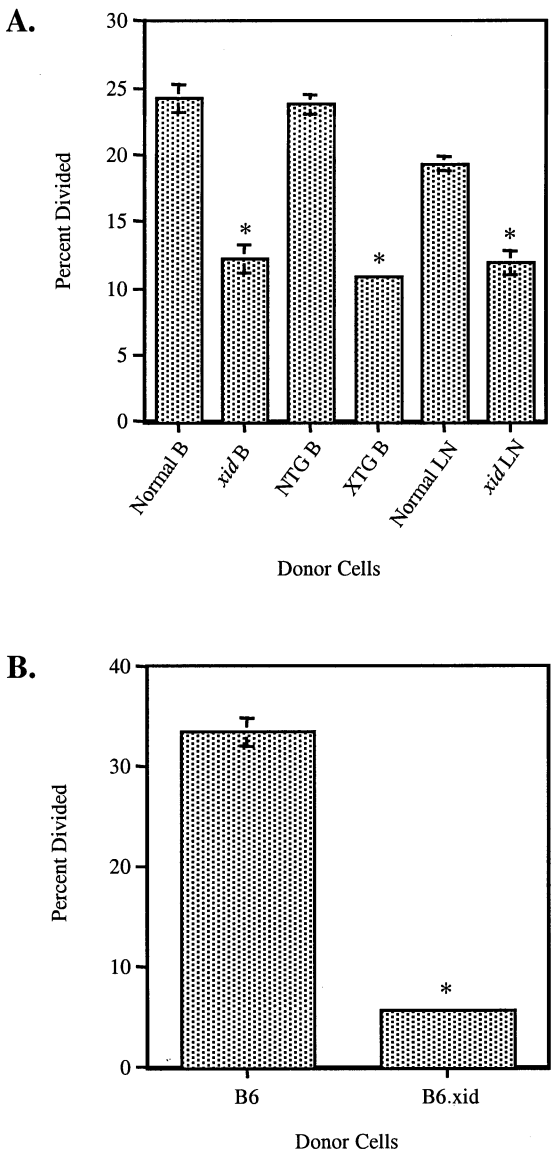


Figure 6. Reduced homeostatic proliferation of *xid* B cells. **A.** $20x10^6$ CFSE labeled small splenic B cells from normal (CBA/Ca), normal/*bcl-2* transgenic (NTG), *xid* (CBA/N), or *xid*/*bcl-2* transgenic (XTG) donors were transferred into *xid* recipients (CBA/N) and analyzed on day 12 after transfer as described in Materials and Methods. In other experiments $40x10^6$ CFSE labeled small total lymph node cells from normal or *xid* (CBA/NxA.By female or male) donors were transferred into *xid* (CBA/NxA.By male) recipients and analyzed on day 14. The mean percent divided ± SE in each donor cell population, gated on $CFSE^+B220^+$ cells. Results are from one to two experiments for each donor cell group, n=4-11. $*p<0.001$ compared to normal B cells. **B.** $20x10^6$ CFSE labeled small splenic B cells from normal (C57BL/6) or *xid* (C57BL/6.*xid* congenics) were transferred into C57BL/6.SCID recipients and analyzed on day 12 after transfer. C57BL/6, n=9 (three experiments); C57BL/6.*xid*, n=4 (one experiment). $*p<0.001$ compared to normal cells.

B cells from c-rel$^{-/-}$ or nfkb1$^{-/-}$ mice are capable of homeostatic proliferation

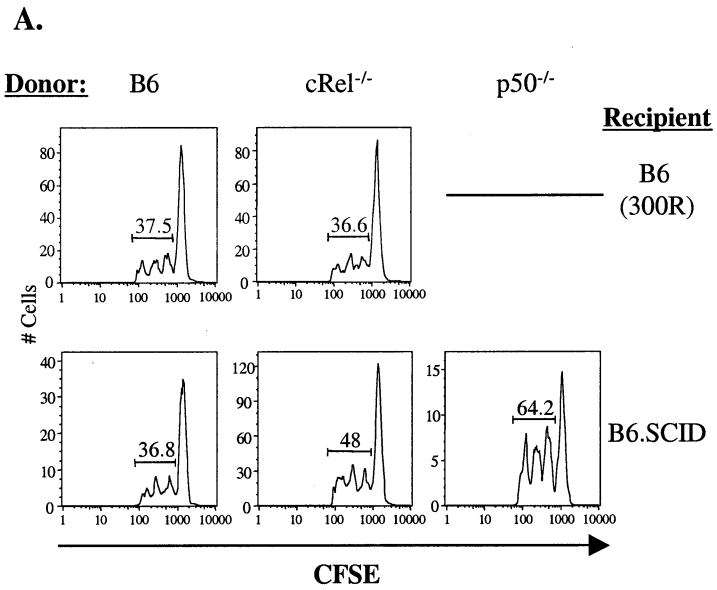
Ligand-receptor interactions leading to cell proliferation require the upregulation or relocalization of transcription factors. For B cells, optimal mitogen and antigen induced replication requires members of the NF-κB/Rel family of transcription factors, some of which act downstream of Btk (149, 150). Targeted mutation of the *c-rel* gene has demonstrated that c-Rel is important for B cell proliferation induced by stimulation through the BCR, CD40, and Toll receptors; but its absence does not affect peripheral B cell numbers or survival *in vivo* (116, 151). In contrast, loss of NF-κB1 (p50, p105) has little effect on proliferation induced by BCR cross-linking, however, survival of peripheral mature B cells is severely compromised (116, 152). These observations led us to hypothesize that c-Rel or NF-κB1 knock-outs could be used to distinguish the signals required for homeostatic proliferation from those necessary for mitogen and/or antigen induced proliferation or peripheral B cell survival.

Small resting CFSE labeled spleen cells from B6, c-rel$^{-/-}$ or nfkb1 (p50)$^{-/-}$ mice were adoptively transferred into irradiated (300R) B6 or unirradiated B6.SCID recipients, and the extent of homeostatic proliferation assessed on day 12 after transfer (Figure 7A). Clearly, lack of c-Rel or NF-κB1 expression had no effect on homeostatic proliferation in B6.SCID (Figure 7B; B6, 39.6±1.3%; c-rel$^{-/-}$, 39.2±3.4%; nfkb1$^{-/-}$, 54.3±6.1%). Similar results were obtained for c-rel$^{-/-}$ B cells in irradiated B6 recipients (B6, 30.8±4.1%; c-rel$^{-/-}$ 27.5±2.0%, Figure 7B). The survival defect of nfkb1$^{-/-}$ donor cells was exacerbated in irradiated B6 recipients, so data could not be obtained for this group. The fact that nfkb1$^{-/-}$ B cells proliferated on transfer (Figure 7A) but exhibited impaired survival *in*

vivo (116) suggests that the signals involved in B cell survival and those for homeostatic proliferation are not identical.

Although, c-rel$^{-/-}$ B cells were unresponsive to anti-Ig+IL-4 or LPS stimulation *in vitro* (data not shown), these cells had the same capacity for homeostatic proliferation as did wild-type cells (Figure 7A and 7B). These data suggest that the surface molecules known to require c-Rel for proliferative responses (e.g. BCR, CD40, Toll receptors) are not exclusively involved in homeostatic proliferation, however it remains possible that combined signaling using one or more of these molecules is important. In fact, it has been shown that using a combination of mitogens that are individually inactive (anti-IgM and LPS) can overcome the proliferative defects of c-rel$^{-/-}$ B cells(116). Overall, these data demonstrate that survival and homeostatic proliferation are separable, and that proliferation is optimally induced by a combination of signals.

Figure 7. B cells from c-rel$^{-/-}$ or p50$^{-/-}$ mice are capable of homeostatic proliferation



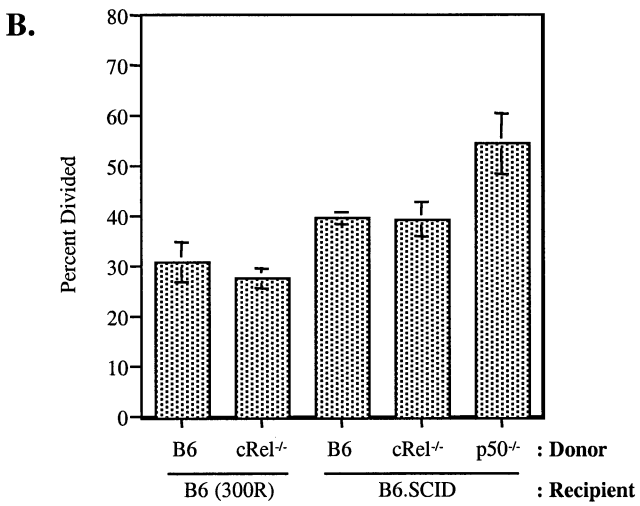


Figure 7. B cells from c-rel$^{-/-}$ or p50$^{-/-}$ mice are capable of homeostatic proliferation. **A.** 35x10^6 CFSE labeled, small resting total spleen cells from B6, c-rel$^{-/-}$, or p50$^{-/-}$ mice were adoptively transferred into the indicated recipients. The percent of B220$^+$ B cells in each donor population was similar. Representative histograms showing the extent of proliferation of CFSE$^+$ B220$^+$ cells on day 12-13 after transfer. Survival of p50$^{-/-}$ cells in irradiated recipients was poor, so the data are not shown. **B.** The mean percent divided ± SE in each donor cell population, gated on CFSE$^+$ B220$^+$ cells. Results were averaged from two independent experiments, n=6-8 mice per group.

Discussion

We show here that immature and mature B cells are subject to homeostatic regulation in the periphery. B cells proliferate in response to a B cell deficient environment in the host and replication is suppressed selectively in a cell dose dependent fashion by mature B cells. B cells undegoing homeostatic proliferation fail to upregulate antigenic markers induced on B cells responding to antigen or mitogen stimulation, and this proliferation is independent of signals delivered by $CD4^+$ T cells. While B cell homeostatic proliferation depends on Btk mediated signaling, it does not rely on transcription factors (c-Rel and p50) critical for conventional proliferation induced by antigen or mitogen (c-Rel), or for peripheral B cell survival (p50), showing the uniqueness of this inductive pathway. These data and the results of similar studies with T cells suggest that homeostatic proliferation is a universal mechanism for effecting peripheral lymphocyte homeostasis.

It was previously shown that CFSE labeled adult B cells replicated when transferred to syngeneic neonatal, but not adult, recipients (101). The host age dependence of B cell replication has also been shown in another model system (153). It was not determined whether the B cells were responding to the selective immunodeficiency of the neonatal host or a generalized stimulatory environment in which lymphocyte replication is promoted. We have found that adult recipients readily support B cell proliferation provided that mature endogenous B cells are reduced in number. Although proliferation is limited in normal unirradiated recipients, it does occur in *xid* recipients, which are deficient in mature IgM^{lo} IgD^{hi} cells (117, 118); sublethally

irradiated (250R) normal recipients, which are primarily B cell deficient (154), and SCID recipients which are deficient in both T and B cells (155). This replication is specific for the deficient subpopulation since *xid* mice have normal numbers of T cells. Moreover, B cell replication is T cell independent since it occurs in SCID and $CD4^{-/-}$ recipients. The finding that homeostatic proliferation is not completely absent in normal unirradiated recipients suggests that this phenomenon may be a normal component of peripheral B cell homeostasis.

Homeostatic proliferation is a property of both immature and mature B cells. Purified immature B cells from auto-reconstituting irradiated donors actively proliferated in *xid* recipients. Mature B cells purified from lymph nodes, which do not contain immature or marginal zone B cells, or B1 cells (130, 131), also actively proliferated. When transferred into *xid* recipients, we observed that mature B cell proliferation was inversely proportional to cell dose, while immature B cells proliferated equally at all cell doses. Replication of immature B cells was suppressed after transfer into unirradiated normal recipients showing their sensitivity to inhibition. While normal and *xid* recipients have comparable numbers of immature and marginal zone B cells, normal mice have significantly more mature B cells. Therefore, it might be reasonable to assume that there is a sensing mechanism present that maintains normal B cell numbers within the host and that the mature B cell is the dominant regulator of both immature and mature B cell homeostatic proliferation.

An inductive signal is involved in homeostatic proliferation because we observe a transient increase in CD69 on virtually every transferred cell. The cells that do divide

also show increases in forward scatter and expression of CD71 which return to resting levels. This signal does not promote differentiation to antibody secretion, as shown by the lack of CD43 and syndecan expression and the maintenance of CD38, IgM, and IgD expression on divided cells. It has been shown that T cells which divide in lymphopenic hosts increase CD44 expression, a marker of memory cells, as well as other memory markers such as Ly6C, CD122, CD132, and LFA-1 (53-56, 58). Memory marker expression and increased antigen responsiveness were a function of an increased number of cell divisions (120, 121). We can easily follow three cell divisions with B cells, and these cells return to a resting phenotype. While we cannot exclude the possibility that cells which divide more than three times differentiate to effector function, our data are consistent with the idea that B cells undergoing homeostatic proliferation receive an inductive signal, but remain naive. Two pieces of evidence demonstrate that this inductive signal is not delivered by $CD4^+$ T cells. First signals delivered by CD40L, IL-4, or IL-6, which are on the surface of or secreted by activated T cells (133-135), are not absolutely required for homeostatic proliferation. Second, B cells from $CD4^{-/-}$ donors transferred into irradiated $CD4^{-/-}$ recipients proliferate as well as B cells transferred into a $CD4^+$ T cell sufficient environment. Thus, while the existence of an inductive signal is clear, the nature of the signal remains unknown.

Our observation that *xid* and *xid*/bcl-2 transgenic B cells are defective for homeostatic proliferation demonstrates that Btk is an important component of the inductive signal(s) driving homeostatic proliferation. Based on the observation that T cell homeostatic proliferation requires interactions between the TCR and peptide-MHC

complexes (53-57, 156), it is tempting to speculate that signals through the BCR may be involved in B cell homeostatic expansion. The Btk dependence of proliferation is consistent with this idea, since *xid* B cells are hyporesponsive to anti-Ig stimulation *in vitro* (141). *xid* B cells, however, also have defects in signaling through a number of other cell surface receptors, including the IL-5 and IL-10 receptors (142, 143), RP-105 (144), and CD38 (145). Thus, we cannot rule out the possibility that these or other molecules are involved in B cell homeostatic proliferation.

We also examined the effects of deficiencies in the NF-κB family members c-Rel or p50 on B cell homeostatic proliferation. c-Rel is interesting because it is required for B cell proliferation in response to a variety of mitogens, including anti-Ig (116, 151). p50, on the other hand, is not required for mitogen induced proliferation, but is involved in regulating B cell survival, since $p50^{-/-}$ B cells undergo more rapid apoptosis *in vitro* and have faster turnover rates *in vivo* (116, 152). Interestingly, we find that homeostatic proliferation is not impaired in $c\text{-}Rel^{-/-}$ or $p50^{-/-}$ B cells. While the c-Rel data would seem to argue against involvement of the BCR in homeostatic proliferation, it has been shown that the defect in anti-Ig induced proliferation can be partially overcome by combinations of activators such as anti-Ig +LPS or anti-IgM+anti-CD40 (116, 157), so it is possible that signals through the BCR along with other signals are involved. The $p50^{-/-}$ result suggests that the signals involved in B cell survival and homeostatic proliferation are not identical, which is consistent with recent data on T cells showing that homeostatic proliferation, but not survival, was impaired in T cells lacking $p56^{lck}$ (114). This does not exclude the possibility, however, that these two processes use some common signaling

pathways. For example, survival and homeostatic proliferation may both use the BCR, with survival requiring one set of additional signals and proliferation another.

Overall, our data are consistent with a model in which immature and naive mature B cells sense the number of mature cells in the periphery and respond to a reduction in these cells by proliferating; this response is exaggerated in B cell deficient hosts. This sensing mechanism may operate in a specific microenvironment since pertussis toxin treatment of donor cells blocks entry into the splenic white pulp and inhibits B cell proliferation and survival in neonatal recipients (158, 159). A model depicting the putative signals involved in driving homeostatic proliferation is shown in Figure 8. Signals through the BCR and other receptors on B cells promote cell survival *in vivo* (41, 44-46). Our observation that mature B cells mediate feedback inhibition of homeostatic proliferation suggests that there is an inhibitory signal delivered by mature B cells that normally suppresses proliferation. Under conditions of B cell deficiency, the inhibitory signal may be removed and stronger survival signals delivered; in addition, other receptors may be engaged that promote B cell proliferation without differentiation to effector function.

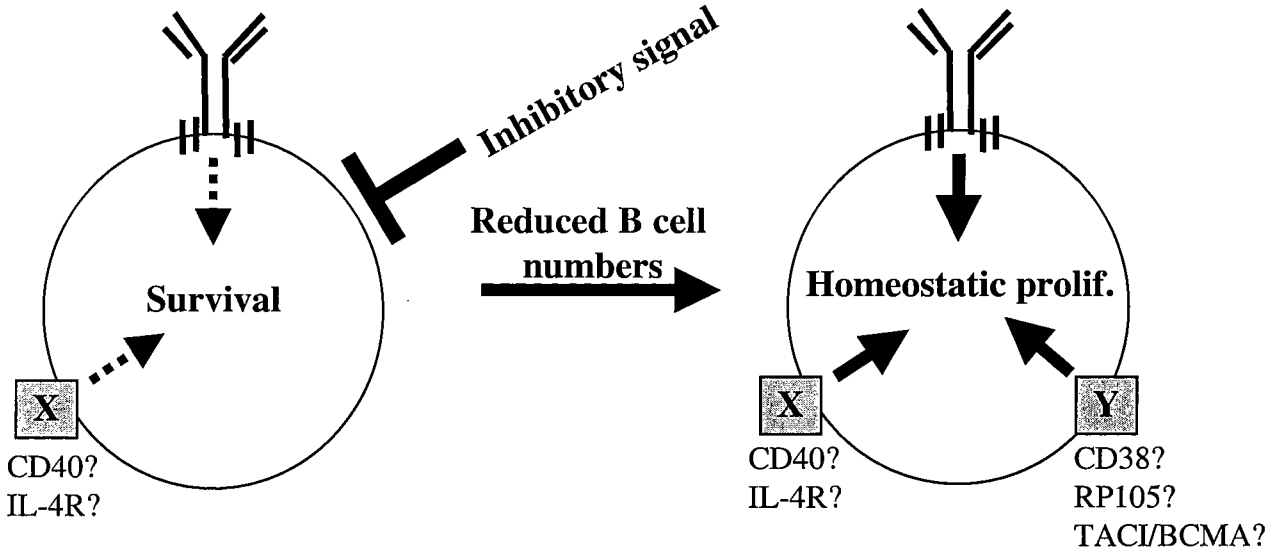


Figure 8. Model for B cell homeostatic proliferation. Signals through the BCR and other receptors on B cells promote cell survival *in vivo*. Furthermore, there may be inhibitory signals that prevent B cells from proliferating in response to these signals. Under conditions of B cell deficiency, stronger survival signals may be delivered, and other receptors engaged that promote B cell proliferation without differentiation to effector function.

Since our data with *xid* B cells are consistent with BCR involvement and the c-rel$^{-/-}$ and p50$^{-/-}$ experiments do not exclude it, we hypothesize that the BCR is one of the B cell surface receptors involved in driving B cell homeostatic proliferation. There is evidence for a single BCR specificity binding a number of peptide ligands, and these ligands differ in their ability to activate B cells depending on the affinity of the interaction (160). So, it is possible that mature B cells bind cross-reactive ligands with low affinity in the periphery. Under conditions of B cell deficiency, these ligands might be more readily available, leading to increased BCR signaling which contributes to B cell proliferation. One prediction of this model is that B cells with different specificities should undergo different degrees of homeostatic proliferation. In fact, it has been shown that T cell homeostatic proliferation is dependent on the TCR specificity, since some TCR transgenics fail to proliferate in lymphopenic hosts (55). We are currently testing this hypothesis using Ig heavy chain transgenic mice.

Another possibility is that the BCR generates a low level signal without being engaged by environmental ligands. In this model, the outcome of BCR signaling is determined not by the degree of BCR cross-linking, but by the composition of the BCR signaling complex. There are several proteins known to positively or negatively regulate BCR signaling, and it is the balance between these proteins that determines the outcome of BCR signaling (161). One example is CD22, which constitutively associates with the BCR in resting B cells and is thought to inhibit spontaneous BCR signaling (162). This idea is supported by the finding that sequestering CD22 away from the BCR using anti-CD22 coated beads lowers the concentration of anti-IgM required to induce B cell

proliferation by 100-fold (163). CD22 binds Sia6LacNAc, a trisaccharide found in many glycoproteins (161). Thus, one can envision a model in which CD22 is associated with the BCR under B cell sufficient conditions; when B cell numbers decrease, more CD22 may be bound by its ligand and sequestered away from the BCR. This would lead to a stronger "spontaneous" signal that might synergize with other signals to drive homeostatic expansion. A knock-out for the enzyme that produces the ligand for CD22 (ST6Gal sialyltranferase) has been made, and these mice show defective humoral immune responses (164). If sequestering CD22 away from the BCR is involved in homeostatic proliferation, one would expect proliferation in irradiated ST6Gal sialyltranferase knock-outs to be reduced.

B cells from c-Rel knock-out mice show impaired anti-immunoglobulin induced proliferation, but this defect can be overcome using combinations of activator such as anti-Ig+LPS (116). Assuming that BCR signals contribute to homeostatic proliferation, the observation that c-Rel$^{-/-}$ B cells are capable of replicating in B-deficient hosts suggests that other factors are important as well. Alternatively, such signals might induce proliferation independently of the BCR. If this is the case, these inductive pathways must be Btk dependent; two possibilities are CD38 and RP105. CD38 is expressed on mature B cells, and cross-linking this molecule in combination with IL-4 or LPS induces B cell proliferation in a Btk dependent manner (145, 165). Interestingly, stimulation through CD38 does not induce nuclear translocation of c-Rel, suggesting that this pathway is not c-Rel dependent (R. Woodland, laboratory observations). The fact that CD38 can induce B cell proliferation in a Btk dependent, c-Rel independent fashion makes it possible that

this pathway is involved in B cell homeostatic proliferation. This hypothesis is testable, since a CD38 knock-out mouse has been developed (165). RP105 is a member of the Toll-like family of proteins that is expressed on mature B cells and is involved in LPS responses (144, 166). Cross-linking this protein induces B cell proliferation in normal, but not Btk-defective *xid* B cells (144). Proliferation to anti-RP105 is also reduced in c-rel$^{-/-}$ and p50$^{-/-}$ B cells, but like BCR dependent proliferation in these cells, the defect can be corrected by the addition of cytokines (IL-2, IL-4, and IL-5) or LPS (116). This suggests that signals through RP105, possibly induced by bacterial cell wall components such as LPS, might contribute to the induction of homeostatic proliferation.

The induction of CD69 essentially all B cells transferred into *xid* recipients suggests that an inductive signal drives proliferation in these hosts. The fact that mature B cells suppress this proliferation indicates that an inhibitory signal in normally involved in controlling B cell replication. Thus, a balance of positive and negative signals likely controls this process. Interestingly, two receptors that are members of the TNF family (BCMA and TACI) and are expressed on B cells in the periphery (167) have recently been shown to provide both positive and negative regulatory signals to B cells. The ligands for TACI and BCMA are BAFF (also known as BLyS) and APRIL, which are expressed on or secreted by monocytes, dendritic cells, and activated T cells (167). Both BAFF/BLyS and APRIL can bind to either BCMA or TACI, but there may be preferential TACI-BAFF/BLyS and BCMA-APRIL binding (168). A number of lines of evidence suggest that this dual receptor-ligand system is involved in maintaining peripheral B cell homeostasis. *In vitro,* BAFF/BLyS enhances B cell proliferation

induced by anti-immunoglobulin treatment (169, 170) and also promotes the survival of all B cell populations, including immature splenic B cells (171). The importance of BAFF/BLyS in peripheral B cell homeostasis is further demonstrated by *in vivo* studies; BAFF transgenic mice show a large increase in the number of peripheral B cells while blocking BAFF/BLyS receptor binding with soluble BCMA leads to a decrease in peripheral B cell numbers (46, 172). Thus, BAFF/BLyS may maintain homeostasis by promoting B cell survival and perhaps proliferation. Interestingly, TACI knock-out mice have increased numbers of hyperproliferative B cells in the spleen, suggesting that TACI delivers inhibitory signals to B cells *in vivo*. These data indicate that the BAFF/BLyS-TACI/BCMA receptor-ligand system has the potential to mediate both positive and negative signaling in B cells. A model can be envisioned in which preferential use of TACI under B cell sufficient conditions tends to suppress B cell replication while in a B cell deficient environment, BAFF/BLyS binds BCMA to promote homeostatic proliferation. Thus, it would be interesting to see if homeostatic proliferation is enhanced by exogenous BAFF/BLyS or inhibited by soluble BCMA. Our data suggest that multiple signals are involved in driving homeostatic expansion, and experiments such as these should allow us to dissect out the important pathways.

The fact that we see a small degree of proliferation after adoptive transfer into unirradiated normal recipients suggests that homeostatic proliferation occurs at a low level and may contribute to normal B cell homeostasis. If, as we speculate, signals through the BCR and BCR specificity are important for this process, homeostatic proliferation may help to determine the naive B cell repertoire. The contribution of

homeostatic proliferation to the repertoire would be greater during reconstitution of immunodeficient hosts and perhaps in neonates. Finally, we have preliminary evidence that the aged environment is less capable of supporting homeostatic proliferation. This may in part explain the defects in B cell-mediated immunity and repertoire changes in older individuals.

Chapter IV

In Vivo Persistence Of Vesicular Stomatitis Virus In B Cells And Suppression Of Reactivation By Antiviral Antibody

Introduction

Persistent virus infections are characterized either by the maintenance of viral genomes in a host cell or organism in a noninfectious state or by continuous low level production of virus after acute infection (1). Establishing persistent infections allows viruses to evade the host immune response and thus be maintained within individuals in a population of susceptible hosts; these types of infections are often associated with diseases such as cancer, immunodeficiency, and disorders of the central nervous system (1). Persistence of RNA viruses such as hepatitis C virus and measles virus has been associated with chronic diseases including hepatitis, hepatocellular carcinoma and subacute sclerosing panencephalititis (173, 174). Vesicular stomatitis virus (VSV) is a negative-strand RNA virus of the Rhabdovirus family (17). Many studies of persistence using RNA viruses such as VSV have been carried out in cell lines; the mechanisms of persistence in these systems generally involve the restriction of virus replication in nonpermissive cells, the emergence of viral mutants or defective interfering particles, and the induction of antiviral factors, such as interferon, by infected cells (21-25, 29, 30). The mechanisms by which RNA viruses persist *in vivo*, however, are not well understood.

One way in which a virus can persist *in vivo* is by infection of nonpermissive cell types, which limits injury to the host and allows the virus to evade the immune response

(1). Lymphocytes act as conditionally permissive host cells for many viruses, including VSV in that resting lymphocytes are non-permissive while antigen or mitogen stimulated cells support full virus replication (32-36). Persistent infections are often characterized by a cyclical pattern of virus replication: periods of little or no infectious virus production followed by periodic production of higher levels of infectious virus. *In vivo*, lymphocytes exist in a continuum of activation states, from resting cells to fully activated effector cells; this allows them to harbor non-replicating virus or support full virus replication *in vivo.*

When VSV infects small, resting primary splenic B cells, no viral proteins or infectious particles are produced and only low levels of viral transcripts can be detected (35, 36). The full viral replicative cycle can be induced, however, by treatment with B cell specific activators, with different B cell activation pathways supporting different levels of virus replication (35, 36). For example, treatment of infected B cells with IL-4 enhances viral mRNA levels, but viral proteins and infectious particles are not produced. Treatment with PMA enhances viral protein production but does not support infectious particle production whereas anti-Ig+IL-4 makes B cells fully permissive for virus replication (36). Signals sufficient to drive B cell replication and differentiation are not required for virus replication because activator combinations such as PMA+IL-4 make B cells fully permissive for virus replication without inducing cell proliferation or antibody secretion (36). Thus, submitogenic signals or signals that promote proliferation but not differentiation may alter B cell permissiveness for VSV *in vivo.*

Mice expressing the anti-apoptotic protein Bcl-2 as a transgene under the control of the Ig heavy chain enhancer have an expanded population of mature B cells in the spleen (97). B cells from these mice (B/bcl-2 cells) show greatly extended survival in culture (97, 146), making them ideal for studying long term virus persistence in primary lymphocytes *in vitro*. VSV is maintained in culture for weeks in small resting B/bcl-2 cells with no viral proteins or infectious virus produced; infectious virus can be reactivated from these cells at any time after the initiation of persistent infection by treatment with B cell activators (M. Schmidt, manuscript in preparation). It is generally thought that RNA viruses persist by low level, non-cytopathic replication in host cells (1). VSV persistence in resting B cells, however, is more akin to latent infection caused by DNA viruses such as EBV, which evades the host immune response by restricting viral gene expression in resting B cells (59). Thus resting B cells may make an ideal reservoir for maintaining *in vivo*, allowing the virus to evade the host immune response. *In vivo*, however, B cells receive of variety of signals, which may alter their activation state. B cells are actively maintained *in vivo* by signals delivered through the BCR and other cell surface receptors (41, 44-46). Furthermore, it is shown in this dissertation that B cells can be induced to proliferate without differentiation to effector function under lymphopenic conditions (Chapter III). Since virus expression is dependent on the activation state of lymphocytes, do these signals effect virus replication in infected B cells? Homeostatic signals delivered to B cells may enable them to make infectious virus or viral proteins. Infected B cells could be killed directly by the virus; alternatively, a

host immune response generated as a consequence of virus expression could clear infected cells or help to maintain persistence

There is evidence to suggest that effector cells of the acquired immune response to viruses are directly involved in maintaining virus persistence. Loss of T cells and NK cells results in reactivation of murine CMV from latently infected mice and $CD8^+$ T cells inhibit reactivation of HSV-1 from latently infected trigeminal ganglia (74, 75). It has also been suggested that antiviral antibody is directly involved in maintaining persistence by stripping viral antigens from the surface of infected cells or by restricting intracellular virus replication (2). Treatment of infected cell lines with measles antiserum or monoclonal antibodies to measles HA protein results in downregulation of both externally and internally expressed viral proteins (77-81). Restriction of measles virus mRNA by antibody treatment has also been observed in a rat model of measles encephalitis and in persistently infected neuroblastoma cells (82, 83). These studies demonstrate the ability of antiviral antibodies to act directly on cells harboring an RNA virus (measles virus) and suppress replication at the levels of transcription, protein synthesis, and infectious virus production without killing the cells. Thus, antibody dependent inhibition of virus replication may play a role in the establishment or maintenance of RNA virus persistence *in vivo*. VSV infection in mice is primarily controlled by neutralizing antibody (90-94) and Type I (α/β) interferons (95, 96), suggesting that these immune mechanisms might play a role in the establishment or maintenance of VSV persistence *in vivo*.

Three questions are addressed in this chapter: 1) can VSV infected B cells be maintained *in vivo* for an extended period of time? 2) can virus be reactivated from persistently infected B cells *in vivo*? 3) does antiviral antibody play a role in maintaining virus persistence in B cells? In order to address these questions, two experimental approaches were taken. Persistence of VSV in B cells *in vivo* was studied by purifying small, resting splenic B cells, infecting them with VSV, and adoptively transferring them into naive recipients. This approach has the following advantages: the cell type of interest (B cells) is specifically targeted for infection; large numbers of infected cells can be transferred making detection of infected cells easier; the cells can be tracked *in vivo* by staining with fluorescent dyes. The effect of antiviral antibody on VSV persistence and reactivation in primary B cells was studied in an *in vitro* system using B cells from bcl-2 transgenic mice. VSV infected, bcl-2 transgenic B cells can be maintained for weeks in culture; this allows one to determine the effects of long term antibody treatment on the maintenance of virus persistence in primary B cells.

These experiments demonstrate that VSV can be maintained in B cells for at least 72 days after adoptive transfer. An antibody response to VSV is seen in infected B cell recipients which is concurrent with the proliferation of a fraction of the transferred cells; this suggests that signals inducing homeostatic proliferation may induce virus expression in some infected B cells. Viral RNA can be detected in the spleens of infected B cell recipients, but infectious virus cannot be isolated from activated spleen cells, indicating that virus replication is suppressed in these animals. *In vitro*, prolonged treatment of VSV infected, bcl-2 transgenic B cells with anti-VSV antibody results in suppression of

viral RNA, protein, and infectious particle production after removal of antibody and stimulation of infected cells; however, virus can be reactivated from antibody treated cells after culturing the cells in the absence of antibody. Taken together, these observations lead to a model of VSV persistence in B cells *in vivo* in which viral genomic RNA is maintained in resting B cells, resulting in evasion of the immune response. Virus expression may be induced in some cells by homeostatic signals that drive proliferation, resulting in an antibody response to the virus. The antiviral antibody, in turn, suppresses virus reactivation and maintains persistence by rendering infected B cells refractory to signals that can induce full VSV replication.

Results

VSV persists in mice receiving infected resting B lymphocytes

It has been demonstrated in this laboratory that VSV persistently infects resting primary B cells from bcl-2 transgenic mice (B/bcl-2 cells), and persistence is maintained for several weeks in culture. These infected B cells produce low levels of viral transcripts, but no detectible proteins or infectious particles; however, virus can be reactivated at any time by treatment of the cells with various B cell activators (35, 36) and (M. Schmidt, manuscript in preparation). The ability of resting B cells to maintain the genome of an RNA virus for extended periods suggests that these cells are ideal hosts for a persisting RNA virus *in vivo*; the fact that viral proteins and infectious particles are not produced in resting B cells means that the virus should be able to evade the host immune response. To test whether B cells can function as a reservoir for VSV *in vivo* without being cleared by the immune system or eliminated by replicating virus, small

resting B cells from *xid*-bcl-2 transgenic mice were infected with VSV, adoptively transferred into irradiated or unirradiated *xid* recipients, and spleens harvested on day 10, 30, or 72 after adoptive transfer for analysis. *xid*-bcl-2 transgenic B cells were used in initial experiments to maximize the chances of maintaining persistently infected cells; the bcl-2 transgene enhances survival (98, 175) while the *xid* mutation renders the cells less sensitive to environmental signals *in vivo* (Chapter III). *xid* recipients were used because the number of mature peripheral B cells in these mice is reduced (117, 176); this allows for the adoptive transfer of cells without irradiation, which could alter the *in vivo* cytokine environment and thus the activation state of the transferred cells. Irradiation could, however, increase the efficiency of uptake of the transferred cells, so both irradiated and unirradiated recipients were used. *xid* mice make normal antibody responses to VSV (177), so persistence could be studied in the context of a normal immune response.

A persistent virus infection was defined as the ability to detect viral RNA by RT-PCR but not infectious particles in a particular tissue, whereas in a productive infection viral RNA and infectious particles should be detected. The RT-PCR assay could detect one VSV infected B cell in the presence of 10^6 uninfected B cells (data not shown). VSV persisted in the spleens of both irradiated (data not shown) and unirradiated xid mice receiving infected B lymphocytes for at least 72 days (Figure 9, Table 1). Similar results were obtained using normal bcl-2 transgenic or non-transgenic B cells as donors, demonstrating that VSV persistence was not unique to *xid* or bcl-2 transgenic B cells (Figure 11 and data not shown). Irradiation of recipients did not increase the frequency

with which viral RNA was detected, indicating that there was sufficient space in unirradiated *xid* recipients to allow for survival of the adoptively transferred B cells (data not shown). These data were consistent with the idea that VSV RNA was maintained in B cells after adoptive transfer, but it was possible that infectious virus was produced by the transferred B cells and maintained in host cells other than B lymphocytes. This possibility was addressed by experiments described below.

VSV persistence was also observed in the brains of some unirradiated infected B cell recipients (Table 1). This is interesting because VSV usually replicates very efficiently upon entering the CNS and causes hind-limb paralysis and death within 7-10 days (178); thus, it seems unlikely that viral RNA was maintained in cells of the CNS. While hind-limb paralysis and death were seen in some irradiated recipients of infected B cells, there were irradiated recipients in which viral RNA was detected in the absence of infectious virus (data not shown). One possibility was that persistently infected B cells may have crossed the blood-brain barrier and may acted as reservoirs for virus in the brain. This would not be unprecedented, since it has been suggested that B cells carry other infectious agents (JC virus or prion proteins) across the blood-brain barrier and into the CNS (179, 180). Future experiments will determine if, in fact, VSV is maintained in B cells in the CNS. If true, this would provide a model in which to study the mechanisms by which persistence is maintained in the CNS and the long-term consequences of such an infection to the host.

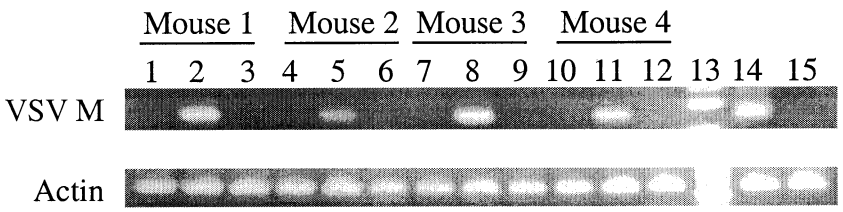


Figure 9. VSV RNA persists in the spleens of infected B cell recipients. Small resting splenic B cells purified from CBA/N-bcl-2 transgenic mice (*xid*) were infected with VSV-SLC at moi=10, extensively washed, and $20x10^6$ cells injected into unirradiated CBA/N (*xid*) recipients via the lateral tail vein. On day 10 after transfer, the indicated tissues were removed, RNA extracted, and nested RT-PCR was performed as described in Materials and Methods; random primers were used for the RT reactions and primers for VSV M or actin for the PCR reactions. PCR products were run on a 2% agarose gel and detected by ethidium bromide staining. Lanes 1, 4, 7, 10 blood; Lanes 2, 5, 8, 11 spleen; Lanes 3, 6, 9, 12 brain; Lane 13 molecular weight markers; Lane 14 infected B cell RNA (positive control); Lane 15 uninfected B cell RNA (negative control).

	Organ			
	Spleen		Brain	
Day Post Transfer	Viral RNA detected	Infectious virus isolated	Viral RNA detected	Infectious virus isolated
10	10/13 (77%)	0/13	1/13 (8%)	0/13
30	7/9 (78%)	0/5	2/5 (40%)	0/5
72	1/4 (25%)	N.D.	1/4 (25%)	N.D.

Table 1. VSV persistence in the spleen and brain of infected B cell recipients. Small resting splenic B cells from *xid*-bcl-2 transgenic mice were infected at moi=10 with VSV-SLC and injected via the lateral tail vein into unirradiated *xid* recipients. Spleens and brains were harvested from recipients on the indicated day after transfer and assayed for the presence of viral RNA by RT-PCR or infectious virus by plaque assay. N.D. not determined.

VSV persists in adoptively transferred B lymphocytes

While these results were consistent with the idea that VSV RNA was maintained in resting B cells *in vivo*, it was possible that the donor cells produced virus upon transfer which was then maintained in another cell type in the spleen of B cell recipients. To examine this possibility, small resting splenic B cells were labeled with the vital fluorescent dye CFSE before infection and adoptive transfer into *xid* recipients. CFSE allows one to track adoptively transferred cells as well as determine their cell division history (101), and the dye does not inhibit production of virus from infected B cells stimulated *in vitro* (laboratory observations). Infected, $CFSE^+$ bcl-2 transgenic or non-transgenic B cells could clearly be seen in recipient spleens two weeks after adoptive transfer, indicating that virus replication did not eliminate infected cells after transfer; the lower intensity $CFSE^+$ peaks demonstrated that a fraction of the transferred B cells had divided, indicating homeostatic proliferation (Figure 10). The fact that some infected B cells divided in *xid* recipients suggested that these cells respond to homeostatic signals in the same way as uninfected B cells (Chapter III). To determine if virus remained in adoptively transferred B cells, $CFSE^+$ and $CFSE^-$ cells were purified from recipient spleens by FACS sorting. RNA was extracted from each population of cells and subjected to RT-PCR; the results clearly showed that VSV RNA was maintained in adoptively transferred B cells (Figure 11, lanes 5 and 6). Whether viral RNA was maintained only in non-dividing cells was not determined because both dividing and non-dividing cells were included in the sort gate. Given the fact that proliferation of VSV infected B cells *in vitro* is associated with the production of infectious virus (36),

however, it is reasonable to hypothesize that some level of virus expression is induced in proliferating B cells *in vivo*. Consistent with this, VSV RNA was detected by RT-PCR in recipient ($CFSE^-$) cells in some experiments, suggesting that virus produced by donor cells was transferred to cells in the host (Table 2). It was also possible, however, that some infected B cells divided to the extent that CFSE staining was lost; thus, some infected donor B cells might be present in the $CFSE^-$ population. Experiments designed to demonstrate the induction of virus expression more directly are described below. Overall, these data are consistent with the idea that VSV can persist in B cells *in vivo* in a quiescent state.

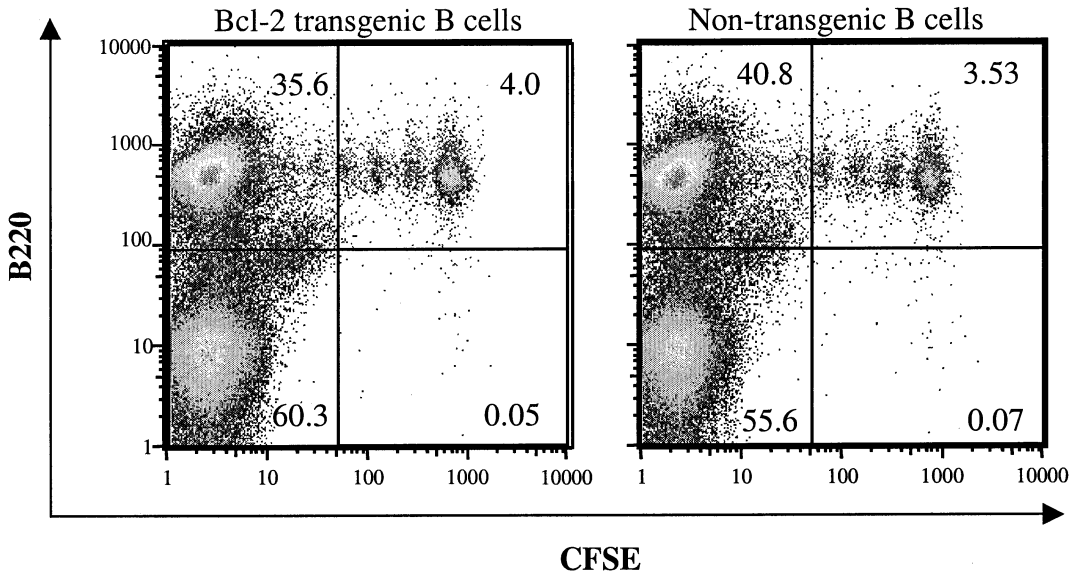


Figure 10. VSV infected B cells survive and proliferate after adoptive transfer into *xid* recipients. Small resting splenic B cells purified from CBA/Ca or CBA/Ca-bcl-2 transgenic (normal) mice were stained with 1 μM CFSE, infected with VSV-SLC at moi=10, and $20x10^6$ cells injected into unirradiated CBA/N (*xid*) recipients via the lateral tail vein. On day 14 after transfer, spleen cell suspensions were made from the recipients as described in Materials and Methods, stained with anti-B220-APC, and analyzed by flow cytometry.

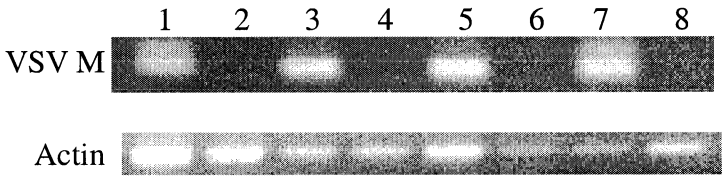


Figure 11. VSV RNA persists in adoptively transferred B cells. Small resting splenic B cells purified from CBA/Ca-bcl-2 transgenic (normal) mice were stained with 1 μM CFSE, infected with VSV-SLC at moi=10, and $20x10^6$ cells injected into unirradiated CBA/N (*xid*) recipients via the lateral tail vein. On day 14 after transfer, spleen cells from 2-3 recipients were pooled and FACS sorted to obtain $CFSE^+$ (donor) and $CFSE^-$ (recipient) populations. RNA was extracted from each cell population and subjected to nested RT-PCR to amplify VSV M or actin sequences as described in Materials and Methods and in the legend for Figure 2. PCR products were run on a 2% agarose gel and detected by ethidium bromide staining. Lanes 1: input infected B cells, 2: uninfected B cells, 3: total spleen, infected B cell recipients, 4: total spleen, uninfected B cell recipients, 5: $CFSE^+$ cells, infected cell recipients, 6: $CFSE^-$ cells, infected cell recipients, 7: positive control RNA, 8: negative control RNA.

	Input Cells	Total Spleen	CFSE (+)	CFSE (-)
Experiment 1				
RT-PCR 1	+	+	+	-
RT-PCR 2	+	+	+	-
Experiment 2				
RT-PCR 1	+	-	+	-
RT-PCR 2	+	+	+	+
Experiment 3				
RT-PCR 1	+	+	-	+
RT-PCR 2	+	+	+	-
RT-PCR 3	+	+	+	+
Total:	7/7 (100%)	6/7 (86%)	6/7 (86%)	3/7 (43%)

Table 2. VSV persists in adoptively transferred B cells. Summary of three independent experiments performed as described in Figure 3. RT-PCR was repeated two to three times on each sample; + indicates VSV M sequences were detected, - indicates no PCR product.

Recipients of infected B cells make an antibody response to VSV

The hypothesis that virus expression was induced in B cells undergoing homeostatic proliferation predicts that an immune response to VSV should be generated in the recipients. This proved to be the case because unirradiated *xid* recipients of VSV infected B cells made anti-VSV IgM and IgG responses that were similar to those in *xid* mice that had received 10^8 PFU of VSV i.v., suggesting that infectious virus or viral proteins were produced by donor cells (Figure 12A). There were two possible explanations for this; (1) non-internalized virus was carried in on the surface of donor cells, or (2) a fraction of donor cells was activated to produce viral antigens *in vivo* after transfer. When infected B cells were treated with mouse anti-VSV serum to neutralize any non-internalized virus prior to transfer, recipient mice still made IgM and IgG responses to VSV that were comparable to recipients of untreated infected cells (Figure 12B). This suggests that antibody was made in response to viral antigens produced by infected B cells, not to virus carried in on the surface of the cells. While there are a small number of virus producing cells before transfer (1-2 infectious centers per 10^3 cells with anti-VSV treatment), the robust nature of the antibody response suggests that some resting, non-virus producing B cells were activated to produce virus or viral proteins upon adoptive transfer. Given the fact that a fraction of infected B cells divided after adoptive transfer into *xid* recipients (Figure 10), one possible source of inductive signaling that would lead to VSV activation is homeostatic proliferation.

Figure 12A. Antibody response to VSV in infected B cell recipients

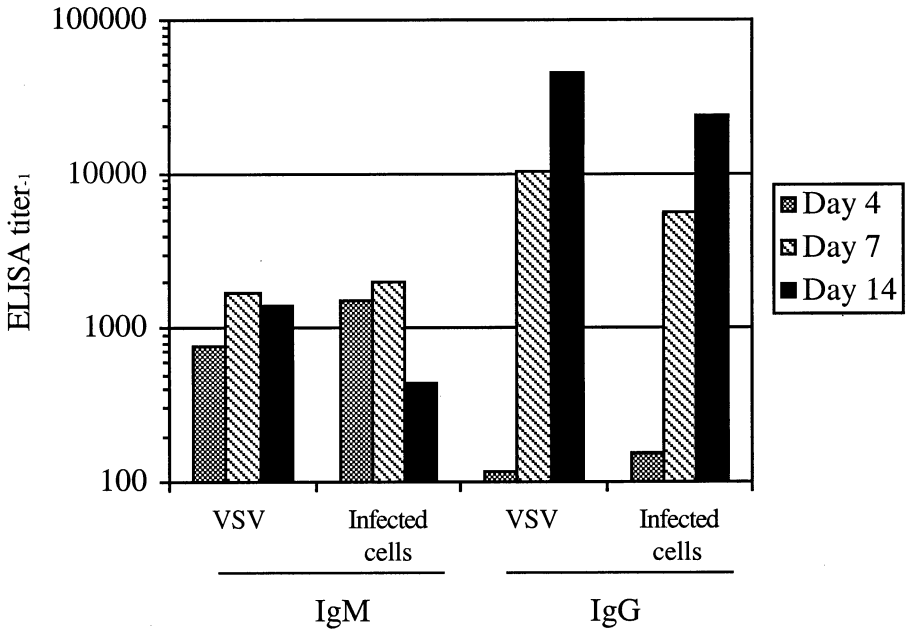


Figure 12B. Pre-treatment of infected cells with anti-VSV serum

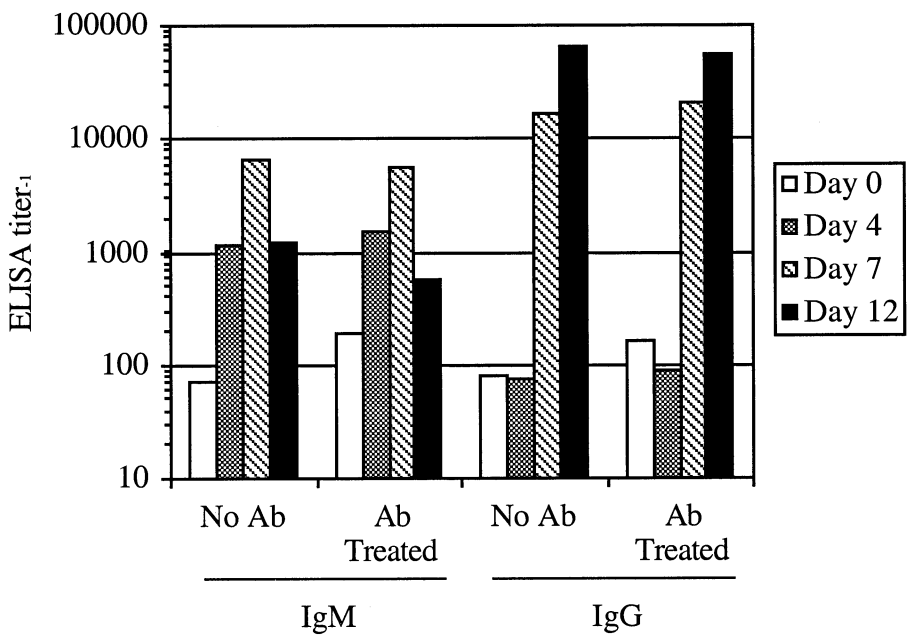


Figure 12. Antibody response to VSV in infected B cell recipients. **A.** Unirradiated CBA/N (*xid*) mice were injected via the lateral tail vein with either 10^8 PFU of VSV or $20x10^6$ small, resting, VSV-SLC infected splenic B cells from CBA/Ca (normal) mice. Levels of VSV specific IgM and IgG were assessed on day 4, 7, and 14 in individual mice by ELISA. Each bar represents the mean ELISA titer of three mice. **B.** Infected B cells were either treated with mouse anti-VSV serum (+ Ab) or not (no Ab) before adoptive transfer into unirradiated *xid* recipients. Levels of VSV specific IgM and IgG were assessed on Day 0, 4, 7, and 12 by ELISA as described above; each bar represents the mean ELISA titer of three mice.

Infectious VSV cannot be recovered from the spleens of infected B cell recipients

Demonstrating maintenance of VSV RNA in adoptively transferred B cells was the first step in developing an *in vivo* model in which to study RNA virus persistence in lymphocytes. An important feature of viral persistence is the ability of virus to periodically reactivate, produce infectious virus, and potentially cause overt disease in the host. This is important in the pathogenesis of persistent viral diseases as well as in the maintenance of virus in a population of potential hosts. In initial experiments, VSV RNA was detected by RT-PCR in the spleens of infected B cell recipients, but infectious virus was not detected by plaque assay. Attempts to activate persistently infected B cells using B cell activators that enhance VSV replication in the *in vitro* system were also unsuccessful. Since *in vitro* experiments demonstrated that infectious virus could be reactivated from resting B/bcl-2 cells after weeks in culture (M. Schmidt, manuscript in preparation), this suggests that persistence *in vivo* was maintained by factors not present *in vitro*.

One of the differences between the *in vitro* and *in vivo* systems was the presence of interferon *in vivo*. VSV is sensitive to type I (α,β and type II (γ) interferons, which restrict virus replication at multiple levels and are important for control of VSV infection in mice (28, 95). Thus, it was possible that infected B cells were exposed to interferons after adoptive transfer which restricted the reactivation of virus from these cells while allowing viral RNA to persist. This idea was tested by using IFN α/β receptor knock-out mice as B cell donors; thus, the infected, adoptively transferred B cells were unresponsive to type I interferons. Attempts to reactivate VSV *in vitro* from infected IFN

α/β $R^{-/-}$ B cells which had been maintained for 7-14 days in wild-type mice were also unsuccessful, despite the fact that adoptively transferred cells could be seen (by CFSE staining) and that viral RNA could be detected in the spleens of recipients by RT-PCR. In other experiments, infected IFN α/β $R^{-/-}$ B cells were maintained in wild-type recipients, then total spleen cells from these primary recipients were transferred into IFN α/β $R^{-/-}$ secondary recipients in which 30-50 PFU of VSV is 100% lethal (95); these recipients survived, further suggesting that VSV reactivation was not suppressed by Type I interferons. One possibility is that IFN γ suppressed reactivation; the fact that IFN $\alpha/\beta R^{-/-}$ mice are highly susceptible to VSV argues against this since IFN γ does not inhibit virus replication in these mice (95). It is also possible that IFN γ is a stronger inhibitor of virus replication in B cells than in other cell types. This, however, seems unlikely because treatment of infected B cells *in vitro* with 60 U/ml of IFN γ does not completely suppress virus replication (laboratory observations). These data indicate that VSV reactivation from adoptively transferred B cells is blocked by an interferon independent mechanism. The finding that VSV persistence was maintained in the presence of an antibody response to the virus (Figure 12A and B) could suggest that antiviral antibodies play a role in restricting VSV reactivation from B cells.

Reactivation of VSV from persistently infected B cells *in vitro* is inhibited by prolonged treatment with anti-VSV serum

Restriction of virus replication by antiviral antibodies is another mechanism by which viral persistence might be maintained *in vitro* and *in vivo*. This mechanism has been reported for RNA viruses such as measles virus, rubella virus, and Sindbis virus (77,

85, 87). This phenomenon has been well-studied in cells of neural origin (80, 83, 86, 181), but there is limited information regarding its importance in cells of the immune system (81, 87). In these systems, when infected cells or infected animals are treated with antibodies to viral proteins, viral mRNA, protein and infectious particle production are suppressed (77, 80-83, 85, 87). Furthermore, the effect is reversible since removal of antibody results in the re-expression of viral mRNAs, proteins and infectious particles (80, 83, 87). *In vivo*, antibodies are thought to exert their antiviral activity in the following ways: neutralization of extracellular virus, killing of infected cells by antibody-dependent cell-mediated cytotoxicity (ADCC), or destruction of infected cells by complement mediated lysis (67). These data suggest, however, that antiviral antibody can also act directly on infected cells by restricting virus replication without killing the cells. Any mechanism of non-cytolytic control of virus replication may be involved in the maintenance of virus persistence.

The fact that VSV was maintained in B cells in the presence of an antibody response to the virus led me to hypothesize that antiviral antibodies were suppressing virus reactivation from infected B cells. To test this idea directly in the absence of other immune effectors such as virus specific T cells, NK cells, or complement, I took advantage of the fact that VSV persistence can be maintained for weeks in cultures of primary B/bcl-2 cells (M. Schmidt, manuscript in preparation). In this way, infected B cells could be treated with antiviral antibody for extended periods and the effects of this treatment on virus reactivation observed. Small resting splenic B/bcl-2 cells were prepared, infected with VSV, and cultured for two to seven days with normal mouse

serum (NMS) or anti-VSV serum at a 1:500 or 1:1000 dilution; these dilutions were used to maximize the amount of antiviral antibody present while minimizing any nonspecific effects of other serum proteins. Following this treatment, antiviral antibody was washed out and virus production was assessed by plaque assay after stimulation of the cultured cells with the B cell specific activators anti-Ig+IL-4.

Short-term treatment with anti-VSV serum (2 days) had no effect on virus reactivation since the levels of infectious virus produced were similar to those seen in acutely infected cells are infected cells treated with NMS for 2 days (Figure 13A). This demonstrated that antibody treatment did not block virus uptake. In contrast, anti-VSV treatment for 7 days resulted in complete inhibition of virus reactivation; less than 1 PFU per 10^3 B cells versus 5800 PFU per 10^3 B cells for NMS treated controls (Figure 13A Table 3). This showed that prolonged exposure of infected cells to antiviral antibody was required to mediate inhibition, suggesting a direct effect of antibody on viral replication within the cells. General inhibition of B cell activation was an unlikely explanation for the lack of virus replication since the persistently infected B cells treated with anti-VSV serum proliferated as well as uninfected cells in response to all activators tested (Figure 13B and data not shown). Persistently infected B cells treated with NMS or acutely infected B cells, on the other hand, showed greatly reduced proliferation after activation; this is likely due to killing of activated cells by the virus.

The maintenance of VSV persistence in BHK cells requires that the virus spreads at a low level from cell to cell; thus treatment with anti-VSV antibody, which inhibits this spread, leads to elimination (or "curing") of virus from the cells (22). Experiments in this

laboratory show that very little cell to cell spread of VSV occurs in long-term cultures of persistently infected resting primary B cells, suggesting that persistence in B cells does not depend on this mechanism (M. Schmidt, laboratory observations). Consistent with this, treatment with anti-VSV serum did not cure the B cells of virus, since in many cases virus could be reactivated from antibody treated cells that had been recultured for 1-2 weeks in the absence of antibody (Figure 13C, Table 4). Overall, these data are consistent with the idea that antibodies to VSV proteins directly inhibit reactivation of virus from persistently infected B cells in a reversible fashion, demonstrating the potential of components of the specific immune response to maintain virus persistence in lymphocytes.

Figure 13A. Inhibition of VSV reactivation from B cells by VSV immune serum

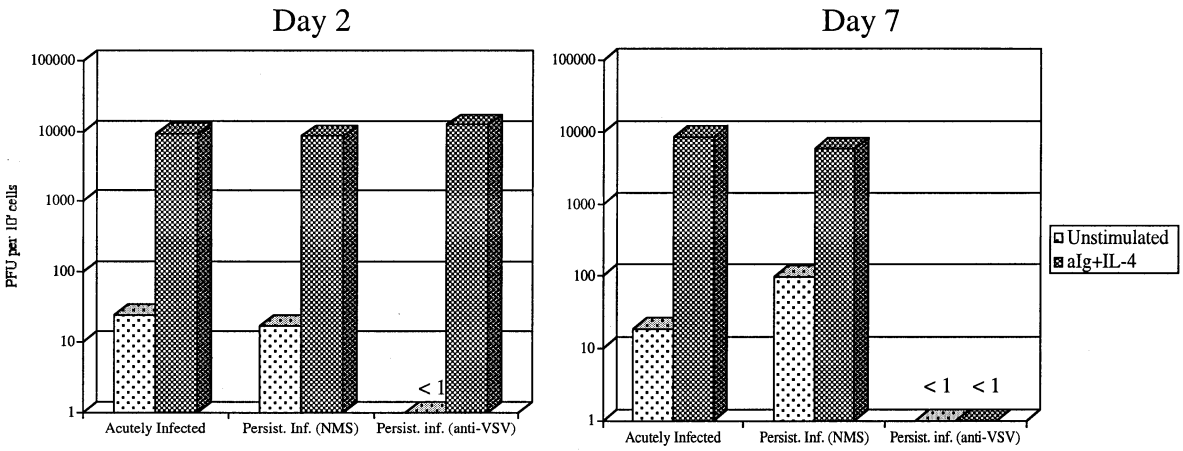


Figure 13B. Proliferation of Persistently Infected B Cells Treated With VSV Immune Serum

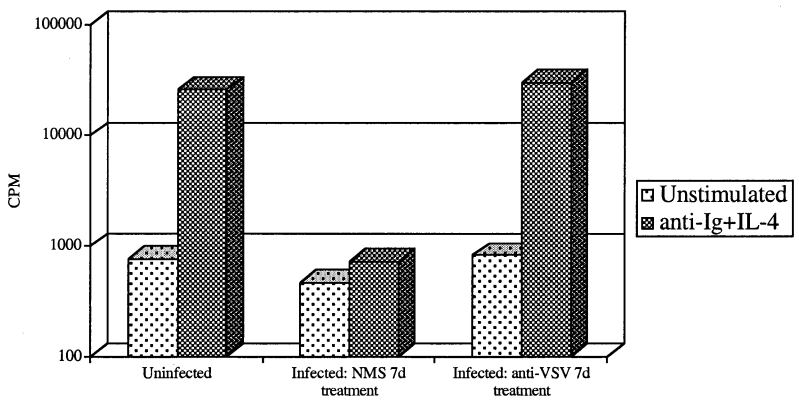


Figure 13C. Antibody Inhibition of VSV Reactivation Is Reversible

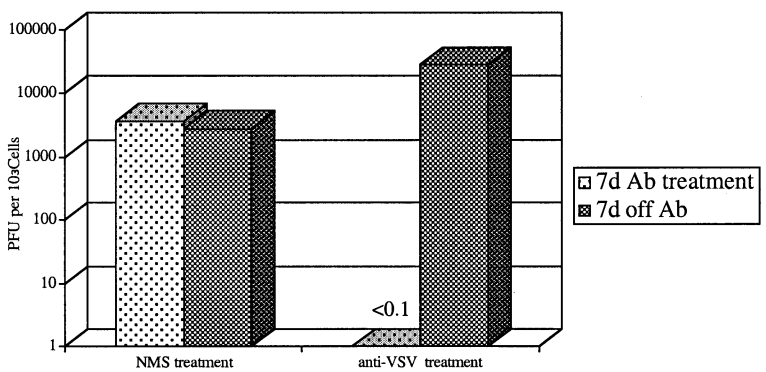


Figure 13. Inhibition of VSV reactivation from B cells by VSV immune serum. **A.** Small, resting splenic B cells from CBA/Ca-bcl-2 transgenic mice were prepared on Percoll step gradients. VSV-SLC infected or uninfected B cells were cultured in the presence of a 1:500 dilution of normal mouse serum (infected cells) or mouse anti-VSV serum (infected and uninfected cells) for 2 or 7 days. On the indicated days, cells were harvested, washed extensively, and uninfected cells were acutely infected with VSV-SLC. Each cell population was then cultured in media alone (unstimulated) or in the presence of anti-Ig+IL-4 as described in Materials and Methods. Virus titers (PFU per 10^3 cultured B cells) were determined after 48h of activation by plating serial dilutions of culture supernatants on CHO monolayers. **B.** Proliferation of uninfected B cells and VSV infected B cells treated with NMS or anti-VSV serum for 7 d was assessed by [^{3}H]TdR incorporation in 8 h pulses at 42 to 48 h after activation. Cells were cultured in media alone (unstimulated) or stimulated with anti-Ig+IL-4. **C.** VSV infected B cells were treated with NMS or anti-VSV serum for 7 days, activated with anti-Ig+IL-4 for 48h after extensive washing, and virus titers measured in culture supernatants. The remaining cells were placed in culture in the absence of NMS or anti-VSV serum for an additional 7 days. After this second culture period, the cells were again harvested, activated with anti-Ig+IL-4, and virus titers determined.

		Frequency of virus inhibition	
		Concentration	
Antibody	**Dilution***	**10 μg/ml**	**1 μg/ml**
NMS	0/9	-	-
anti-VSV serum	9/10 (90%)	-	-
Control ascites	0/4	-	-
1E9 ascites (neutralizing)	5/6 (83%)	-	-
8E11 ascites (non-neutralizing)	2/4 (50%)	-	-
NMS IgG	-	0/3	
anti-VSV IgG	-	3/3 (100%)	2/3 (67%)
anti-VSV IgG $F(ab')_2$	-	3/3 (100%)	2/3 (67%)
anti-VSV IgG Fab	-	4/4 (100%)	0/4

Table 3. Summary of virus inhibition experiments. The frequency of inhibition for each antibody was determined by dividing the number of experiments in which the antibody inhibited infectious virus production by the total number of experiments. Inhibition was defined as at least a 10-fold reduction in virus titer compared to NMS or control ascites after activation with anti-Ig+IL-4 for 48h. Monoclonal antibodies: 1E9 (neutralizing anti-VSVG, IND); 8E11 (non-neutralizing anti-VSVG, IND). * NMS and anti-VSV serum were used at 1:500-1:1000, ascites fluids at 1:100-1:500.

VSV reactivation from persistently infected B cells is inhibited by intact IgG, $F(ab')_2$ and Fab fragments purified from anti-VSV serum

While the data presented above suggested that anti-VSV antibody in the immune serum was directly suppressing virus reactivation, it was possible that other serum components were involved. For example, interferons are induced by virus infections and inhibit virus replication while complement forms complexes with antiviral antibodies to lyse infected cells (28, 67). In order to directly demonstrate that inhibition of VSV reactivation was mediated by antibodies and not other components of immune serum, IgG was purified from the normal mouse serum or VSV immune serum used in previous experiments (NMS IgG or anti-VSV IgG). VSV infected B/bcl-2 cells were treated NMS IgG or anti-VSV IgG for 7 days; thereafter, the cells were washed to remove antibody and virus reactivation was assessed after stimulation of the cells with anti-Ig+IL-4 and titering culture supernatants on CHO cells. Intact anti-VSV IgG inhibited virus reactivation at 1 and 10 μg/ml, but not at 0.1 μg/ml while NMS IgG did not inhibit at any concentration (Figure 14A, Table 3 and data not shown). While this demonstrated that antibodies in VSV immune serum were responsible for inhibition, the mechanism underlying this effect was not determined.

Cross-linking of Fcγ receptor inhibits anti-immunoglobulin induced B cell activation at several levels including blastogenesis, proliferation, *c-myc* mRNA induction, and antibody secretion (182-184). Thus, it was possible that simultaneous binding of VSV G and Fc receptors on the cell surface by antiviral antibodies mediated inhibition of B cell activation when the cells were treated with anti-Ig+IL-4. One argument against

this hypothesis was that IL-4 overcomes Fc-mediated inhibition of B cell activation (185, 186). Another was that infected B cells treated with anti-VSV antibody proliferated in response to all B cell activators tested (Figure 13B and data not shown). To directly look at the involvement of Fc mediated effects in VSV inhibition, $F(ab')_2$ fragments prepared from the anti-VSV IgG, which are bivalent antibodies lacking the Fc portion, were used to treat infected B cells for seven days, and virus reactivation was determined as described above. This preparation inhibited virus reactivation as efficiently as intact IgG, providing further evidence that Fc signaling was not required for this process (Figure 14A and Table 3).

A second mechanism by which antiviral antibodies might inhibit virus reactivation is through generation of an intracellular signal as a consequence of cross-linking viral proteins on the surface of infected cells, resulting in the inhibition of cellular pathways required to support virus replication. This could occur through direct signaling by viral proteins or indirect signaling through cellular proteins associated with viral proteins. It has been shown that treatment of measles virus infected cells with anti-measles antibodies triggers the breakdown of phosphoinositide, a component of numerous signaling pathways (187), suggesting that viral proteins can be coupled to cellular signaling pathways. To determine if cross-linking viral proteins was required for the inhibition observed in VSV infected B lymphocytes, persistently infected B cells were treated for seven days with intact, $F(ab')_2$, or Fab fragments of anti-VSV IgG or with intact NMS IgG; virus reactivation was then assessed as described above. Although anti-VSV Fab fragments inhibited virus reactivation, they were less efficient than intact

IgG or $F(ab')_2$ fragments since inhibition was only seen at 10 μg/ml, whereas intact or $F(ab')_2$ fragments worked at a ten-fold lower concentration (Figure14A). This inhibition occurred despite the fact that aggregates in the Fab preparation, which could cross-link viral proteins, were removed by high speed centrifugation. The fact that Fab fragments inhibit virus reactivation less efficiently than bivalent antibodies suggests the following: generation of a putative inhibitory signal occurs at some level with monovalent antibodies at high concentration, but signaling is optimal when viral proteins are cross-linked. This may be due to stabilization of antibody-protein interactions by cross-linking. Overall, these data demonstrated 1) that antibody in VSV immune serum mediated inhibition of virus reactivation; 2) that Fc mediated inhibition of B cell activation was not involved in virus suppression; 3) that cross-linking of viral proteins was not essential, but made virus inhibition more efficient.

Figure 14A. Inhibition of VSV reactivation by $F(ab')_2$ and Fab fragments of anti-VSV

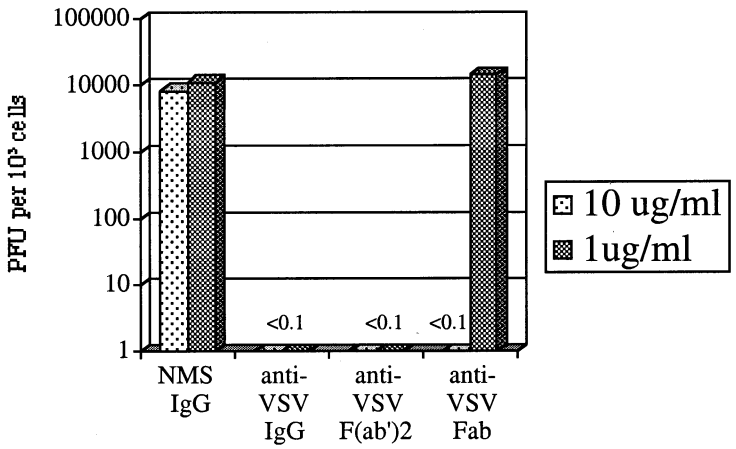


Figure 14B. Inhibition of VSV reactivation monoclonal antibodies

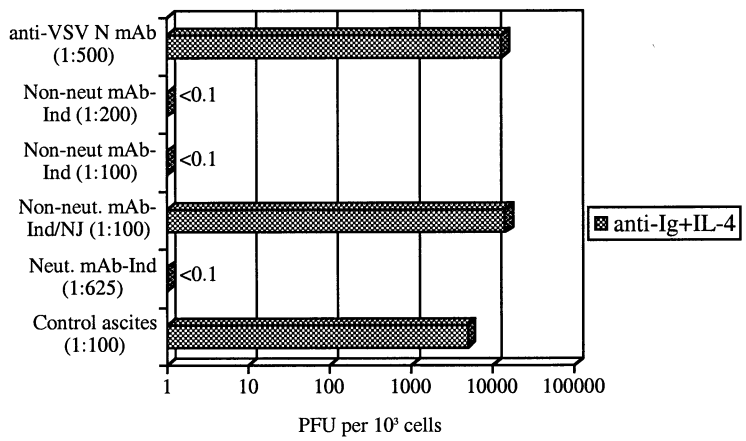


Figure 14. Inhibition of VSV reactivation from persistently infected B cells by anti-VSV $F(ab')_2$, Fab fragments, and monoclonal antibodies. **A.** VSV infected bcl-2 tg B cells were cultured in the presence of purified IgG from NMS, anti-VSV serum, or anti-VSV $F(ab')_2$ or Fab fragments for 7 d at the indicated concentrations. The cells were then harvested, washed extensively, activated with anti-Ig+IL-4 for 48 h, and virus titers measured. **B.** The experiment was performed essentially as described in 6A. VSV infected B cells were treated with control ascites or ascites containing the following monoclonal antibodies at the indicated dilutions: 1E9 (neutralizing, IND specific); 5E4D8 (non-neutralizing, IND/NJ cross-reactive); 8E11F2 (non-neutralizing, IND specific); 1OG4 (anti-VSV N).

Inhibition of VSV reactivation from persistently infected B cells by neutralizing and non-neutralizing monoclonal antibodies

Since anti-VSV serum contained antibodies specific for all viral proteins, it was possible that interactions between antibody and one or more viral proteins were required to inhibit VSV reactivation. It has been shown, however, that treatment of cells infected with measles virus (HeLa cells, C6 rat glioma cells) or Sindbis virus (rat dorsal root ganglion neurons) with monoclonal antibodies to viral glycoproteins inhibits viral protein and infectious particle production; in the case of measles virus, neutralizing activity was required for this effect (79, 80, 181). These studies suggest that antibody binding to viral glycoproteins on the cell surface is sufficient to inhibit virus replication.

To determine if a similar mechanism operated in persistently infected primary B cells, these cells were treated with monoclonal antibodies specific for VSV G, the viral glycoprotein, or VSV N, the nucleocapsid protein which is associated with viral genomic RNA (17). A neutralizing monoclonal antibody specific for VSV G-Indiana (1E9) inhibited virus reactivation in most experiments while an antibody specific for VSV N (10G4) did not, suggesting that binding to VSV G was sufficient to inhibit (Figure 14B and Table 3). It seems likely that neutralization is not required since a non-neutralizing antibody specific for VSV G-Indiana could inhibit; a second monoclonal antibody, which was non-neutralizing and Indiana-New Jersey cross-reactive did not inhibit virus reactivation (Figure 14B and Table 3). This was in contrast to the requirement for neutralizing activity in measles virus inhibition (80) but consistent with the observation that HSV-1 reactivation from trigeminal ganglia was inhibited by nonneutralizing

monoclonal antibodies (86). Why do nonneutralizing antibodies inhibit less efficiently? VSV specific neutralizing and nonneutralizing monoclonal antibodies bind distinct epitopes (105, 106), so it was possible that antibody binding to different epitopes had slightly different biological effects. Alternatively, it has been proposed that the main difference between neutralizing and non-neutralizing antibodies is that neutralizing antibodies bind envelope or capsid proteins with higher affinity, coat virions more effectively, and block attachment or entry (188). Thus, it was also possible that the differences in virus inhibition were related to differences in the affinity of binding between antibody and viral protein. The idea that avidity or affinity of binding to viral proteins was important for virus inhibition was supported by the finding that monovalent Fab fragments inhibited virus reactivation less efficiently than bivalent intact IgG or $F(ab')_2$ fragments (Figure 14A, Table 3). The binding of one non-neutralizing antibody to VSV G can enhance the binding of a second non-neutralizing antibody (106). Thus, it would be useful to look at the ability of pooled non-neutralizing antibodies to inhibit virus replication. In fact, in the HSV-1 experiments, pooled non-neutralizing antibodies inhibited virus reactivation much more efficiently than individual antibodies (86). Taken together, these data suggested that antibodies that recognize VSV G were sufficient to inhibit virus reactivation from persistently infected B cells, that neutralizing activity was not absolutely required for this effect, and that the affinity or site of binding to VSV G was an important factor. Whether the antibody recognizes VSV G on the surface of the infected cell or inside the cell is not known.

Effect of anti-VSV serum or monoclonal antibody treatment on viral RNA and protein levels in infected B cells

Treatment of measles virus infected cells (HeLa, rat C6 glioma, mouse neuroblastoma cells) with anti-measles serum or monoclonal antibodies to HA protein inhibits not only infectious particle production, but also suppresses viral proteins and mRNAs (77-80, 83). This shows that antiviral antibodies can inhibit measles virus replication at multiple levels in cell lines. The results described above demonstrated that anti-VSV antibodies inhibited infectious virus production in primary B cells. To see if VSV genomic RNA, mRNAs, and proteins were also suppressed by antiviral antibody treatment, infected B cells were treated with NMS or anti-VSV serum for seven days; thereafter, the cells were activated with anti-Ig+IL-4 for 48h, then the cells were either labeled with ^{35}S-methionine for protein analysis by immunoprecipitation or harvested for RNA analysis by Northern blot. After seven days of NMS treatment, activated B cells synthesized viral genomic RNA, messenger RNA, viral proteins and infectious particles (Figures 15A and B, lane 1). With anti-VSV serum treatment, on the other hand, viral RNA and proteins were reduced to undetectable levels as assessed by Northern blot or immunoprecipitation of ^{35}S-methionine labeled proteins (Figures 15A and B, lane 2). Viral mRNA transcription did continue at a very low level because VSV M mRNA could be detected by RT-PCR (data not shown). This demonstrates that antibody treatment did not "cure" persistently B cells of the virus, which is expected as virus replication can be restored after cessation of the antibody treatment (Figure 13C).

Experiments described above showed that a neutralizing monoclonal antibody to VSV G (1E9) completely inhibited infectious virus production in persistently infected B cells (Figure 14B, Table 3). It was possible that binding of antibody to VSV G only inhibited virus assembly or release; however, when infected B cells were treated with 1E9 for seven days, all levels of virus replication were inhibited (genomic RNA, mRNA, protein, and infectious virus production, Figures 15A and B, lane 4). In contrast, treatment with control ascites or a non-neutralizing antibody specific for VSV G (5E4D8) did not affect virus replication at any level (Figures 15A and B, lanes 3+5). In some experiments treatment of infected B cells with another non-neutralizing monoclonal antibody to VSV G (8E11) inhibited infectious virus production (Figure14B, Table 3). I have not looked at viral RNA or proteins in cells in which 8E11 inhibited infectious particle production, so it remains to be determined if neutralizing activity is required for inhibition of all levels of virus replication. Thus, as with anti-VSV serum, suppression of infectious virus production by a neutralizing anti-VSV G monoclonal antibodies was associated with a reduction in viral genomic RNA, mRNA and protein levels. These results are interesting because they suggest that recognition of only VSV G by antibody results in global suppression of virus replication.

Figure 15. Effect of anti-VSV serum or anti-VSV monoclonal antibody treatment on viral RNA and protein levels

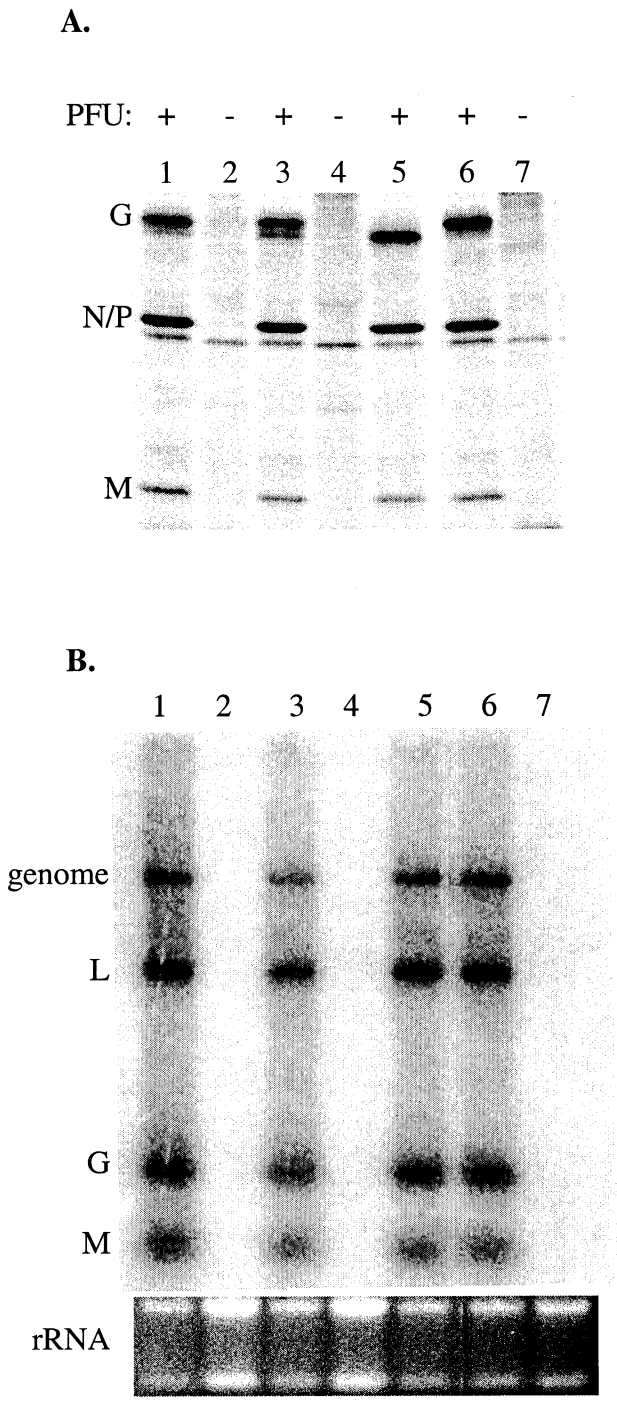


Figure 15. Effect of anti-VSV serum or anti-VSV monoclonal antibody treatment on viral RNA and protein levels. B cells from bcl-2-tg mice were infected with VSV-SLC and cultured in the presence of the indicated antibodies for 7 days. Cells were then washed extensively and treated with anti-Ig+IL-4 to induce virus replication. After 48h of activation, cells were either pulsed with ^{35}S-methionine for 4h or harvested for RNA isolation. VSV proteins were immunoprecipitated from ^{35}S-methionine pulsed cell extracts using rabbit anti-VSV serum and run on a 10% SDS-PAGE gel. Viral genomic and messenger RNAs were detected by Northern blotting using pooled probes for VSV M, G, and L sequences. PFU analysis: (+) indicates virus was detected (>10,000 PFU/10^3 B cells); (-) indicates no virus was detected (<0.1 PFU/10^3 B cells). A. VSV protein and B. RNA in infected cells treated with diluted serum or monoclonal antibodies. Lanes: 1: normal mouse serum (1:1000), 2: anti-VSV serum (1:1000), 3: control ascites (1:200), 4: 1E9 ascites (neutralizing, 1:625), 5: 5E4D8 ascites (non-neutralizing, 1:200), 6: 10G4 ascites (anti-VSV N, 1:500), 7: uninfected B cells.

Fc is not required for suppression of viral genomic RNA and mRNA

In previous experiments, anti-VSV $F(ab')_2$ fragments were shown to inhibit infectious particle production as efficiently as intact IgG from VSV immune serum, suggesting that the Fc portion of antibody was not required for infectious virus suppression (Figure 14A and Table 3). It was possible, however, that an Fc-mediated signal was required for suppression of viral genomic RNA, mRNA, or protein. This idea was tested by treating VSV infected B cells with NMS IgG, anti-VSV IgG or anti-VSV $F(ab')_2$ fragments as described above. At 10 μg/ml, both intact anti-VSV IgG and anti-VSV $F(ab')_2$ fragments suppressed viral mRNAs and proteins to below the level of detection (Figures 16A and 16B, lane 2 vs. lane 5). The suppression of mRNA was seen even after overexposure of the Northern blot; interestingly, viral genome was still detectable in both cell populations (Figure 16B, lane 2^* vs. lane 5^*). The maintenance of viral genome in these cells is consistent with the idea that treatment of infected B cells with antiviral antibody does not "cure" the cells of virus. In other experiments, antibody-treated cells which had no detectible viral mRNA by Northern blot analysis had low levels of VSV M mRNA that was detectible by RT-PCR (data not shown). Thus, viral genome and low levels of mRNA were likely maintained in cells treated with anti-VSV IgG or anti-VSV $F(ab')_2$ fragments at 10 μg/ml.

In this particular experiment, intact anti-VSV IgG at 1 μg/ml did not suppress viral RNA, protein, or infectious particle production (Figures 16A and B, lane 3). However, inhibition of infectious virus production was seen at this concentration in 2 out of 3 experiments, indicating that suppression by this antibody preparation could occur at

1 μg/ml but was less efficient (Figure 14A and Table 3). A similar efficiency of inhibition was seen with anti-VSV $F(ab')_2$ fragments at 1 μg/ml (2 out of 3 experiments), suggesting that intact antibody and $F(ab')_2$ fragments inhibit infectious virus production by the same mechanism. Thus, any suppression of viral RNA or protein levels seen with 1 μg/ml of anti-VSV $F(ab')_2$ would probably also be seen with intact antibody under conditions where infectious particle production was inhibited. Treatment of infected B cells with 1 μg/ml of anti-VSV $F(ab')_2$ fragments had little effect on viral protein levels (except for a slight decrease in M protein), but reduced viral RNA levels (genome and message) and inhibited infectious virus production completely relative to NMS IgG treatment (Figures 16A and B, lane 4- $F(ab')_2$ vs. lane 1-NMS IgG). This suggests that Fc is not required to suppress viral RNA levels; to determine if Fc signaling is required for inhibition of protein synthesis, an experiment will have to be performed in which both intact anti-VSV IgG and $F(ab')_2$ fragments inhibit infectious virus production at 1 μg/ml.

Figure 16. Effect of anti-VSV IgG, anti-VSV F(ab')$_2$, or anti-VSV Fab fragments on viral RNA and protein levels

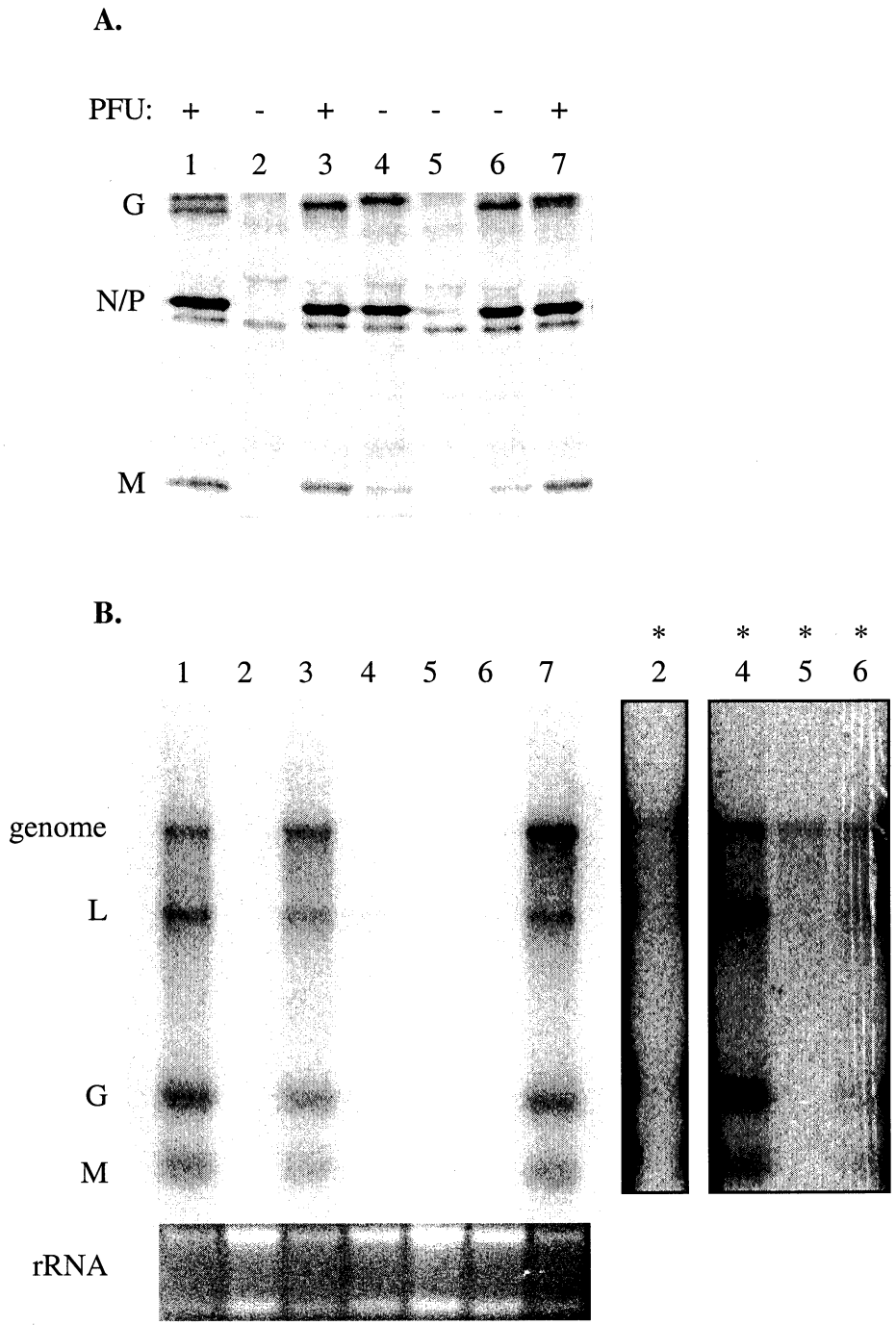


Figure 16. Effect of anti-VSV IgG, anti-VSV $F(ab')_2$, or anti-VSV Fab fragments on viral RNA and protein. The experiment was performed as described in Figure 13. PFU analysis: (+) indicates virus was detected (>10,000 PFU/10^3 B cells); (-) indicates no virus was detected (<0.1 PFU/10^3 B cells). **A.** VSV protein and **B.** RNA in infected cells treated with IgG purified from NMS or anti-VSV serum. Lanes: 1: normal mouse serum IgG (10 μg/ml), 2: anti-VSV IgG (10 μg/ml), 3: anti-VSV IgG (1 μg/ml), 4: anti-VSV $F(ab')_2$ (1 μg/ml), 5: anti-VSV $F(ab')_2$ (10 μg/ml), 6: anti-VSV Fab (10 μg/ml), 7: anti-VSV Fab (1 μg/ml). * Longer exposure of lanes 2 and 4-6.

Does signal strength determine the level at which VSV replication is inhibited in anti-VSV treated B cells?

There are two possible mechanisms by which antiviral antibodies could inhibit all levels of virus replication in B cells. First, antibodies might enter cells, interact with their target proteins and directly inhibit their function in virus replication. This possibility is minimized by the finding that a monoclonal antibody to VSV G inhibits all levels of virus replication in B cells (Figure 15A and B, lane 4). Since VSV G is only involved in attachment to cells, assembly, and release of virus from cells (17), it is difficult to envision how antibody binding to this protein would suppress viral RNA and protein levels. This leads to the hypothesis that interactions between VSV G and antibody generate intracellular signal(s) that specifically inhibit cellular pathways required to induce VSV replication in B cells. VSV replication is regulated at multiple levels in primary B cells, and different cellular signaling pathways likely regulate each stage of virus replication (36). By analogy with antigen receptors, in which the strength of signal has different functional consequences (189), one might predict that the level at which virus replication is inhibited depends on the strength of the putative inhibitory signal(s) generated by antiviral antibodies. The strength of signaling generated by antibodies to cellular receptors depends on the concentration of antibody and its ability to cross-link; high concentrations of antibody and bivalent binding generate stronger signals (189). Consistent with the idea that signal strength is important in the suppression of VSV replication, the ability of anti-VSV antibodies to cross-link viral proteins was important for efficient inhibition of infectious particle production (Figure 14A and Table 3). Cross-

linking may enhance the stability of antibody-viral protein interactions leading to a stronger signal.

Cross-linking was also required for efficient suppression of viral RNA and protein levels. At 10 μg/ml, anti-VSV Fab fragments inhibited infectious particle production; while this treatment also reduced genomic and mRNA levels relative to NMS IgG treatment (Figure 16B lane 6 vs. lane 1), mRNA levels were higher compared to treatment with intact anti-VSV IgG or $F(ab')_2$ fragments at the same concentration (Figure 16B, lane 6^* vs. lanes 2^* and 5^*). Thus, a further reduction in mRNA levels required a stronger "signal" induced by cross-linking. Consistent with this idea, viral genomic and mRNA levels are much lower in cells treated with 10 μg/ml of anti-VSV $F(ab')_2$ compared to 1 μg/ml (Figure 16B, lane 4 vs. lane 5). This suggests that a stronger inhibitory signal is delivered by $F(ab')_2$ fragments at higher concentration. In contrast to genomic and mRNA levels, viral protein levels were essentially unaffected by treatment with 10 μg/ml of anti-VSV Fab compared to intact anti-VSV and $F(ab')_2$ fragments used at the same concentration (Figure 16A, lane 6 vs. lanes 2 and 5). The only exception was a slight decrease in VSV M protein, which was also seen with anti-VSV $F(ab')_2$ at 1 μg/ml, suggesting that M protein may have been selectively destabilized under some conditions of antibody treatment (Figure 16A, compare lanes 4 and 6 to lane 1). These data suggested that suppression of viral protein levels required a very strong inhibitory signal (high concentrations of bivalent antibody).

Overall the results with intact anti-VSV IgG, $F(ab')_2$, and Fab fragments are consistent with the idea that the strength of the putative inhibitory signal(s) generated by

antiviral antibody treatment differentially inhibits virus replication in infected B cells. The data summarized in Table 4 suggest the following: 1) Fc is not required for inhibition of viral RNA (genomic or message); 3) inhibition of infectious virus production requires a weak inhibitory signal; 4) suppression of viral genomic or mRNA is more dependent on signal strength; 5) suppression of viral protein levels (except VSV M) requires a very strong signal. Thus, antiviral antibody inhibits VSV replication at multiple levels in B cells and the extent of inhibition may depend on the strength of inhibitory signal(s) induced by the antibody.

Inhibitory signal:	Strong	Weak		None
	10 μg/ml bivalent	1 μg/ml bivalent	10 μg/ml monovalent	1 μg/ml monovalent
PFU	++++	++++	++++	-
mRNA	++++	++	+++	-
Genome	+++	++	+++	-
Protein	++++	-	-	-

Table 4. Inhibition of VSV replication at multiple levels in anti-VSV treated B cells. Summary of the data presented in Figures 14A and 14B demonstrating the extent to which the indicated antibody preparations inhibited each stage of virus replication. The degree of inhibition ranged from complete (++++) to none (-). The putative inhibitory signal(s) generated by antiviral antibodies were classified as strong (high concentrations of bivalent antibody), weak (low concentrations of bivalent antibody or high concentrations of monovalent antibody), or none (low concentrations of monovalent antibody).

Reactivation of infectious virus after recovery from antibody treatment

A hallmark of persistent infection is the ability of infected cells to periodically produce infectious virus. *In vivo* studies described earlier in this chapter demonstrated that VSV persistence was maintained in B cells in a type I interferon independent manner in the presence of high levels of anti-VSV antibody. It is proposed here that suppression of virus reactivation from persistently infected B cells by antiviral antibody is a mechanism for maintaining VSV persistence in B cells. In order to demonstrate that antiviral antibodies maintained persistence and did not clear virus from infected B cells, the following experiment was performed: persistently infected B cells were treated with antiviral antibodies for 7 days; the cells were then washed extensively and re-cultured for 7-14 days in the absence of antibody. After this culture period, the cells were stimulated with anti-Ig+IL-4 for 48h and virus titers in culture supernatants determined. Treatment of infected B cells with anti-VSV serum for 7 days resulted in complete inhibition of infectious virus production which was reversed after reculturing the cells without anti-VSV serum for 7 days (Figure 13C). Recovery of virus from anti-VSV serum treated cells was not a consistent finding (4 of 6 experiments, Table 5). Virus could not be recovered from B cells treated with purified anti-VSV IgG, and only rarely from anti-VSV $F(ab')_2$ treated cells (0 of 4 and 1 of 5 experiments respectively, Table 5). Importantly, viral genome is still present in anti-VSV IgG or anti-VSV $F(ab')_2$ treated B cells even when infectious virus is suppressed, demonstrating that virus is not completely cleared from the cells (Figure 16B lanes 2^*, 4^*, and 5^*). Cultures in which infectious virus production was initially inhibited by anti-VSV Fab fragments (10 μg/ml), on the

other hand, could always be activated to produce infectious virus 7-14 days after antibody removal (3 of 3 experiments, Table 5). Since Fab fragments inhibited virus reactivation less efficiently than intact IgG or $F(ab')_2$ fragments (Figure 14A, Table 3), this suggested that the initial "strength" of inhibition determined whether virus could be recovered from antibody treated cells. It was possible that a strong inhibitory signal required a longer recovery period following antibody removal. Overall, these data demonstrate that inhibition of virus replication by antiviral antibody is reversible, providing a mechanism by which VSV could be reactivated from persistently infected B cells *in vivo*.

Antibody	Recovery of virus after treatment
anti-VSV serum	4/6 (67%)
anti-VSV IgG	0/4
anti-VSV $F(ab')_2$	1/5 (20%)
anti-VSV Fab	3/3 (100%)

Table 5. Summary: Reactivation of virus after recovery from antibody treatment. Small, resting bcl-2 transgenic B cells were infected with VSV-SLC and treated for one week with the indicated antibody. Cultures in which virus production was suppressed were washed to remove antibody and then re-cultured for one to two weeks in the absence of antibody. Percentages reflect the number of experiments in which virus could be reactivated after recovery from antibody treatment out of the total number of experiments.

Inhibition of transcription from ΔG-HA VSV in B cells by anti-VSV IgG

In resting B cells infected with VSV, viral protein synthesis is undetectable by immunoprecipitation of ^{35}S-methionine labeled cell extracts with polyclonal rabbit anti-VSV serum (36). This leads to the following question: if treatment of persistently infected, resting B cells with antibody recognizing VSV G inhibits virus reactivation, where does the VSV G protein come from? It was possible that a very low level of viral proteins were continuously made in these cells; alternatively, VSV G from input virions may remain on the cell surface, possibly associated with host cell proteins. In order to determine if *de novo* synthesis of VSV G was required for antibody inhibition, an experiment was performed using a virus (ΔG-HA VSV) generously provided by Dr. Jack Rose. In this virus, the gene encoding VSV G has been deleted and replaced with sequences encoding influenza HA (99). This virus was grown in a VSV G expressing cell line so that infectious virions containing G protein were produced, but once this virus enters cells, it can only produce influenza HA and not VSV G; thus, any VSV G found in infected cells is derived from input virions. Small resting splenic B cells were infected with a low-titer stock of ΔG-HA VSV at moi=0.1 and treated with either NMS IgG or anti-VSV IgG for seven days, and the cells activated with anti-Ig+IL-4 for 48h before analysis. The use of a low multiplicity of infection was not likely to effect the results because I have shown in experiments with wild-type VSV that inhibition of infectious particle production occurs at a moi of 0.1 (data not shown). Since this treatment was shown to inhibit viral transcription in cells infected with wild-type VSV (Figure 16B, lane 2) and few infected cells were available for analysis, the ability of anti-VSV to

inhibit transcription from this virus was assessed by RT-PCR. RNA from equivalent numbers of cells in each group was subjected to an RT reaction using oligo-dT primers. Serial ten-fold dilutions were then made of each RT reaction and subjected to PCR using primers for VSV M or actin to determine relative levels of mRNA. Amplification of actin cDNA from these serial dilutions demonstrated that there were essentially equivalent amounts of this mRNA in the two cell populations, since PCR product was lost at the same dilution (Figure 17). In contrast, the VSV M PCR product was lost at a 10-fold lower dilution in anti-VSV IgG treated cells compared to NMS IgG treatment (Figure 17A, 10^{-3} vs. 10^{-4} for 35 cycles or 17B, 10^{-2} vs. 10^{-3} for 20 cycles), demonstrating inhibition of VSV transcription by antiviral antibody in the absence of *de novo* VSV G synthesis.

This result supports the idea that VSV G from input virions is stable in cells infected with ΔG-HA VSV, and that antibody recognition of this protein is sufficient for inhibition of viral transcription. There is, however, another potential explanation for this result. The anti-VSV IgG preparation presumably contains antibodies to other viral proteins; thus, it may be that these antibodies, and not those specific for VSV G, are responsible for the inhibition of viral transcription seen. This is an interesting possibility since VSV G is the only viral protein expressed on the surface of infected cells; any mechanism invoking other specificities would require intracellular inhibition of virus replication by antibodies. Sendai virus and rabies virus replication can be inhibited by antiviral IgA and IgG respectively; it has been suggested that these antibodies enter infected cells and directly inhibit intracellular virus replication (190, 191). Thus, it is

possible that anti-VSV antibodies inhibition virus replication in B cells by this mechanism. More detailed experiments with monoclonal antibodies specific for VSV proteins will be required to determine if antibodies recognizing VSV G inhibit viral transcription if ΔG-HA VSV infected B cells.

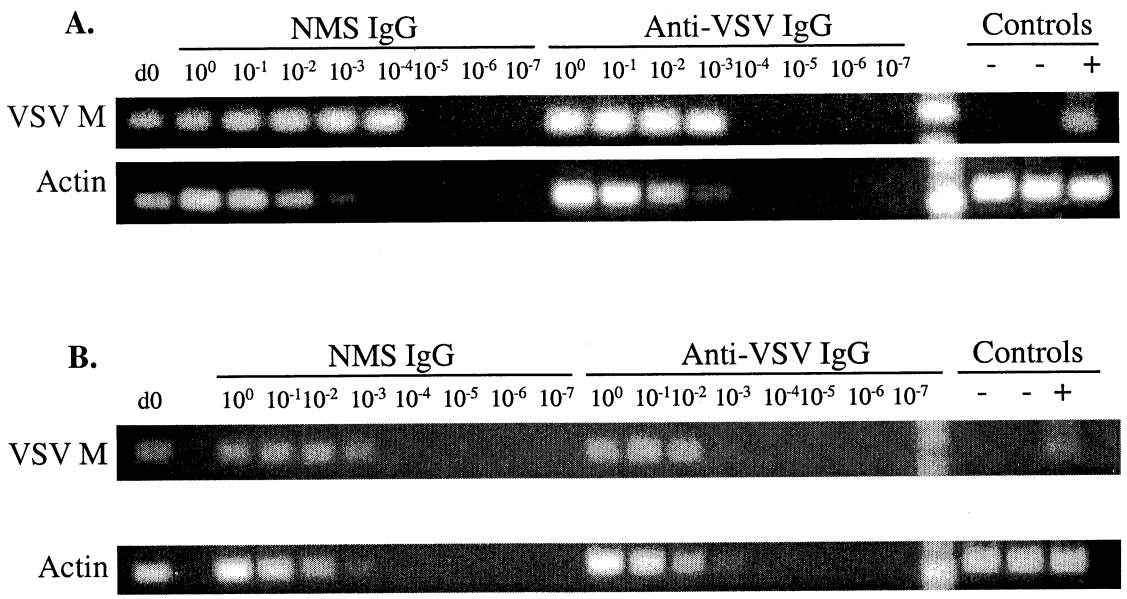


Figure 17. Inhibition of transcription from ΔG-HA VSV by anti-VSV IgG. B cells from bcl-2 tg mice were infected with ΔG-HA VSV at moi=0.1 and cultured for 7d in the presence of NMS IgG or anti-VSV IgG at 10 μg/ml. RNA was prepared from each cell population and subjected to RT-PCR using oligo-dT primers in the RT reactions and either VSV M or actin primers in PCR reactions as described in Materials and Methods. The d0 sample is RNA from cells harvested immediately after infection; random primers were used in the RT reaction for this sample. PCR products were run on 2% agarose gels and detected by ethidium bromide staining. **A.** Nested PCR for VSV M (35 cycles per round), one round of PCR for actin (35 cycles). **B.** Nested PCR for VSV M (first round, 35 cycles; second round 20 cycles) and one round of PCR for actin (35 cycles).

Chapter V: Discussion

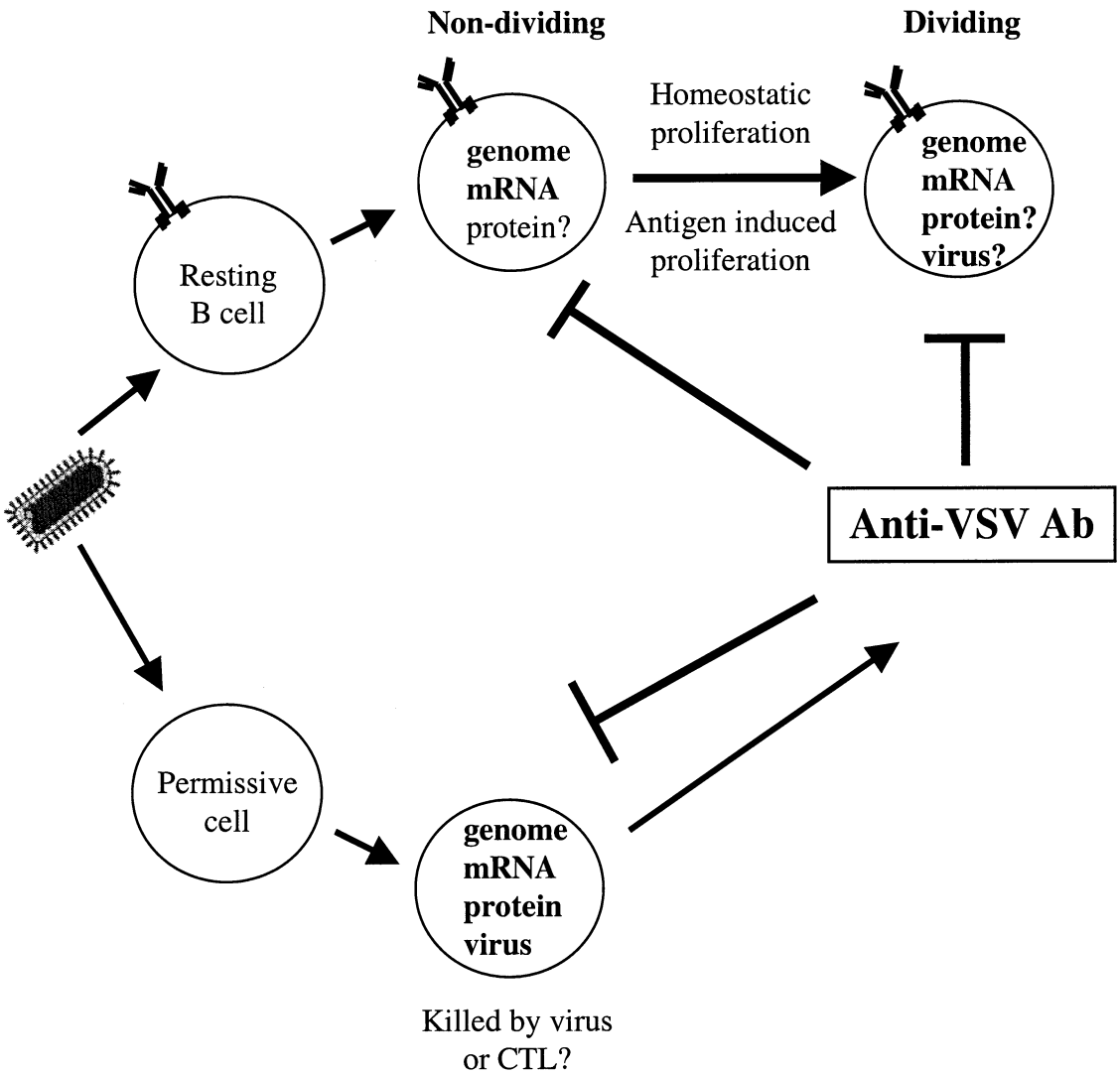
Model of VSV persistence in B cells *in vivo*

Many RNA viruses are capable of establishing persistent infections which are often associated with disease (2). Virus persistence has been studied extensively using *in vitro* culture systems; however, the mechanisms by which these viruses persist *in vivo* are not well understood. Persistent infections of lymphocytes are particularly important, since these types of infections are often associated with immunodeficiency (9). Furthermore, lymphocytes are an ideal reservoir for RNA viruses such as VSV because they are non-permissive for virus replication when resting, but support full virus replication upon activation (32-36). This would allow virus to be maintained in resting lymphocytes so as to evade the immune response and at the same time provide the virus with opportunities to periodically reactivate to reinfect new cells or be transmitted to new hosts. In this chapter, an *in vivo* model system for studying persistence of a negative-stranded RNA virus (vesicular stomatitis virus) is described, and a role for antiviral antibody in the maintenance of virus persistence is proposed.

Based on the data presented in this chapter, I propose the model for VSV persistence in B cells *in vivo* depicted in Figure 18. This model shows events that likely occur during the course of an infection with virus as opposed to after adoptive transfer of infected cells. When virus enters the body, it encounters both permissive cells (activated lymphocytes and other cells) and non-permissive cells such as resting B cells. Infected resting B cells express low levels of viral genome and mRNA with little or no *de novo*

protein expression; these resting B cells maintain viral RNA for an extended period without producing infectious virus. A fraction of the B cells may also undergo homeostatic proliferation, driving the production of viral antigens in these cells. Viral antigens produced by these cells and other permissive cells, in turn, induce an antibody response to the virus. The cells producing viral antigens may eventually be killed by the virus or by virus specific CTL; alternatively, virus replication might be inhibited by antiviral antibody. The non-dividing cells, on the other hand, contain only viral genome, mRNA, and perhaps proteins at low levels; these cells are the reservoir for virus during persistence. Virus persistence is maintained not only by the restriction of virus replication in resting B cells, but also by antiviral antibody, which can suppress virus reactivation from these cells. By this model, one would predict that reactivation should occur when antibody titers drop and when infected B cells are induced to proliferate by homeostatic signals or during a response to antigen.

Figure 18. Model for VSV Persistence in B Cells *in vivo*



VSV infected, resting B cells contain low levels of viral genome and mRNA, little or no *de novo* protein expression, no detectable infectious virus during persistence *in vitro* (35, 36). The idea that VSV is maintained in a quiescent state in B cells *in vivo* is supported by the finding that viral RNA can be detected for at least 72 days in infected B cell recipients in the absence of infectious particles (Table 1). In addition, CFSE stained, infected B cells can be seen by FACS at day 10 after transfer, and these cells are positive for VSV M RNA by RT-PCR (Figures 8 and 9). This demonstrates that infected B cells are not cleared by the immune response or killed by virus, and that viral RNA is maintained in B cells.

In these experiments, the infected B cells are transferred into B cell deficient *xid* recipients, so some of them undergo homeostatic proliferation (Figure 10) as described in Chapter III. The idea that homeostatic signals induce virus expression in B cells is supported by three observations: first, proliferation of VSV infected B cells is associated with virus production *in vitro* (36); second, RT-PCR analysis demonstrates viral RNA in recipient cells, suggesting that infectious virus was produced by the donor B cells; third, a robust antibody response is generated after transfer of VSV infected B cells even when extracellular virus is neutralized with anti-VSV serum before transfer; this further suggests that viral proteins or infectious virus were produced by some of the infected B cells.

While these data are consistent with the idea that viral antigens are produced by proliferating infected B cells after transfer, the hypothesis needs to be directly tested. *In vitro*, the induction of B cell proliferation is associated with optimal expression of viral

mRNA, genome, protein, and infectious particles (36). Thus, one prediction is that viral mRNA levels, protein levels, or production of infectious virus should increase in B cells undergoing homeostatic proliferation. While infectious virus has not been detected in recipients of infected B cells, it should be possible to develop assays to measure relative levels of viral mRNA or protein in adoptively transferred B cells. One approach would be to FACS sort CFSE stained donor cells into divided and undivided fractions and perform quantitative RT-PCR on the two populations. Viral protein levels in the two populations could be compared by performing Western blots on the sorted populations; alternatively, viral protein levels in individual cells (either divided or undivided) could be determined by flow cytometric analysis.

Another prediction of this hypothesis is that the antibody response seen after adoptive transfer of infected B cells should be reduced or absent when homeostatic proliferation is limited. Homeostatic proliferation is greatly reduced in normal, B cell sufficient recipients and in *xid* donor B cells (Chapter III). Thus, adoptive transfer of infected *xid* B cells into normal unirradiated recipients should reduce or eliminate the antibody response to VSV if homeostatic proliferation is required. The data presented in Chapter III suggest that the signals for homeostatic proliferation and those for survival are distinct; this is demonstrated by the fact that B cells from nfkb1$^{-/-}$ mice, which have impaired survival *in vivo*, proliferate in B cell deficient hosts. Thus, an alternative hypothesis is that survival signals induce virus expression. In this case, homeostatic proliferation would not be required for induction of viral mRNA, protein, or infectious virus in adoptively transferred B cells. It is also possible that survival signals and

proliferation signals induced different stages of virus replication; this would be consistent with *in vitro* studies in which VSV replication in resting B cells is differentially regulated by various B cell activators (36). Since NF-κB1 deficiency selectively effects survival signals, it would be interesting to see how virus expression is regulated in B cells from nfkb1$^{-/-}$ mice *in vivo*. Together, these experiments should determine if lymphocyte homeostatic signals (survival or proliferative) can induce virus expression in infected B cells.

The model shown in Figure18 proposes that VSV persistence in B cells is maintained not only by restricted virus replication in resting B cells, but also by the suppression of virus reactivation by antiviral antibodies. Support for the latter mechanism comes from the observation that prolonged treatment of infected, resting B cells with anti-VSV antibodies *in vitro* inhibits virus reactivation in a reversible manner (Figure 13A and C). The fact that virus suppression is reversible is a key point, because it means that virus reactivation *in vivo* is possible, which in turn allows for occasional infection of new cells in the host or transfer to new hosts. Experiments performed to date have failed to reactivate virus from infected B cells maintained *in vivo*. Since type I interferons do not seem to play a role (see Results), I have performed preliminary experiments to determine if antiviral antibody suppresses virus reactivation *in vivo*. Passive transfer of VSV immune serum suppresses the endogenous antibody response normally seen in infected B cell recipients (data not shown). Under these conditions, infected B cells are exposed to antiviral antibody, but antibody secreting cells are not generated. Thus, spleen cells from these recipients can be transferred into secondary

recipients without transferring antiviral antibody; this is analogous to the *in vitro* experiments in which the anti-VSV antibody can be washed out of the cultures. When infected B cells are maintained in secondary recipients in the absence of anti-VSV antibody for 7-14 days, virus reactivation (by activation of spleen cells *in vitro* or adoptive transfer into susceptible IFN$\alpha/\beta R^{-/-}$ mice) is not seen. The most likely explanation for this is that the recovery period after antibody exposure was not sufficient. *In vitro*, reactivation of infectious virus after antibody treatment is only seen in some experiments after a 7-14 day recovery period (Table 5), suggesting that recovery periods of more than two weeks may be necessary. Thus, while the *in vitro* data are consistent with the idea that anti-VSV antibodies restrict virus reactivation from B cells *in vivo* further experiments are required to demonstrate this directly.

Another consideration in this model is the fate of the infected B cells that proliferate. It is proposed that these cells express viral antigens or produce infectious particles; thus, one might argue that these cells should be killed by virus replication or virus specific CTL. The data suggest, however, that these proliferating cells are maintained in infected B cell recipients for at least 14 days. One possibility is that some of the cells that proliferate after transfer were never infected in culture; thus, infected cells that proliferated are killed by virus or CTL while uninfected cells that proliferated remain. This question could be addressed by sorting the cells that have divided and performing RT-PCR to detect VSV RNA. Assuming that the proliferating B cells are infected and expressing viral antigens, how might these cells be maintained? A likely explanation is that infected B cells that proliferate produce infectious virus early after

transfer, but this virus replication is suppressed by innate or specific immune responses at later times in a way that preserves these cells; this might be accomplished by antiviral cytokines such as interferons (192) or by antiviral antibody as described in this chapter. The role of interferons could be tested by seeing if proliferating, infected B cells are maintained when the cells are unresponsive to type I interferons by using B cells from IFNα/βR$^{-/-}$ mice. If type I interferons are required to maintain the proliferating cells, then only non-dividing B cells should be seen after adoptive transfer of infected IFNα/βR$^{-/-}$ B cells. The requirement for antiviral antibody in maintaining these proliferating B cells will be more difficult to determine. The obvious experiment would be to perform transfers into recipients that are incapable of making an antibody response (such as SCID mice); these recipients, however, would quickly succumb to the virus, making analysis of the transferred cells difficult. Another approach would be to see if treatment with anti-VSV antibodies during activation of acutely infected B cells *in vitro* enhances the survival of proliferating infected cells. Clearly, further experiments are required to show that proliferating B cells are infected and to determine how they are maintained.

Model for the inhibition of VSV reactivation from B cells by anti-VSV antibodies

In the model depicted in Figure 18, it is proposed that antiviral antibodies act directly on infected, resting B cells to suppress virus reactivation. This is demonstrated in vitro by the fact that treatment of infected B cells with anti-VSV serum for 7 days inhibits virus replication after activation of the cells with anti-Ig+IL-4 (Figure 13A). Antibody inhibition is not due to blocking of virus entry since cells treated with antibody for two days can be activated to produce virus. Rather, inhibition is associated with

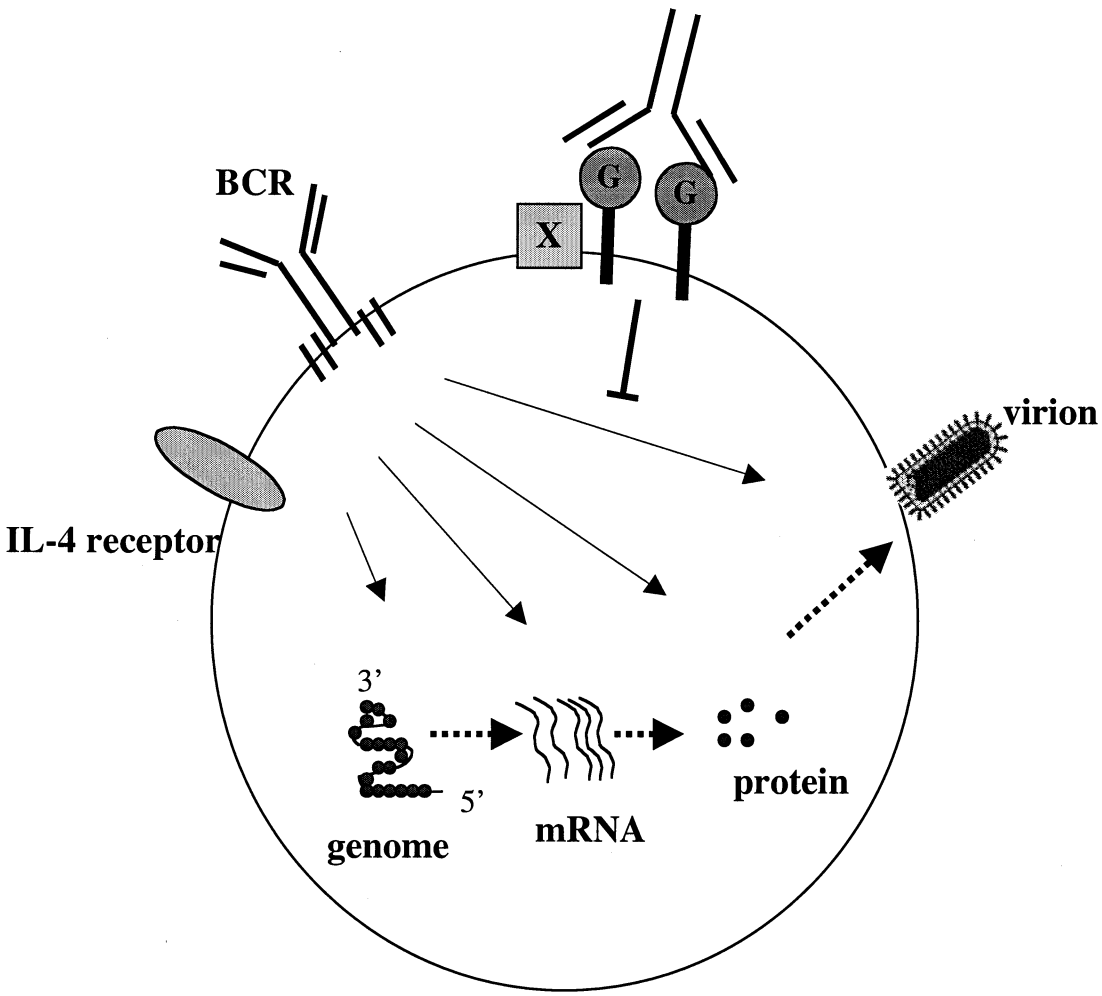
longer term (7 days) antibody treatment (Figure 13A). Treatment of VSV persistently infected cell lines with antiviral antibodies can result in curing of virus from the cells (22, 25); in these cell lines, persistence is maintained by continual reinfection of cells and antibody blocks the spread of virus in the cultures. Four pieces of evidence argue against curing in primary B cell cultures. First, there is little if any infectious virus produced in cultures of primary resting B cells (M. Schmidt, manuscript in preparation), suggesting that continual reinfection of cells is not required to maintain persistence. Second, a nonneutralizing monoclonal antibody to VSV G is capable of suppressing infectious virus production (Figure 14B); this antibody, by definition, cannot block reinfection. Third, viral genome is maintained in antibody treated cells even when viral mRNAs, proteins, and infectious virus are undetectable (Figure 16B, lanes 2+5). Fourth, virus can be reactivated from antibody treated cells after culturing the cells in the absence of antiviral antibodies for 7-14 days in some experiments (Figure 13C).

Another possibility is that antibody treatment of infected B cells suppresses B cell activation in general by Fc-mediated inhibition. Cross-linking of Fcγ receptor inhibits anti-immunoglobulin induced B cell activation at several levels including blastogenesis, DNA synthesis, *c-myc* mRNA induction, and antibody secretion (182-184). Thus, it is possible that simultaneous binding of VSV G and Fc receptors on the cell surface by antiviral antibodies mediates inhibition of B cell activation when the cells are treated with anti-Ig+IL-4. One argument against this hypothesis is that IL-4 overcomes Fc-mediated inhibition of B cell activation (185, 186); indeed, infected, anti-VSV treated cells proliferate in response to anti-Ig+IL-4. One might argue, then, that an inhibitory

signal is generated through Fc receptors that specifically inhibits virus replication without affecting proliferation. The observation that $F(ab')_2$ fragments of anti-VSV lacking the Fc region inhibit virus reactivation as well as intact antibody, however, further demonstrates that a signal through the Fc receptor is not required.

The model I favor for VSV reactivation from B cells by antiviral antibody is in Figure 19. When VSV enters resting B cells, little if any *de novo* protein synthesis occurs, but VSV G protein from the virion is stable in the cell and may be found on the surface. Alternatively, low levels of VSV G may be synthesized and exported to the cell surface. VSV G interacts with an unknown protein at the cell surface that has signaling properties. When antibody binds to G, intracellular signals are generated that inhibit pathways required to support VSV replication in B cells; the "strength" of the signal determines which stage of replication is suppressed and the degree of inhibition. When the B cells are subsequently activated, these pathways are unavailable, and the virus cannot replicate despite the presence of viral genome in the cells. This suppression of virus replication is not permanent because virus can be reactivated from the cells after a recovery period in the absence of antiviral antibody. This model explains how antiviral antibody can inhibit subsequent virus reactivation in resting B cells, in which VSV does not actively replicate and it provides for the periodic reactivation of virus from the cells.

Figure 19. Model for inhibition of VSV replication by anti-VSV antibodies



The idea that VSV G from input virions is sufficient to mediate suppression by antiviral antibody is supported by the observation that VSV M mRNA is reduced about 10-fold by anti-VSV IgG treatment in resting B cells infected with VSV ΔG-HA (Figure 17); in cells infected with this virus, G protein can only be derived from input virions. This is an important point because resting B cells do not produce detectable levels of VSV proteins (35, 36). Thus, the fact that virion derived G is sufficient for antibody inhibition provides a source of protein in resting B cells. This does not rule out, however, that in resting B cells infected with wild-type VSV, a small amount of VSV G is synthesized *de novo* and contributes to the pool of protein recognized by antiviral antibodies. It merely suggests that *de novo* synthesis is not absolutely required. Since anti-VSV IgG, which presumably contains antibodies specific for other viral proteins, was used in this experiment, further experiments with monoclonal antibodies to VSV G will be required to show that recognition of G suppresses VSV transcripts in VSVΔG-HA infected B cells.

What is the evidence that virus suppression is mediated by signals generated by antibody binding to VSV G? Treatment of infected B cells with anti-VSV serum or IgG purified from suppresses VSV genome, mRNA, protein, and infectious particle production (Figure 15A and B, lane 2; Figure 16A and B, lane 2). Since these preparations likely contain antibodies specific for all of the viral proteins, it is possible that the functions of multiple viral proteins are directly inhibited by antibody binding. The fact that a monoclonal antibody specific for VSV G also inhibits all levels of virus replication (Figure 15A and B, lane 4), however, is not consistent with this idea.

Inhibition of VSV G function should only affect assembly or release of infectious virus, not transcription or protein synthesis. This suggests that antiviral antibody does not act by directly inhibiting the function of viral proteins, but rather it interferes with cellular signaling pathways required for VSV replication in B cells by generating a signal through VSV G. In the measles virus system, it has been proposed that when antibodies to measles HA bind the protein on the surface of infected cells, a signal is generated in the cell that suppresses viral proteins inside the cell (79). Treatment of measles virus infected rat C6 glioma cells with measles antiserum or a monoclonal antibody to the viral glycoprotein HA results in the breakdown of phosphatidylinositide (187), suggesting that the measles HA protein is coupled to cellular signaling molecules. Therefore, it is reasonable to hypothesize that antibody binding to viral proteins on the cell surface generates an intracellular signal.

VSV G has a short cytoplasmic domain of 29 amino acids (17); a protein data base search did not reveal any signaling motifs in this domain, making it unlikely that G has any direct capacity for signaling. A more likely explanation is that G interacts with a cellular signaling molecule (shown as "X" in the model) either in the cytoplasm or on the plasma membrane; it is proposed in the model that binding of antibody to G generates signals indirectly through this cellular protein. VSV G is thought to interact with VSV M protein through its cytoplasmic tail (17), so it is not unreasonable to think that G could interact with cellular proteins as well. Demonstrating interactions between G and cellular proteins might be difficult in resting B primary B cells because protein levels are likely to low in these cells. An alternative would be to overexpress G in these cells using an

adenovirus vector. A mouse transgenic for the human coxsackie/adenovirus receptor (hCAR) has been developed in this laboratory that allows for the expression of foreign genes in primary lymphocytes (193). Thus, it might be possible to infect resting B cells from the hCAR mice with a VSV G expressing adenovirus and then perform coimmunoprecipitation experiments to determine which cellular proteins G interacts with in resting B cells.

While I have not presented direct evidence for signals induced by antiviral antibodies suppress virus reactivation, there is some evidence to suggest that this is the case. It is important to remember that in these experiments, infected, resting B cells are being treated with antiviral antibodies; in these cells, VSV replication is restricted such that only low levels of viral genome and mRNA are present (35, 36). Since the antiviral antibody treatment acts on resting B cells, it is unlikely to directly inhibit virus replication. VSV replication is regulated at multiple levels in primary B cells, and different cellular signaling pathways likely regulate each stage of virus replication (36). For example, the transcription and genome replication of VSV is regulated by phosphorylation of the viral P protein by cellular protein kinases (194). I propose that the putative signals generated by anti-VSV antibody in infected B cells inhibit cellular signaling pathways required for VSV replication so that these pathways are unavailable after cell activation.

By analogy with antigen receptors, in which the strength of signal has different functional consequences (189), one might predict that the level at which virus replication is inhibited depends on the strength of the putative inhibitory signal(s) generated by

antiviral antibodies. The strength of signaling through antigen receptors depends on the concentration of antigen and the avidity of binding; high concentrations of antigen and multivalent binding generate stronger signals (189). Differential effects on virus replication are seen in an experiment comparing intact anti-VSV IgG, $F(ab')_2$ fragments, and Fab fragments, which presumably have different capacities to generate a signal. In Table 4, it is proposed that high concentrations of bivalent antiviral antibody (intact or $F(ab')_2$ fragments) generate a strong signal, low concentrations of bivalent antibody or high concentrations of monovalent (Fab fragments) generate a weaker signal, and low concentrations of monovalent antibody generate no signal. When a "weak" signal is generated, only infectious virus production is suppressed completely. Viral RNA levels are reduced to a much greater extent with a "strong" signal than with a "weak" signal. In contrast, viral protein levels are only suppressed by a "strong" signal. These data are consistent with the idea that inhibitory signals are generated by treatment of infected B cells with antiviral antibody and that the "strength" of signal determines the level of suppression.

The hypothesis that interactions between viral proteins and antiviral antibodies generate inhibitory signals in infected B cells is testable in several ways. It is proposed that these inhibitory signals interfere with cellular activation pathways required for VSV replication in B cells. Infected B cells in which virus reactivation is suppressed by antiviral antibody proliferate in response to anti-Ig+IL-4 and other B cell activators, suggesting that pathways leading to proliferation are not blocked. B cell activation, however, is associated with numerous other events such as changes in surface marker

expression and differentiation to effector function that depend on different components of activation pathways (195). Thus, it would be interesting to see if treatment of infected B cells with antiviral antibody inhibits anti-Ig+IL-4 induced differentiation to antibody secretion or upregulation of B cell activation markers such as MHC class II, CD25, CD69, B7-1, and B7-2 (126). Stimulation of B cells through the antigen receptor induces numerous signaling events; activation of protein tyrosine kinases such as Lyn, Syk, and Btk; induction of the PLCγ, Ras, and PI3-kinase pathways; induction of Ca^{2+} flux; translocation of nuclear transcription factors such as NF-κB (196). If the hypothesis that antiviral antibody treatment leads to selective inhibition of cellular signaling pathways is true, it should be possible to see changes in one or more of these signaling events. The system proposed for looking at interactions of VSV G with cellular proteins might also be useful for studying the signaling potential of G. Thus, VSV G could be over expressed from an adenovirus vector in resting B cells from hCAR mice. The effect of cross-linking VSV G on B cell activation pathways could then be determined by looking the induction of activation markers and the activity of signaling mediators as described above.

Summary

The data presented in this chapter describe a powerful model system for studying the complex mechanisms by which RNA virus persistence is established and maintained in lymphocytes *in vivo*. The ability of lymphocytes to support virus persistence depends on the interplay between lymphocyte homeostatic signals, which tend to induce virus expression, and the specific immune response (antibody in this case), which tend to

suppress virus expression. In Chapter I, a model for EBV persistence in B cells *in vivo* was described in which it was proposed that EBV proteins aid in the establishment of persistence by inducing the differentiation of resting naive B cells into memory cells, which are thought to maintain virus during latency (62). Another viral protein (LMP2A) is thought to promote the survival of infected memory B cells and to inhibit reactivation of virus by interfering with BCR signaling (63-66). Thus, this complex DNA virus is able to manipulate lymphocyte activation and homeostatic signals to establish and maintain persistence. Since VSV, a simple RNA virus, does not encode such regulatory proteins, it must take advantage of normal lymphocyte physiology and the immune response to the virus to establish and maintain persistence. There are, however, some interesting parallels between the two viruses. VSV can be maintained in a quiescent state for extended periods in resting B cells *in vitro*, and the evidence suggests that it does so *in vivo* as well. While EBV forces the B cell it infects to differentiate into a cell type in which it can persist, VSV establishes persistence in a cell type that is naturally non-supportive of virus replication. The end result in both cases is that the virus is maintained without significant production of viral antigens so that the infected cells evade clearance by the immune system. The proposed function of EBV LMP2A (inhibition of virus reactivation) may be played by antiviral antibody in the case of VSV. This comparison illustrates how two dissimilar viruses use different mechanisms to accomplish the same goal; the establishment and maintenance of a persistent infection in resting lymphocytes.

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