Immunity, Vol. 10, 753–760, June, 1999, Copyright ©1999 by Cell Press

SHIP Recruitment Attenuates FcγRIIB-Induced B Cell Apoptosis

Roger N. Pearse,* Tsutomu Kawabe,† Silvia Bolland,* Rodolphe Guinamard,* Tomohiro Kurosaki,‡ and Jeffrey V. Ravetch*§

*Laboratory of Molecular Genetics and Immunology
The Rockefeller University
1230 York Avenue
New York, New York 10021

†First Department of Internal Medicine
Nagoya University School of Medicine
65 Tsurumai-cho, Showa-ku
Nagoya 466
Japan

‡Department of Molecular Genetics
Kansai Medical University
10-15 Fumizono-cho, Moriguchi
Osaka 570
Japan

Summary

FcγRIIB is an inhibitory receptor that terminates activation signals initiated by antigen cross-linking of the BCR through the recruitment of SHIP. FcγRIIB can also signal independently of BCR coligation to directly mediate an apoptotic response, requiring only an intact transmembrane domain. Failure to recruit SHIP, either by deletion of SHIP or mutation of FcγRIIB, results in enhanced FcγRIIB-triggered apoptosis. Thus, in the germinal center, where ICs are retained by FDCs, FcγRIIB may be an active determinant in the negative selection of B cells whose BCRs have reduced affinity for antigen as a result of somatic hypermutation. Selection of B cells may represent the sum of opposing signals generated by the interaction of ICs with the BCR and FcγRIIB through pathways modulated by SHIP.

Introduction

During the course of an immune response, the affinities of antibodies progressively increase in a process known as affinity maturation (Jerne, 1951; Eisen and Siskind, 1964; Siskind and Benacerraf, 1969). These hypermutated antibodies are crucial to the formation of the memory B cell pool, insuring that reexposure to antigen elicits a rapid, efficient, and specific response (Berek et al., 1985; Cumano and Rajewsky, 1986; Foote and Milstein, 1991). Dissection of the pathway by which naive B cells become memory cells has focused on the germinal center (GC). Within this anatomic compartment, B cells of differing affinities for antigen undergo mutation and selection (Berek et al., 1991; Jacob et al., 1991; MacLennan, 1994; Kelsoe, 1996). Antigen, in the form of an immune complex (IC), is retained on follicular dendritic cells (FDCs) where it drives the selection of B cells that have undergone V region hypermutation (Nossal et al., 1964; Mandel et al., 1980). Since the most likely outcome of random V region mutation is a B cell receptor (BCR) with reduced affinity for antigen (Tonegawa, 1983), mechanisms must exist that discriminate between those B cells bearing BCR with reduced affinity and those bearing BCR with higher affinity for the antigen. This discrimination is attributed to preferential antigen stimulation of those B cells bearing higher-affinity BCRs and to cognate T cell interactions facilitated by the preferential uptake of antigen by these higher-affinity BCRs (Nossal, 1994; Rajewsky, 1996; Healy and Goodnow, 1998). In the absence of these stimulatory signals, cells bearing lower-affinity BCRs undergo apoptotic death and are removed by macrophages residing in the GC.

This model takes into account the role of the BCR in driving positive selection by delivering activation signals and by providing a means of internalization and presentation of antigen to cognate T cells. However, it neglects to consider that the interaction of retained ICs with the B cell is potentially mediated by both antigen binding to the BCR and Fc binding to FcγRIIB. The unique FcR expressed on B cells is FcγRIIB (Ravetch et al., 1986), a low-affinity receptor for ICs that contains the inhibitory ITIM motif in its cytoplasmic domain (Amigorena et al., 1992; Muta et al., 1994). Coengagement of BCR and FcγRIIB results in the tyrosine phosphorylation of the ITIM (Muta et al., 1994) and the recruitment of the SH_2-containing inositol polyphosphate 5-phosphatase SHIP (Chacko et al., 1996; Ono et al., 1996; Kiener et al., 1997; Tridandapani et al., 1997). SHIP, by hydrolyzing PIP_3 (phosphatidylinositol [3,4,5]trisphosphate) (Damen et al., 1996), leads to the dissociation of Bruton's tyrosine kinase (Btk) from the membrane and the inhibition of calcium influx into the cell (Bolland et al., 1998; Scharenberg et al., 1998). Thus, FcγRIIB can modulate both BCR-induced B cell activation and antigen internalization through the recruitment of SHIP. In addition, engagement of FcγRIIB on B cells has been found capable of generating an apoptotic signal (Ashman et al., 1996; Ono et al., 1997). This response to FcγRIIB engagement has been shown in DT40 cells to be independent of SHIP recruitment, suggesting that this receptor may directly couple to an apoptotic pathway in the absence of BCR-induced signaling (Ono et al., 1997). We now report that cross-linking of FcγRIIB alone is sufficient to induce apoptosis in B cells and that this response requires an intact FcγRIIB transmembrane domain. The signaling pathway induced by FcγRIIB aggregation leads to the activation of Jun kinase, is independent of the expression of lyn, syk, or phospholipase C-γ2 (plcγ2), and requires the expression of Btk. This profile of signaling requirements is similar to that observed for apoptosis induced by osmotic shock or γ irradiation (Uckun et al., 1996; Qin et al., 1997a, 1997b; Goodman et al., 1998), suggesting that aggregation of FcγRIIB in the membrane is registered by the cell as a stress to which it responds

§To whom correspondence should be addressed (e-mail: ravetch@rockefeller.edu).

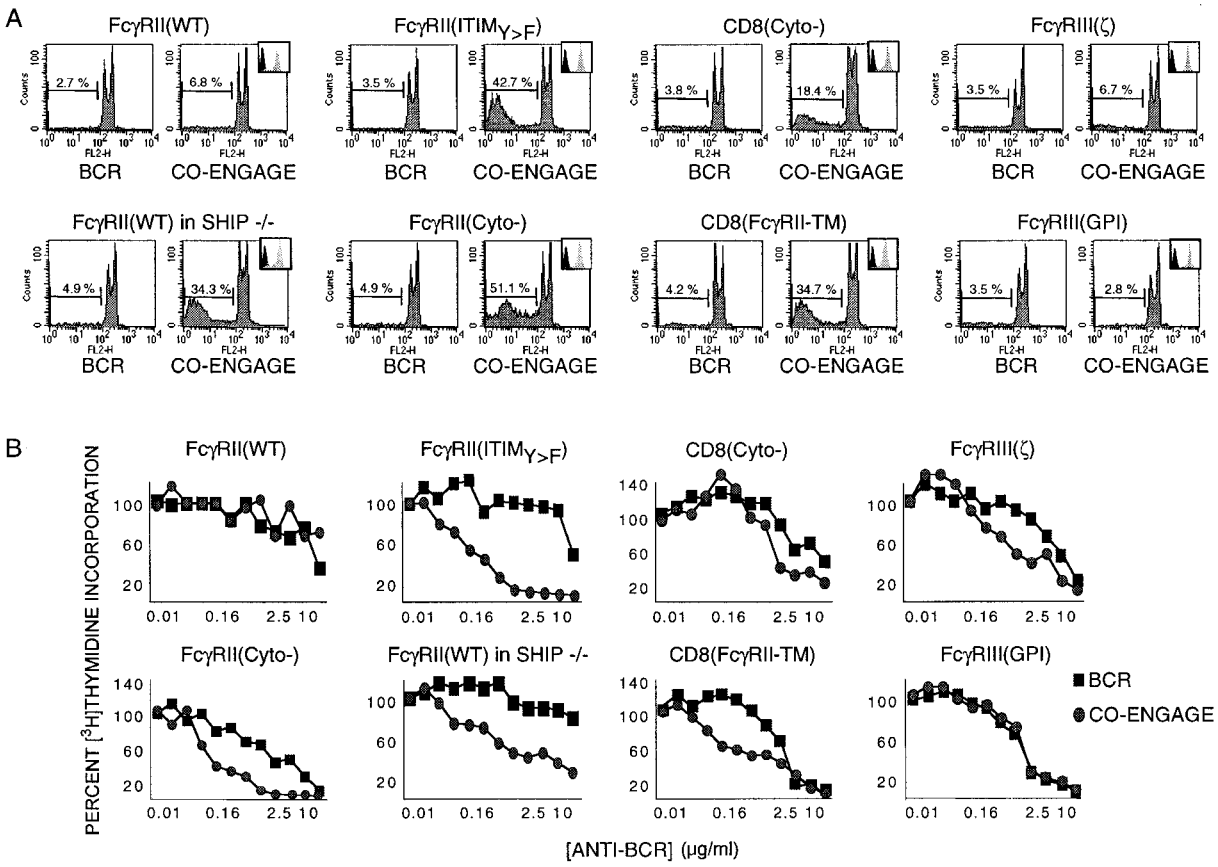

Figure 1. Apoptosis Elicited by Coengagement of the BCR with Mutant FcγRII

(A) Representative FACS analyses of propidium iodide staining (FL2-H) following BCR stimulation (M4 at 2.5 μg/ml) either alone or with coengagement to the indicated surface-expressed receptor. The percent hypodiploid cells after 18 hr of stimulation are indicated. Surface expression of the transfected construct is also presented (inset). (B) Incorporation of [^{3}H]thymidine following either BCR stimulation or coengagement of the BCR with the indicated surface-expressed receptor. DT40 transfectants were stimulated for 18 hr with M4 at concentrations ranging from 0.1 to 20 μg/ml. [methyl-^{3}H]thymidine was added for the last 6 hr of stimulation. The amount of [^{3}H] incorporated at each dose of M4, with or without coengagement, is presented (Chen et al., 1982; Ono et al., 1997).

by apoptosis. SHIP recruitment rescues this stress response by a mechanism that requires an intact phosphatase domain.

These results suggest that ICs retained on the FDC can trigger either apoptotic or proliferative responses depending on the strength of interaction with FcγRIIB or BCR, respectively, and that these responses can be modulated by the recruitment of SHIP. The apoptotic activity associated with FcγRIIB could provide a mechanism for the active elimination of those B cells whose hypermutated BCRs bear lower affinity for antigen. Selection in the germinal center may thus reflect the net result of both positive and negative signaling pathways mediated through the BCR and FcγRIIB, respectively.

Results

SHIP Modulates FcγRIIB Enhancement of BCR-Triggered Apoptosis

The B cell line DT40 undergoes apoptosis upon cross-linking of its BCR (Takata et al., 1995). We have previously demonstrated that coengagement of FcγRIIB and BCR does not affect this apoptotic response under conditions of coligation that result in the inhibition of calcium influx (Ono et al., 1997). Disruption of SHIP recruitment, however, either by mutation of the SH_2 binding site on the ITIM of FcγRIIB (ITIM$_{Y>F}$) or by deletion of SHIP (SHIP$^{-/-}$) results in enhanced apoptosis as measured by DNA fragmentation (Figure 1A) or arrest of proliferation (Figure 1B). This ability to confer enhanced apoptosis is retained upon elimination of the complete cytoplasmic tail of FcγRIIB (Cyto-) and can be transferred to a second molecule by transferring the transmembrane domain of FcγRIIB (CD8(FcγRII-TM)). Coengagement of the BCR with human FcγRIII possessing either a GPI anchor or the transmembrane domain of CD3ζ does not result in an enhanced apoptotic response, suggesting that this effect is specific to the transmembrane domain of FcγRIIB.

SHIP recruitment abrogates the enhanced apoptotic response observed upon coligation with FcγRIIB. SHIP is a multidomain molecule, with inositol polyphosphate 5-phosphatase, phosphotyrosine binding (PTB), proline-rich, and src homology 2 (SH_2) domains. To determine which regions of the molecule are required for the abrogation of the apoptotic enhancement seen upon

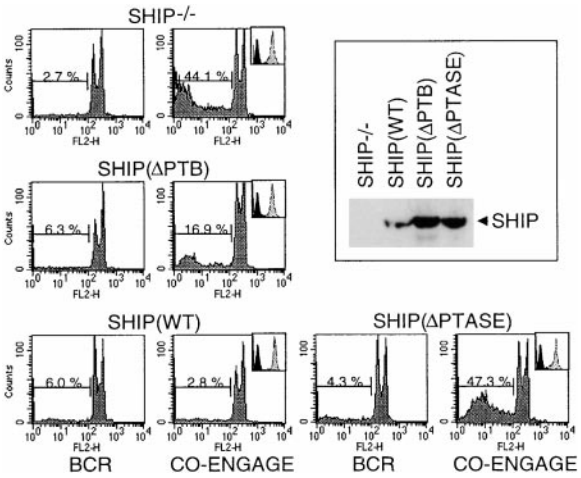

Figure 2. Complementation of SHIP$^{-/-}$ DT40 Cells: SHIP Requires Inositol Phosphatase Activity to Block Apoptosis Triggered by Coengagement of BCR with FcγRII

DT40 cells that had been deleted of endogenous SHIP (SHIP$^{-/-}$) were transfected with wild-type mFcγRIIB and complemented with either wild-type (WT), PTB mutant (ΔPTB), or inositol phosphatase mutant (ΔPtase) murine SHIP. Shown are representative FACS analyses of propidium iodide staining (FL2-H) following either BCR or BCR-mFcγRIIB stimulation, as described for Figure 1A. Large inset: Western blot of WT or Δ SHIP expression by the different DT40 cell lines. FcγRIIB surface expression is shown for each DT40 cell line (small insets).

FcγRIIB coengagement, SHIP$^{-/-}$ DT40 cells were transfected with either wild-type (WT), phosphatase mutant (ΔPtase), or PTB mutant (ΔPTB) versions of SHIP. While mutation within the PTB domain results in a molecule that is less efficient than wild-type SHIP at inhibiting FcγRIIB-triggered apoptosis, only the phosphatase domain is clearly required for the protection mediated by SHIP recruitment (Figure 2).

Jun N-terminal kinase (JNK) is activated upon the induction of the apoptotic response (Ip and Davis, 1998). As such, it can be viewed as a surrogate marker for apoptotic signaling. In DT40 cells, BCR coengagement with FcγRIIB results in enhanced JNK activity when SHIP recruitment is abrogated by mutation of the ITIM of FcγRIIB (Figure 3). BCR stimulation alone, or BCR coengagement with wild-type FcγRIIB, results in reduced levels of JNK, consistent with the observations presented in Figure 1.

FcγRIIB Cross-Linking Alone Is Sufficient to Induce Apoptosis

Since the enhancement of apoptosis mediated by FcγRIIB is abrogated by SHIP recruitment, we investigated whether aggregation of FcγRIIB under conditions that would not result in phosphorylation of the ITIM and thereby SHIP recruitment would itself result in an apoptotic response. Clustering of wild-type FcγRIIB by rat anti-mFcγRII (2.4G2), followed by goat anti-rat IgG, results in a concentration-dependent inhibition of thymidine incorporation (Figure 4). 2.4G2 alone does not trigger this apoptotic response, indicating that dimerization is insufficient to trigger an apoptotic signal. Clustering of receptor does not result in tyrosine phosphorylation or inhibition of calcium influx (data not shown), indicating that the apoptotic response triggered by this receptor is distinct from the SHIP-dependent inhibition of calcium influx and cellular activation.

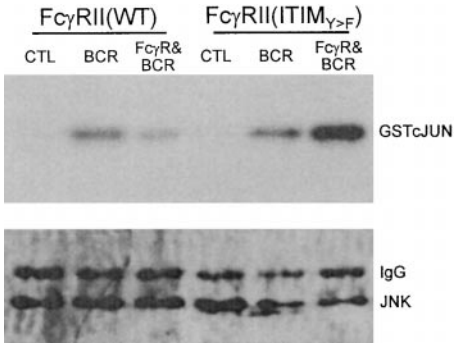

Figure 3. Coengagement of FcγRII(ITIM$_{Y>F}$) with BCR Augments c-Jun NH_2-Terminal Kinase Activation

DT40 cells, transfected with wild-type (WT) or ITIM mutant (ITIM$_{Y>F}$) mFcγRIIB, were stimulated as in Figure 1A with anti-BCR (M4 at 2.5 μg/ml) plus or minus mFcγRIIB coengagement. Following 20 min of stimulation, JNK1 was immunoprecipitated and the precipitates assayed for kinase activity using GST-c-Jun as substrate. The kinase reaction products were resolved by 12.5% SDS-PAGE. Upper panel: autoradiogram of [^{32}P]phosphorylated GST-c-Jun. Lower panel: Western blot of the immunoprecipitates using anti-JNK1, goat anti-mIgG, and ECL.

FcγRIIB-Induced Apoptosis Requires Expression of Btk

The signaling requirements for FcγRIIB-mediated apoptosis were investigated in a series of DT40 mutants that were transfected with wild-type FcγRIIB and then cross-linked using immobilized rat anti-mFcγRII (Figure 5). The apoptotic response to FcγRIIB clustering was not affected by deletion of syk, lyn, or plcγ2. However, deletion of Btk reduced the induction of apoptosis by >50%. This dependence on Btk is consistent with the requirement of an intact phosphatase domain for the antiapoptic activity of SHIP. Btk has been identified as an indirect target for SHIP inositol phosphatase activity

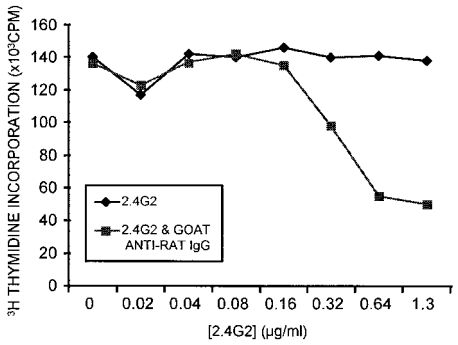

Figure 4. Growth Arrest of DT40 Cells following FcγRII Aggregation

DT40 cells expressing wild-type mFcγRIIB were incubated for 18 hr with rat IgG anti-mFcγRII (2.4G2) at concentrations ranging from 0.02 to 2.5 μg/ml, with or without cross-linking by goat anti-rat IgG. [methyl-^{3}H]thymidine was added for the last 6 hr of incubation, and the amount of [^{3}H] incorporated is presented.

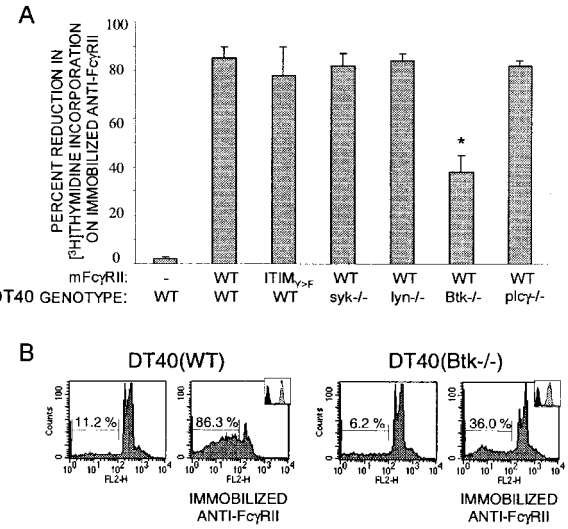

Figure 5. Role of BTK in FcγRII-Induced Apoptosis

DT40 cells, either wild-type (WT) or deleted of the genes encoding syk, lyn, Btk, or plcγ2, were transfected with wild-type (WT) or ITIM mutant ($ITIM_{Y>F}$) mFcγRIIB. (A) Cells from each DT40 cell line were incubated for 18 hr on plates coated with rat anti-mFcγRII. [methyl-^{3}H]thymidine was added for the last 6 hr of incubation, and the amount of [^{3}H] incorporated after mFcγRIIB cross-linking (anti-FcγRII-coated plates) was compared to the amount of [^{3}H] incorporated without mFcγRIIB cross-linking. The data presented are the percent decrease in [^{3}H] incorporation following mFcγRIIB cross-linking and are the mean and standard error of six determinations. Statistical significance between WT and $Btk^{-/-}$ DT40 cells was determined using the Student's t test where $P < E\text{-}05$ (*). (B) Propidium iodide staining of WT and $Btk^{-/-}$ cells following 18 hr of mFcγRIIB cross-linking by immobilized anti-FcγRII. The percentage of hypodiploid nuclei are indicated. mFcγRIIB surface expression by the two clones is also presented (inset).

as hydrolysis of PIP_3 by SHIP leads to the dissociation of Btk from the membrane (Damen et al., 1996; Bolland et al., 1998; Scharenberg et al., 1998). Thus, the apoptotic signal generated by FcγRIIB clustering may be mediated by enhanced Btk association with the membrane, which is reversed upon SHIP recruitment. This pattern of signaling sensitivity is reminiscent of the requirements for γ irradiation or osmotic shock-induced apoptosis (Uckun et al., 1996; Qin et al., 1997a, 1997b; Goodman et al., 1998) and suggests that clustering of FcγRIIB results in a membrane perturbation that triggers a stress response in the cells.

FcγRIIB-Triggered Apoptosis in Murine Splenocytes

The enhanced apoptotic response seen upon FcγRIIB clustering in the chicken B cell line DT40 is also observed in primary murine B cells. Murine splenocytes proliferate in response to LPS or IL-4. Clustering of FcγRIIB in LPS- or IL-4-treated splenocyte cultures results in an increase in the percent of hypodiploid nuclei at 36 hr (Figures 6A and 6B) and arrest of [^{3}H]thymidine incorporation (Figure 6C). In contrast, splenocytes isolated from $Fc\gamma RIIB^{-/-}$ mice did not exhibit enhanced apoptosis under these conditions, confirming that the response is specific and selective.

Discussion

The interaction of antibody with Fc receptors can result in either cellular activation, inhibition, or apoptosis (Daeron, 1997; Ravetch, 1997). Activation responses are mediated by FcRs, which are associated with ITAM sequences found in FcγRI and FcγRIII, while inhibitory responses are transduced through the ITIM sequence found associated with FcγRIIB. For cells that express both activation and inhibitory receptors, such as macrophages, neutrophils, and mast cells, the outcome of an interaction with an immune complex (IC) is determined by the relative levels of activation and inhibitory receptors. Mice deficient in the activation receptors are protected from IC-triggered inflammation (Sylvestre and Ravetch, 1994; Clynes et al., 1998, 1999), while animals deficient in the inhibitory receptor have enhanced sensitivity to these same stimuli (Takai et al., 1996; Clynes et al., 1999; Yuasa et al., 1999).

The B cell expresses only the inhibitory Fc receptor FcγRIIB without a corresponding activation FcR. However, coligation of FcγRIIB and an activation counterpart, the BCR, does occur during interaction with ICs, when antigen binds to the BCR and the Fc domains interact with FcγRIIB. This coengagement suggests a mechanism whereby ICs function as feedback regulators of B cell activation. In vitro, ICs are potent inhibitors of B cell activation stimulated through the BCR (Chan and Sinclair, 1971; Phillips and Parker, 1983, 1984; Bijsterbosch and Klaus, 1985). However, in vivo, mice deficient in FcγRIIB have modestly elevated serum antibody titers to both thymic-dependent and thymic-independent antigens (Takai et al., 1996). Interpretation of those results is complicated by the fact that the primary site for the interaction of B cells with ICs is in the germinal center, where ICs are retained by follicular dendritic cells (FDCs). Indeed, the presence of FDCs in an in vitro B cell proliferation assay converts the inhibitory IC into a stimulatory molecule (D. Qin et al., submitted). Further, FcγRIIB is the exclusive FcR on FDCs; its level of expression is induced during the course of the secondary immune response, and it plays a significant role in facilitating presentation of ICs to B cells, resulting in B cell activation. Thus, FcγRIIB on FDCs appears to have a positive regulatory role in development of the germinal center reaction (Liu et al., 1996). Consequently, deletion of FcγRIIB from both B cells and FDCs may result in compensatory defects, with a net minimal phenotype.

The present study expands the role of FcγRIIB from an inhibitor of activation requiring coengagement with an activating receptor to that of an autonomous receptor capable of independently triggering an apoptotic response upon clustering. Previous studies have suggested that FcγRIIB on B cells is capable of both inhibiting BCR-mediated activation and generating an apoptotic signal in mixed populations of splenocytes (Ashman et al., 1996; Yamashita et al., 1996). While those studies primarily investigated the augmentation of an apoptotic signal from BCR through FcγRIIB coengagement, the results may be pertinent to the present study as well.

The receptor requirements for the proapoptotic signaling response reside exclusively in the transmembrane domain of FcγRIIB. Substitution of a GPI anchor

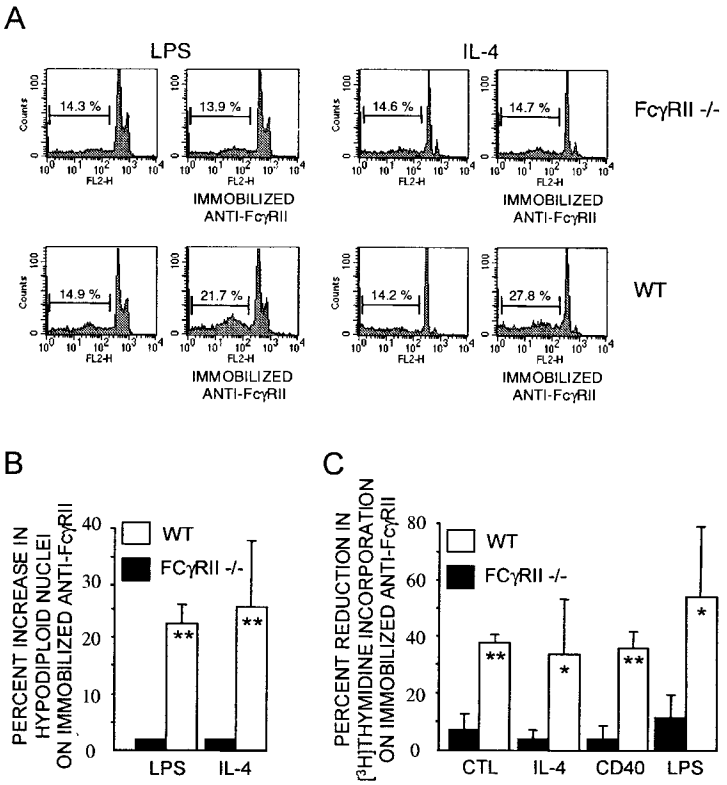

Figure 6. Apoptosis Elicited by Cross-Linking of FcγRIIB on Primary Murine Lymphocytes

B lymphocytes were isolated from WT and FcγRII$^{-/-}$ mice and cultured either on immobilized rat anti-FcγRIIB or nonspecific rat IgG. (A) Representative FACS analyses of propidium iodide staining (FL2-H) following 36 hr of incubation in the presence of LPS (10 μg/ml) or IL-4 (5 IU/ml). (B) The percent hypodiploidy observed following 36 hr of incubation on immobilized anti-FcγRIIB was compared to that seen following 36 hr of incubation on nonspecific rat IgG. The data presented are the percent increase in hypodiploid nuclei associated with anti-FcγRII exposure and are the mean and standard error of three determinations. (C) Percent reduction in [methyl-^{3}H]thymidine incorporation as a result of FcγRIIB cross-linking. WT and FcγRII$^{-/-}$ B lymphoctes were incubated for 36 hr in the presence of LPS (10 μg/ml), IL-4 (5 IU/ml), anti-CD40 (0.5 μg/ml), or no added mitogen (CTL). [methyl-^{3}H]thymidine was added for the last 6 hr of incubation, and the amount of [^{3}H] incorporated with mFcγRIIB cross-linking (anti-FcγRII coated plates) was compared to the amount of [^{3}H] incorporated without. The data presented are the percent decrease in [^{3}H] incorporation following mFcγRIIB cross-linking and are the mean and standard error of three determinations. Statistical significance between WT and FcγRII$^{-/-}$ populations was determined using the Student's t test where $P < 0.05$ (*) and $P < 0.005$ (**).

or a charged transmembrane domain, as found in CD3ζ, destroyed the apoptotic response. Since the apoptotic response resembles the stress response seen upon osmotic shock or γ irradiation, the mechanism by which FcγRIIB clustering generates an apoptotic signal may result from a general membrane perturbation associated with this transmembrane domain. The apoptotic signal generated by this membrane perturbation requires Btk and is blocked by SHIP. As Btk associates with the membrane via its plextrin homology (PH) domain (Li et al., 1995), it is likely that SHIP, by hydrolyzing PIP_3, blocks apoptosis by eliminating the target of Btk PH binding (Bolland and Ravetch, 1998). The mechanism of Btk activation by FcγRIIB in lymphocytes is unclear at present. However, β_2 integrins have been implicated in FcγRII-mediated signaling in eosinophils (Kim et al., 1999) and neutrophils (Graham et al., 1993) and may contribute to FcγRIIB signaling in lymphocytes as well.

SHIP recruitment appears to be a determining factor in the choice between apoptosis and survival, as illustrated in the model in Figure 7. When FcγRIIB is engaged in the absence of SHIP recruitment, an apoptotic signal is generated. In contrast, when FcγRIIB-BCR coengagement results in SHIP recruitment, this apoptotic pathway

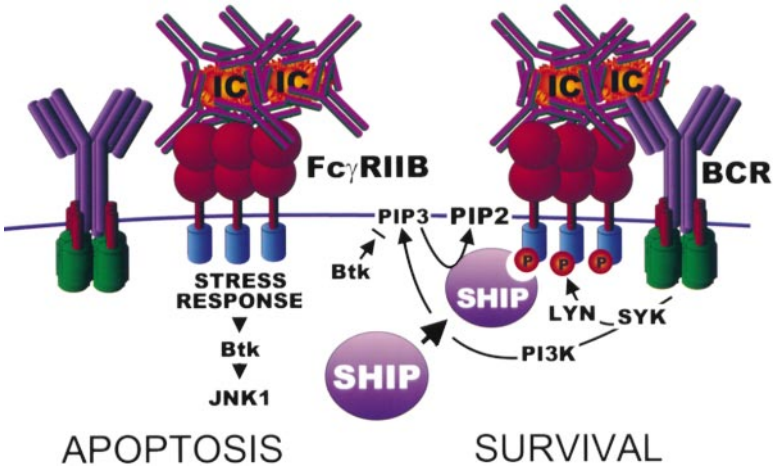

Figure 7. SHIP as Arbiter of B Cell Fate

B cells within the germinal center encounter antigen in the form of ICs retained on FDCs. This B cell interaction with ICs, potentially mediated by antigen binding to the BCR, C3d binding to CD21, and Fc binding to FcγRIIB, drives the selection of B cells that have undergone V region hypermutation. As FcγRIIB is clustered on B cells, a potentially lethal stress response, mediated by Btk, is triggered. Those B cells bearing high-affinity BCR also engage antigen, resulting in activation of syk and lyn, phosphorylation of the FcγRIIB ITIM, and the recruitment of SHIP. SHIP, through its inositol phosphatase activity, aborts the apoptotic signal, possibly due to dissociation of Btk from the plasma membrane.

is blocked, leaving as the dominant response the balance between concurrent activation and inhibitory signals. This molecular switch is likely to be crucial to the fate of B cells in the germinal center, where B cells encounter ICs bound to the surface of FDCs, a state that may provide the avidity necessary to promote sufficient FcγRIIB cross-linking to trigger apoptosis.

In addition to inhibiting FcγRIIB-mediated apoptosis, SHIP appears capable of functioning to stimulate apoptosis by inhibiting the activation of the antiapoptotic molecule PKB in B lymphocytes (Aman et al., 1998) and myeloid cells (Liu et al., 1999). Thus, the result of SHIP recruitment is the net outcome of its impact on both pro- and antiapoptotic signaling cascades and is likely to be influenced by the context in which these signals occur. The enhanced apoptosis seen upon FcγRIIB engagement with the BCR (Ashman et al., 1996; Yamashita et al., 1996) may be the result of either PKB inhibition or of FcγRIIB signaling. Further, the apoptotic response of murine splenocytes to FcγRIIB cross-linking, most apparent during culture in the presence of LPS or IL-4 (Figure 6), may be due to the selective stimulation of a subset of splenocytes sensitive to the effects of FcγRIIB engagement or it may reflect modulation of antiapoptotic signals by these cytokines. Clearly, the regulation of SHIP expression may be a potent means to control B cell responses.

The generation of memory cells and their selection in the germinal center is thought to involve the positive selection of B cells bearing higher-affinity BCRs through the binding of antigen retained as ICs on FDCs. As the present study demonstrates, the interaction of the IC with the B cell can result in either a stimulatory, inhibitory, or apoptotic response. If the latter process dominates, the result of IC binding by FcγRIIB would lead to an active process of negative selection. Somatic hypermutation occurs in the germinal center and can generate both higher- and lower-affinity BCRs. Those B cells that engage ICs preferentially through FcγRIIB would be predicted to undergo apoptosis, while B cells that engage the BCR preferentially would be positively selected. In addition, since SHIP is essential for mediating the inhibitory response as well as overriding the apoptotic response, modulation of SHIP levels in germinal center B cells may represent another point at which the outcome of the interaction of ICs and B cells can be regulated. In support of this model, it is worth noting that mice deficient in SHIP are lymphopenic (Helgason et al., 1998), suggesting that enhanced apoptosis may be occurring in the absence of the ability of FcγRIIB to recruit this signaling molecule. In vivo studies testing this model will be needed to determine the significance of this apoptotic pathway in the generation and selection of memory cells.

Experimental Procedures

Expression Constructs

Cloning of hFcγRIII(GPI), mFcγRIIB, and mFcγRIIB(ITIM$_{Y>F}$) into the expression vector pcEXV-3 (Miller and Germain, 1986) has been described (Kurosaki and Ravetch, 1989; Muta et al., 1994). Cloning of hFcγRIII(ζ), a chimera of the extracellular domain of human FcγRIIIA (amino acids 1–212), the transmembrane domain of the human TCR ζ chain (amino acids 30–58), and rat plcγ2, has been described (Ishiai et al., 1999). FcγRIIB(Cyto-), which contains no cytoplasmic residues beyond the third juxtamembrane lysine, was made by restriction digestion of pcEX(mFcγRIIB) using Apa1 and EcoR1, with subsequent introduction of an Apa1/EcoR1-digested PCR fragment generated from mFcγRIIB using the following primers: 5′ACTACTAC TGCAAAGGAAGTC3′ and 5′CTGGAATTCCTACTTTTTCTTGAGATA GACCAAGGA3′. CD8(Cyto-), which contains no cytoplasmic residues beyond the third juxtamembrane arginine, was made by restriction digestion of pHβAPr(huCD8) (kindly provided by Dan Littman, NYU) using EcoRV and BamH1, with subsequent introduction of a BamH1/EcoRV-digested PCR fragment generated from huCD8 using the following primers: 5′TTGGATCCTAACGTCTTCGGTTCCT GTGGTTGCA3′ and 5′CAGCCCCTGTCCCTGCGCCCA3′. CD8(FcγRII-TM) was constructed by digesting pHβAPr(huCD8) with EcoRV and BamH1, filling in the resulting overhangs with klenow fragment, and introducing a HaeIII-digested PCR fragment generated from mFcγRIIB using the following primers: 5′ACTACTACTGCAAAG GAAGTC3′ and 5′CTGGAATTCCTACTTTTTCTTGAGATAGACCAA GGA3′. SHIP(WT) contains the entire coding sequence of murine SHIP cloned into the pRc/CMV expression vector (Invitrogen). SHIP (ΔPtase) contains mutations at P671A, D675A, and R676G within the inositol 5-phosphatase domain that were introduced by polymerase chain reaction (PCR) using the following mismatched primers: 5′TTCACCCACCTCTTCTGGCTTG3′ and 5′CATGATGTCACTGGTA CTGCCA3′. SHIP(ΔPTB) contains mutations at Y917F and Y1020F within the PTB domain that were introduced by PCR using the following mismatched primers: 5′CAATCCAAACTTCATTGGTATGGG GCCTTTTGGAC3′ and 5′CCATACCAATGAAGTTTGGATTGATCAT CTCATTG3′ and 5′GAGAACCCACTGTTTGGATCCGTGAGTTCCTT CCC3′ and 5′CTCACGGATCCATACAGTGGGTTCTCAAACATCTC3′. All constructs were verified by DNA sequence analysis.

Cell Culture

Wild-type and mutant DT40 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 1% chicken serum, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, and antibiotics. The generation of DT40 mutants deficient in either SHIP (Okada et al., 1998), Btk (Uckun et al., 1996), plcγ2 (Takata et al., 1995), syk (Takata et al., 1994), or lyn (Takata et al., 1994) has been described previously. Transfection of DT40 cells was carried out using electroporation at 250 V and 960 μF in phosphate-buffered saline (10^7 cells in 0.5 ml) with 20 μg of expression construct and 2 μg of pBabepuro′ vector (Morgenstern and Land, 1990). Transfectants were selected in 0.5 mg/ml puromycin 24 hr after electroporation. Expression of WT or mutant mFcγRII was confirmed by fluorescence-activated cell sorting (FACS) of fluorescein isothiocyanate (FITC)–conjugated 2.4G2 (monoclonal rat anti-mFcγRII/III; PharMingen) staining. Expression of FcγRIII(GPI) or FcγRIII(ζ) was confirmed by FACS analysis of 3G8-FITC (monoclonal mouse anti-huFcγRIII; PharMingen) staining. Expression of CD8(Cyto-) or CD8(FcγRII-TM) was confirmed by FACS analysis of monoclonal mouse anti-huCD8-FITC (PharMingen) staining. Expression of WT or mutant mSHIP was confirmed by Western blot using rabbit anti-mSHIP (Ono et al., 1997), horseradish peroxidase-conjugated goat anti-rabbit antibody (Amersham Pharmacia Biotech), and enhanced chemiluminescence (ECL; Amersham). Female wild-type and FcγRIIB$^{-/-}$ (Takai et al., 1996) BALB/c mice were purchased from Taconic Farms (Germantown, NY) and housed in a SPF facility. Primary murine B lymphocytes were isolated from the spleens of 8-week-old mice using MACS (Miltenyi Biotec) according to the manufacturer's specifications to isolate CD43-negative cells. More than 95% of the resulting cell population was positive for CD19. B lymphocytes were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, and antibiotics. LPS (Sigma-Aldrich) was used at 10 μg/ml, anti-mCD40 (PharMingen) was used at 0.5 μg/ml, and IL-4 (R & D Systems) was used at 5 IU/ml.

Apoptosis Analysis

Apoptosis upon coligation of BCR with surface-expressed receptors was assayed as described previously (Ono et al., 1997). In brief, DT40 cells were incubated at a density of 10^5/ml in 100 μl of medium containing various amounts of M4, a monoclonal murine IgM anti-chicken IgM (Chen et al., 1982), and 15 μg/ml of rabbit anti-mouse

IgM (Pierce). Rat anti-mFcγRII/III (2.4G2 at 10 μg/ml; PharMingen) was used to block coengagement of the BCR with transfected mFcγRIIB constructs. Mouse anti-hFcγRIII (3G8 at 10 μg/ml; PharMingen) was used to block coengagement of the BCR with transfected hFcγRIII constructs. Mouse IgM anti-hCD8 (Accurate Chemical) was used to coengage the BCR with transfected CD8 constructs. Apoptosis was also assayed following 18 hr of mFcγRII ligation by soluble 2.4G2, with or without 10 μg/ml of goat anti-rat IgG (Pierce). Apoptosis of DT40 cells following mFcγRIIB ligation with immobilized 2.4G2 was assayed in 96-well plates (Costar) that had been coated overnight with either 2.4G2 or nonspecific rat IgG (Pierce) at 10 μg/ml. Plates were then washed and coated overnight with 30% FCS. Prior to use, FCS was aspirated and cells were plated at a density of 10^5/ml. To assay growth arrest, cells were labeled for the last 6 hr of incubation with 0.5 μCi [methyl-^{3}H]thymidine (74 GBq/mmol, Amersham) and harvested 18 hr after stimulation. Apoptosis of murine splenocytes following mFcγRIIB ligation with immobilized 2.4G2 was assayed in 96-well plates (Costar) that had first been coated with 50 μg/ml goat anti-rat IgG. Plates were then washed and coated with either 2.4G2 or nonspecific rat IgG (Pierce) at 50 μg/ml. Plates were then washed and coated overnight with 30% FCS. Prior to use, FCS was aspirated, and cells were plated at a density of 10^6/ml. To assay growth arrest, cells were labeled for the last 6 hr of incubation with 0.5 μCi [methyl-^{3}H]thymidine and harvested 36 hr after stimulation. [^{3}H]thymidine incorporation was measured by liquid scintillation (Wallac). Nuclear DNA fragmentation was assayed after 12 hr of stimulation by staining with propidium iodide (20 μg/ml) in hypotonic lysis buffer (0.1% sodium citrate and 0.1% Triton X-100). Percent hypodiploid cells was determined by FACS analysis.

Jun Kinase Assay

c-Jun NH_2-terminal kinase activity was assayed as described previously (Derijard et al., 1994). In brief, 5×10^6 cells were lysed in 500 μl of lysis buffer (20 mM Tris-HCl [pH 7.5], 137 mM NaCl, 5 mM EDTA, 2% Triton X-100, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 25 mM B-glycerophosphate, 50 mM NaF, 10 mM NaPPi, 15% (v/v) glycerol, 2 mM benzamidine, 10 μg/μl leupeptin, and 2 μg/μl aprotinin) for 30 min on ice. Precleared lysates were subjected to immunoprecipitation for 2 hr at 4°C using 1 μg of anti-JNK1 mAb (PharMingen) and Protein-G Sepharose (40 μl of a 50% slurry; Amersham Pharmacia Biotech). The resulting immunoprecipitates were washed twice with lysis buffer and twice with kinase assay buffer (25 mM Hepes [pH 7.4], 25 mM B-glycerophosphate, 25 mM $MgCl_2$, 10 mM $MnCl_2$, 2 mM dithiothreitol, and 0.1 mM Na_3VO_4) and resuspended in 30 μl of kinase assay buffer containing 1 μg GST-c-Jun (1–79) (generously provided by Dr. H. Hanafusa, The Rockefeller University), 0.5 μCi γ-[^{32}P]ATP (>3000 Ci/mmol; NEN Life Science Products), and 20 mM ATP. The reactions were allowed to proceed for 20 min at 30°C, following which proteins in the reactions were resolved by SDS PAGE (Laemmli, 1970), transferred to PVDF membrane (Bio-Rad), and exposed to film. The amount of JNK1 in the immunoprecipitates was determined by immunoblot using anti-JNK1, horseradish peroxidase-conjugated goat anti-mIgG, and ECL.

Acknowledgments

This work was supported by grants from the National Institutes of Health (J. V. R.), from the SLE Foundation (S. B.), from the Irvington Institute for Immunological Research (R. G.), and from the Ministry of Education, Science, and Culture of Japan (T. K.). We would like to thank Michel Nussenzweig, Brian Wong, and Yongwon Choi for helpful discussions.

Received January 29, 1999; revised May 5, 1999.

References

Aman, M.J., Lamkin, T.D., Okada, H., Kurosaki, T., and Ravichandran, K.S. (1998). The inositol phosphatase SHIP inhibits Akt/PKB activation in B cells. J. Biol. Chem. *273*, 33922–33928.

Amigorena, S., Bonnerot, C., Drake, J.R., Choquet, D., Hunziker, W., Guillet, J.G., Webster, P., Sautes, C., Mellman, I., and Fridman, W.H. (1992). Cytoplasmic domain heterogeneity and functions of IgG Fc receptors in B lymphocytes. Science *256*, 1808–1812.

Ashman, R.F., Peckham, D., and Stunz, L.L. (1996). Fc receptor off-signal in the B cell involves apoptosis. J. Immunol. *157*, 5–11.

Berek, C., Griffiths, G.M., and Milstein, C. (1985). Molecular events during maturation of the immune response to oxazlone. Nature *316*, 412–418.

Berek, C., Berger, A., and Apel, M. (1991). Maturation of the immune response in germinal centers. Cell *67*, 1121–1129.

Bijsterbosch, M.K., and Klaus, G.G. (1985). Crosslinking of surface immunoglobulin and Fc receptors on B lymphocytes inhibits stimulation of inositol phospholipid breakdown via the antigen receptor. J. Exp. Med. *162*, 1825–1836.

Bolland, S., and Ravetch, J.V. (1998). Inhibitory pathways triggered by ITIM-containing receptors. Adv. Immunol. *72*, 149–177.

Bolland, S., Pearse, R.N., Kurosaki, T., and Ravetch, J.V. (1998). SHIP modulates immune receptor responses by regulating membrane association of Btk. Immunity *8*, 509–516.

Chacko, G.W., Tridandapani, S., Damen, J.E., Liu, L., Krystal, G., and Coggeshall, K.M. (1996). Negative signaling in B lymphocytes induces tyrosine phosphorylation of the 145-kDa inositol polyphosphate 5-phosphatase, SHIP. J. Immunol. *157*, 2234–2238.

Chan, P.L., and Sinclair, N.R.S.C. (1971). Regulation of the immune response: V. An analysis of the function of the Fc portion of antibody in suppression of an immune response with respect to interaction with components of the lymphoid system. Immunology *21*, 967–987.

Chen, C.L.H., Lehmeyer, J.E., and Cooper, M.D. (1982). Evidence for an IgD homologue on chicken lymphocytes. J. Immunol. *129*, 2580–2585.

Clynes, R., Dumitru, C., and Ravetch, J.V. (1998). Uncoupling of immune complex formation and kidney damage in autoimmune glomerulonephritis. Science *279*, 1052–1054.

Clynes, R., Maizes, J.S., Guinamard, R., Ono, M., Takai, T., and Ravetch, J.V. (1999). Modulation of immune complex-induced inflammation in vivo by the coordinate expression of activation and inhibitory Fc receptors. J. Exp. Med. *189*, 179–185.

Cumano, A., and Rajewsky, K. (1986). Clonal recruitment and somatic mutation in the generation of immunological memory to the hapten NP. EMBO J. *5*, 2459–2468.

Daeron, M. (1997). Fc receptor biology. Annu. Rev. Immunol. *15*, 203–234.

Damen, J.E., Liu, L., Rosten, P., Humphries, R.K., Jefferson, A.B., Majerus, P.W., and Krystal, G. (1996). The 145-kDa protein induced to associate with Shc by multiple cytokines is an inositol tetraphosphate and phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase. Proc. Natl. Acad. Sci. USA *93*, 1689–1693.

Derijard, B., Hibi, M., Wu, I.H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R.J. (1994). JNK1: a protein kinase stimulated by UV light and Ha-ras that binds and phosphorylates the c-Jun activation domain. Cell *76*, 1025–1037.

Eisen, H.N., and Siskind, G.W. (1964). Variations in affinities of antibodies during the immune response. Biochem. *3*, 996–1008.

Foote, J., and Milstein, C. (1991). Kinetic maturation of an immune response. Nature *352*, 530–532.

Goodman, P.A., Niehoff, L.B., and Uckun, F.M. (1998). Role of tyrosine kinases in induction of the c-jun proto-oncogene in irradiated B-lineage lymphoid cells. J. Biol. Chem. *273*, 17742–17748.

Graham, I.L., Lefkowith, J.B., Anderson, D.C., and Brown, E.J. (1993). Immune complex-stimulated neutrophil LTB4 production is dependent on beta 2 integrins. J. Cell. Biol. *120*, 1509–1517.

Healy, J.I., and Goodnow, C.C. (1998). Positive versus negative signaling by lymphocyte antigen receptors. Annu. Rev. Immunol. *16*, 645–670.

Helgason, C.D., Damen, J.E., Rosten, P., Grewal, R., Sorensen, P., Chappel, S.M., Borowski, A., Jirik, F., Krystal, G., and Humphries, R.K. (1998). Targeted disruption of SHIP leads to hemopoietic perturbations, lung patholgy, and a shortened life span. Genes Dev. *12*, 1610–1620.

Ip, Y.T., and Davis, R.J. (1998). Signal transduction by the c-jun

N-terminal kinase (JNK)—from inflammation to development. Curr. Opin. Cell Biol. *10*, 205–219.

Ishiai, M., Kurosaki, M., Pappu, R., Okawa, K., Ronko, I., Fu, C., Shibata, M., Iwamatsu, A., Chan, A.C., and Kurosaki, T. (1999). BLNK required for coupling syk to plcγ2 and Rac1-JNK in B cells. Immunity *10*, 117–125.

Jacob, J., Kelsoe, G., Rajewsky, K., and Weiss, U. (1991). Intraclonal generation of antibody mutants in germinal centers. Nature *354*, 389–392.

Jerne, N.K. (1951). A study of avidity based on rabbit skin responses to diptheria toxin antitoxin mixtures. Acta Pathol. Microbiol. Scand. *87*, 1–183.

Kelsoe, G. (1996). Life and death in germinal centers (redux). Immunity *4*, 107–111.

Kiener, P.A., Lioubin, M.N., Rohrschneider, L.R., Ledbetter, J.A., Nadler, S.G., and Diegel, M.L. (1997). Co-ligation of the antigen and Fc receptors gives rise to the selective modulation of intracellular signaling in B cells. J. Biol. Chem. *272*, 3838–3844.

Kim, J.T., Schimming, A.W., and Kita, H. (1999). Ligation of FcγRII pivotally regulates survival of human eosinophils. J. Immunol. *162*, 4253–4259.

Kurosaki, T., and Ravetch, J.V. (1989). A single amino acid in the glycosyl phosphatidylinositol attachment domain determines the membrane topology of FcγRIII. Nature *342*, 805–807.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680–685.

Li, T., Tsukada, S., Satterthwaite, A., Havlik, M.H., Park, H., Takatsu, K., and Witte, O.N. (1995). Activation of Bruton's tyrosine kinase by a point mutation in its pleckstrin homology domain. Immunity *2*, 451–460.

Liu, Y.J., Grouard, G., de Bouteiller, O., and Banchereau, J. (1996). Follicular dendritic cells and germinal centers. Int. Rev. Cytol. *166*, 139–179.

Liu, Q., Sasaki, T., Kozieradzki, I., Wakeham, A., Itie, A., Dumont, D.J., and Penninger, J.M. (1999). SHIP is a negative regulator of growth factor receptor-mediated PKB/Akt activation and myeloid cell survival. Genes Dev. *13*, 786–791.

MacLennan, I.C. (1994). Germinal centers. Annu. Rev. Immunol. *12*, 117–139.

Mandel, T.E., Phipps, T.E.R., Abbot, A., and Tew, J.G. (1980). The follicular dendritic cell: long term antigen retention during immunity. Immunol. Rev. *53*, 29–59.

Miller, J., and Germain, R.N. (1986). Efficient cell surface expression of class II MHC molecules in the absence of associated invariant chain. J. Exp. Med. *164*, 1478–1489.

Morgenstern, J.P., and Land, H. (1990). Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res. *18*, 3587–3596.

Muta, T., Kurosaki, T., Misulovin, Z., Sanchez, M., Nussenzweig, M.C., and Ravetch, J.V. (1994). A 13-amino-acid motif in the cytoplasmic domain of Fc gamma RIIB modulates B-cell receptor signaling. Nature *368*, 70–73.

Nossal, G.J. (1994). Negative selection of lymphocytes. Cell *76*, 229–239.

Nossal, G.J., Ada, G.L., and Austin, C.M. (1964). Antigens in immunity. IV. Cellular localization of ^{125}I and ^{131}I-labelled flagella in lymph nodes. Austral. J. Exp. Biol. Med. Sci. *42*, 311–330.

Okada, H., Bolland, S., Hashimoto, A., Kurosaki, M., Kabuyama, Y., Iino, M., Ravetch, J.V., and Kurosaki, T. (1998). Role of the inositol phosphatase SHIP in B cell receptor-induced Ca2+ oscillatory response. J. Immunol. *161*, 5129–5132.

Ono, M., Bolland, S., Tempst, P., and Ravetch, J.V. (1996). Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor Fc-gamma-RIIB. Nature *383*, 263–266.

Ono, M., Okada, H., Bolland, S., Yanagi, S., Kurosaki, T., and Ravetch, J.V. (1997). Deletion of SHIP or SHP-1 reveals two distinct pathways for inhibitory signaling. Cell *90*, 293–301.

Phillips, N.E., and Parker, D.C. (1983). Fc-dependent inhibition of mouse B cell activation by whole anti-mu antibodies. J. Immunol. *130*, 602–606.

Phillips, N.E., and Parker, D.C. (1984). Cross-linking of B lymphocyte Fc gamma receptors and membrane immunoglobulin inhibits anti-immunoglobulin-induced blastogenesis. J. Immunol. *132*, 627–632.

Qin, S., Minami, Y., Hibi, M., Kurosaki, T., and Yamamura, H. (1997a). Syk-dependent and -independent signaling cascades in B cells elicited by osmotic and oxidative stress. J. Biol. Chem. *272*, 2098–2103.

Rajewsky, K. (1996). Clonal selection and learning in the antibody system. Nature *381*, 751–758.

Ravetch, J.V. (1997). Fc receptors. Curr. Opin. Immunol. *9*, 121–125.

Ravetch, J.V., Luster, A.D., Weinshank, R., Kochan, J., Pavlovec, A., Portnoy, D.A., Hulmes, J., Pan, Y.C., and Unkeless, J.C. (1986). Structural heterogeneity and functional domains of murine immunoglobulin G Fc receptors. Science *234*, 718–725.

Scharenberg, A.M., El-Hillal, O., Fruman, D.A., Beitz, L.O., Li, Z., Lin, S., Gout, I., Cantley, L.C., Rawlings, D.J., and Kinet, J.P. (1998). Phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P3)/Tec kinase-dependent calcium signaling pathway: a target for SHIP-mediated inhibitory signals. EMBO J. *17*, 1961–1972.

Siskind, G.W., and Benacerraf, B. (1969). Cell selection by antigen in the immune response. Adv. Immunol. *10*, 1–50.

Sylvestre, D.L., and Ravetch, J.V. (1994). Fc receptors initiate the Arthus reaction: redefining the inflammatory cascade. Science *265*, 1095–1098.

Takai, T., Ono, M., Hikida, M., Ohmori, H., and Ravetch, J.V. (1996). Augmented humoral and anaphylactic responses in Fc gamma RII-deficient mice. Nature *379*, 346–349.

Takata, M., Sabe, H., Hata, A., Inazu, T., Homma, Y., Nukada, T., Tamamura, H., and Kurosaki, T. (1994). Tyrosine kinases Lyn and Syk regulate B cell receptor-coupled Ca2+ mobilization through distinct pathways. EMBO J. *13*, 1341–1349.

Takata, M., Homma, Y., and Kurosaki, T. (1995). Requirement of phospholipase C-gamma-2 activation in surface immunoglobulin M-induced B cell apoptosis. J. Exp. Med. *182*, 907–914.

Tonegawa, S. (1983). Somatic generation of antibody diversity. Nature *302*, 575–581.

Tridandapani, S., Kelley, T., Pradhan, M., Cooney, D., Justement, L.B., and Coggeshall, K.M. (1997). Recruitment and phosphorylation of SHIP and Shc to the B-cell Fcgamma ITIM peptide motif. Mol. Cell. Biol. *17*, 4305–4311.

Uckun, F.M., Waddick, K.G., Mahajan, S., Jun, X., Takata, M., Bolen, J., and Kurosaki, T. (1996). BTK as a mediator of radiation-induced apoptosis in DT-40 lymphoma B cells. Science *273*, 1096–1100.

Yamashita, Y., Miyake, K., Miura, Y., Kaneko, Y., Yagita, H., Suda, T., Nagata, S., Nomura, J., Sakaguchi, N., and Kimoto, M. (1996). Activation mediated by RP105 but not CD40 makes normal B cells susceptible to anti-IgM-induced apoptosis: a role for Fc receptor coligation. J. Exp. Med. *184*, 113–120.

Yuasa, T., Kubo, S., Yoshino, T., Ujike, A., Matsumura, K., Ono, M., Ravetch, J.V., and Takai, T. (1999). Deletion of FcγRIIB renders H-2^{b} mice susceptible to collagen-induced arthritis. J. Exp. Med. *189*, 187–194.