Enhancement and suppression of signaling by the conserved tail of IgG memory–type B cell antigen receptors

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Immunological memory is characterized by heightened immunoglobulin (Ig) G antibody production caused in part by enhanced plasma cell formation conferred by conserved transmembrane and cytoplasmic segments in isotype-switched IgG B cell receptors. We tested the hypothesis that the IgG tail enhances intracellular B cell antigen receptor (BCR) signaling responses to antigen by analyzing B cells from Ig transgenic mice with IgM receptors or chimeric IgMG receptors containing the IgG tail segment. The IgG tail segment enhanced intracellular calcium responses but not tyrosine or extracellular signal–related kinase (ERK) phosphorylation. Biochemical analysis and crosses to CD22-deficient mice established that IgG tail enhancement of calcium and antibody responses, as well as marginal zone B cell formation, was not due to diminished CD22 phosphorylation or inhibitory function. Microarray profiling showed no evidence for enhanced signaling by the IgG tail for calcium/calcineurin, ERK, or nuclear factor κB response genes and little evidence for any enhanced gene induction. Instead, almost half of the antigen-induced gene response in IgM B cells was diminished 50–90% by the IgG tail segment. These findings suggest a novel "less-is-more" hypothesis to explain how switching to IgG enhances B cell memory responses, whereby decreased BCR signaling to genes that oppose marginal zone and plasma cell differentiation enhances the formation of these key cell types.

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Abbreviations used: BCR, B cell antigen receptor; ERK, extracellular signal–related kinase; HEL, hen egg lysozyme; ITAM, immunoreceptor tyrosine–based activation motif; MAP, mitogen-activated protein; MEK, MAP kinase/ERK kinase; SHP-1, Src homology domain 2–containing protein tyrosine phosphatase.

Immunological memory—a defining feature of adaptive immunity—is characterized by recall antibody responses that are more rapid, of a higher titer, and dominated by IgG (1, 2). Two factors contribute to this capability: an increased frequency of B cells and T cells with high affinity antigen receptors, and the differentiation of "memory cells" with heightened response capacity. Memory B cells have usually undergone an irreversible rearrangement of their Ig heavy chain genes (isotype switching), which removes the constant region exons encoding IgM and IgD B cell antigen receptors (BCRs) found on naive B cells and replaces them with constant region exons encoding membrane-bound IgG. Although isotype switching preserves antigen specificity, it substantially changes the transmembrane and cytoplasmic segments of the BCR. Membrane IgM and IgD have only three cytoplasmic amino acids and transmit signals into B cells through immunoreceptor tyrosine–based activation motifs (ITAMs) in the cytoplasmic tails of an associated CD79a–CD79b heterodimer (3). Although IgG BCRs also associate and signal through CD79 heterodimers, each of the IgG subtypes has an extended, highly conserved cytoplasmic tail that has long been speculated to confer important signaling differences (3). The nature of IgG BCR signaling differences nevertheless remains obscure.

Genetic manipulation in mice has established that the IgG BCR tail is both necessary and sufficient for the dramatically enhanced secretion of IgG that characterizes immunological memory. Truncation of the IgG cytoplasmic tail or the homologous tail in IgE through gene targeting in mice diminished the secretion of antigen-specific IgG1 or IgE by >10–20-fold

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The Journal of Experimental Medicine

during primary and secondary immunization (4, 5). This result shows that the tail is necessary for high-titer IgG memory responses. Conversely, addition of the IgG1 transmembrane and extended cytoplasmic segments to a hen egg lysozyme (HEL)–specific IgM expressed in B cells of Ig gene transgenic mice (in place of the corresponding IgM tail) increased antibody titers and the formation of plasma cells by 10–50-fold during a primary immune response independent of any change in B cell precursor frequency or affinity (6). These complementary in vivo studies clearly established that the cytoplasmic tail of class-switched BCRs dramatically enhances B cell antibody responses, although the mechanism by which the tail enhances responses or changes BCR signaling remains to be defined.

Recently, the extended IgG tail was found to increase BCR signaling to intracellular calcium and extracellular signal–related kinase (ERK) by bypassing the inhibitory coreceptor CD22 (7). CD22 is a 140-kD type I membrane protein on B cells, comprising extracellular Ig domains with lectin-like binding to sialic acid–modified proteins and an intracellular domain containing three immunoreceptor tyrosine–based inhibition motifs and two ITAM-like regions (8, 9). CD22 is physically associated with not only IgM but also IgD or IgG (10, 11). After the engagement of BCR, CD22 is tyrosine phosphorylated (12, 13). Studies using Lyn-deficient mice suggested that Lyn is mainly involved in CD22 tyrosine phosphorylation after BCR cross-linking (14, 15). Phosphorylated CD22 recruits a negative regulator, Src homology domain 2–containing tyrosine phosphatase 1 (SHP-1) (16). Consequently, B cells from CD22-deficient mice exhibit an augmented calcium response upon BCR cross-linking. Strikingly, CD22 phosphorylation failed to occur in B cell lymphoma cell lines expressing hapten-specific IgG BCRs or chimeric IgMG receptors with IgG cytoplasmic tails, whereas CD22 was normally phosphorylated when IgM BCRs were expressed by the cells (7). The failure to phosphorylate CD22 was accompanied by the failure to recruit SHP-1 and dramatically augmented calcium response and ERK activation. These studies have suggested that the heightened secretion of IgG in memory responses in vivo can be explained by the sequestration of CD22 from IgG, resulting in a generalized increase in BCR signaling to antigen. However, the extent to which in vivo antibody responses are negatively regulated by CD22 is unclear (17–20). Two groups showed a decreased proliferative response to BCR stimulation in CD22-deficient B cells, one group found no difference, and a fourth study found hyperresponsiveness. One study showed augmented antibody formation upon T cell–dependent immunization of CD22-deficient mice, whereas others found a normal magnitude of response. Hence, it is important to investigate whether or not the IgG tail confers heightened antibody responses by exaggerating BCR signaling through a CD22 bypass mechanism.

In this paper, we analyze the effect of the IgG memory tail on BCR signaling in primary HEL antigen-specific B cells, as well as the role of CD22 in the heightened antibody production conferred by the IgG tail. The data show that the IgG tail confers a higher Ca^{2+} response and augmented

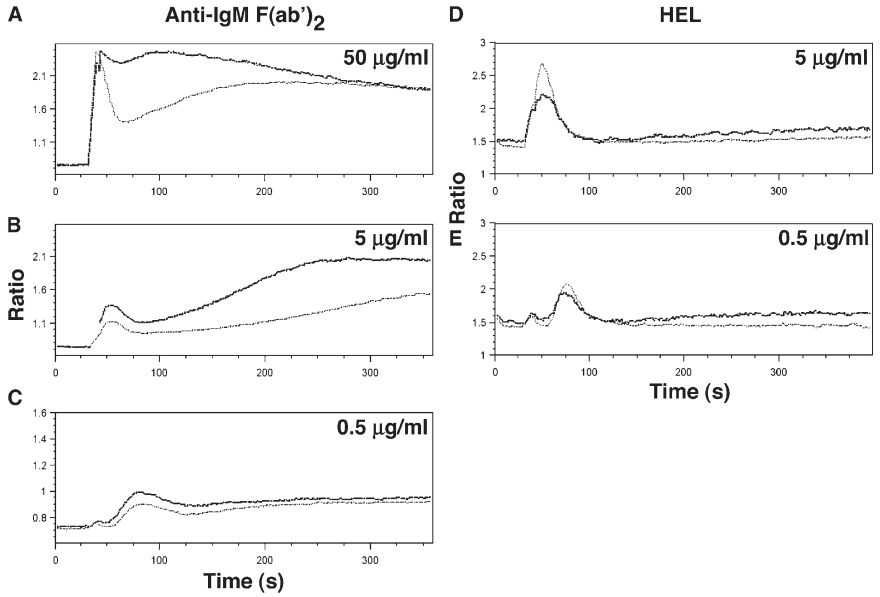


Figure 1. IgG membrane tail BCR induces exaggerated Ca^{2+} influx. RBC-depleted splenocytes from IgMG (continuous line) and IgM (dotted line) mice were labeled with 1 μM Indo-1 for 30 min at 37°C. The cells were counterstained with antibodies against B220 and CD21 for the final 10 min of Indo-1 staining. The stained cells were first acquired for 30 s on an LSR flow cytometer and then stimulated with anti-IgM $F(ab')_2$ antibody (A, 50 μg/ml; B, 5 μg/ml; and C, 0.5 μg/ml) or HEL (D, 5 μg/ml; and E, 0.5 μg/ml; both represent saturating concentrations), and the increase in intracellular calcium was revealed by the change in the mean Indo-1 violet/blue ratio of the $B220^{+}CD21^{medium}$ follicular B cell population. Data are representative of three experiments.

antibody production in a CD22-independent manner. Moreover, the IgG tail does not enhance BCR signaling to ERK or to global gene expression, but instead half of IgM-induced genes are poorly induced by BCRs with the IgG tail. These findings reveal surprising qualitative differences in BCR signaling conferred by the IgG tail, provide an explanation for the IgG tail–induced differences in immature and marginal zone B cell development, and suggest novel explanations for the heightened antibody secretion in immunological memory.

RESULTS

IgG membrane tail BCR induces exaggerated Ca^{2+} influx

To analyze the effect of the IgG tail on BCR signaling in primary B cells, we took advantage of two well-matched Ig-transgenic strains of mice in which the B cells express identical BCRs with homogeneously high affinity for HEL, with the sole difference being that in the IgM strain the BCR is native IgM, whereas in the IgMG strain the coding portions of the transmembrane and cytoplasmic exons are from IgG1 (21). Secreted IgM is identical in the two strains, as are all of the intronic and 3′ untranslated regions. We have previously shown that this precise substitution in the membrane tail of the BCR is sufficient to confer the in vivo antibody response–enhancing effects seen in follicular B cells from a third set of Ig-transgenic strains expressing the native $IgG1^{HEL}$ BCR (6). Because the B cells in the IgM and IgMG mice are so closely matched in all respects other than the BCR tail, they provided an ideal experimental system to investigate how the IgG tail alters BCR signaling.

We first examined intracellular calcium mobilization after BCR stimulation. Cross-linking by $F(ab')_2$ anti-IgM antibody induced a rapid calcium influx, followed by a sustained elevation of intracellular calcium level in IgM B cells (Fig. 1, A–C). IgMG B cells showed a slightly larger initial calcium spike in the high dose of stimulation (Fig. 1 A) and a higher sustained calcium level in both high and low doses of stimulation (Fig. 1, A–C) compared with IgM B cells. A similar trend was found for HEL stimulation (Fig. 1, D and E). Exaggerated calcium response to BCR engagement was also consistently found in IgMG B cell responses to other BCR ligands (anti-kappa antibody or HEL conjugated to dextran), in immature $B220^{low}$ B cells from the bone marrow, and in B cells from IgG1 transgenic mice in which the entire constant region is switched (unpublished data). The IgG tail thus enhances intracellular calcium response across a broad range of antigen receptor occupancies and degrees of receptor cross-linking.

In contrast to the exaggerated calcium response, however, the IgG tail did not alter tyrosine phosphorylation of total proteins and CD22, nor was there any increase in phosphorylation of ERK (Fig. 2). Cross-linking of BCR with $F(ab')_2$ anti-IgM antibody, at an optimal concentration that caused a marked difference in intracellular calcium, induced comparable patterns and magnitudes of tyrosine phosphorylation of total cellular proteins in IgM and IgMG B cells (Fig. 2 A). CD22 was tyrosine phosphorylated in IgM and IgMG B cells with similar kinetics and extent in response to $F(ab')_2$ anti-IgM or HEL stimuli that caused heightened calcium responses (Fig. 2 B and Fig. S1, available at htttp://www.jem.org/cgi/content/full/jem.20061923/DC1). ERK activation upon BCR ligation was measured by intracellular staining with a phospho-ERK–specific antibody. Phosphorylation of ERK was detected clearly after BCR ligation with $F(ab')_2$ anti-IgM or HEL with identical kinetics and magnitude in IgM and IgMG B cells (Fig. 2 C and not depicted). The induction of ERK phosphorylation was almost completely inhibited by the mitogen-activated protein (MAP) kinase/ERK kinase (MEK) inhibitors PD98059 or U0126 (Fig. 2 D), indicating the specificity of the antibody used for the experiment. These results indicate that the IgG tail does not dramatically alter the broad pattern of BCR signaling in primary B cells and that the effect on calcium appears quite specific.

IgG tail BCR interferes with the induction of antigen response genes

To obtain a global view of the extent to which the IgG tail changes BCR signaling to antigen, we employed a microarray strategy previously used to reveal qualitative differences in

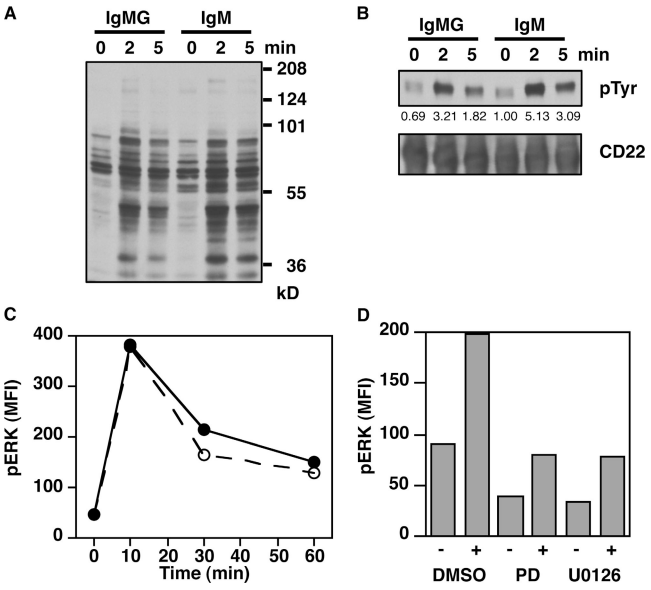
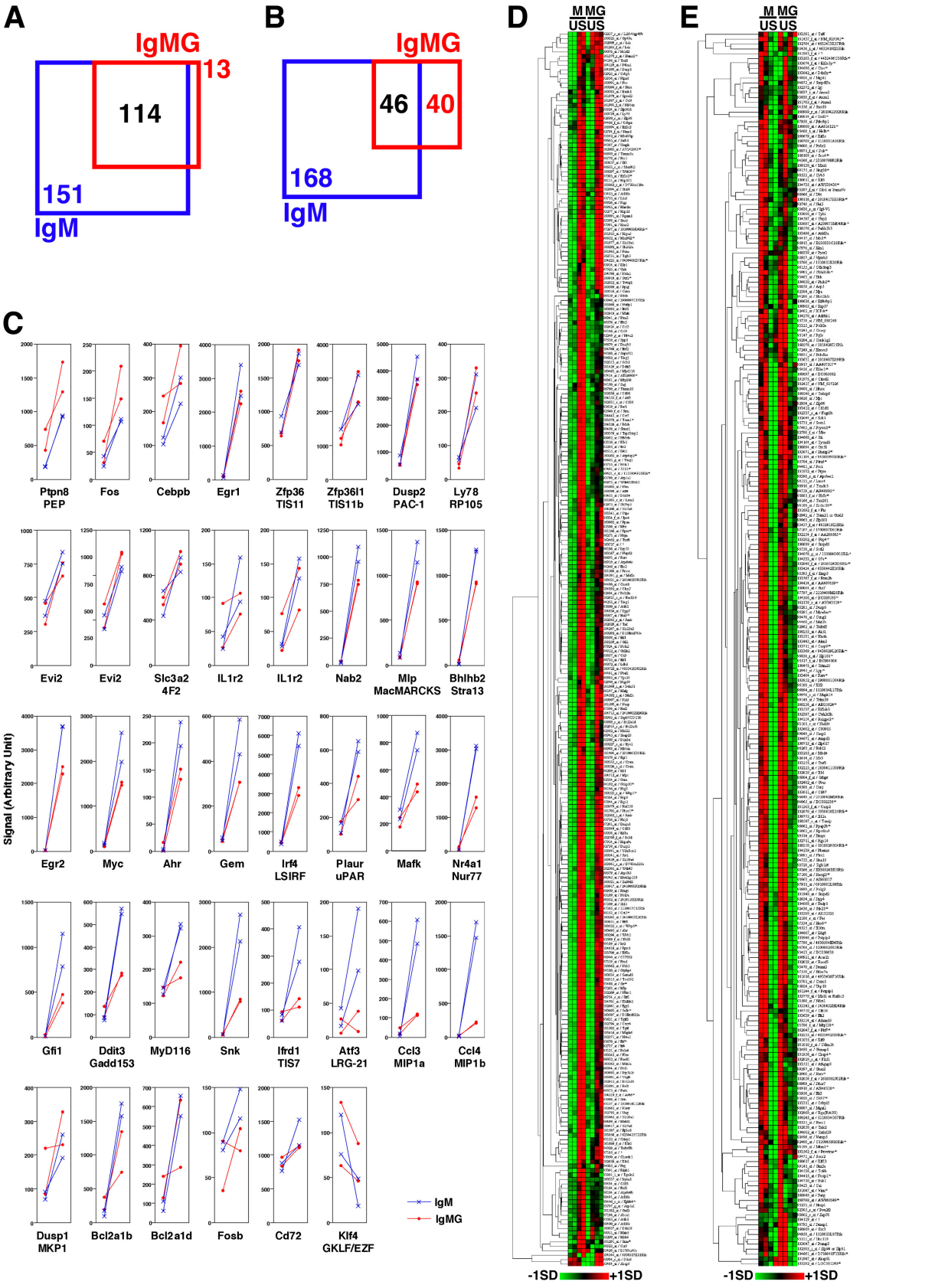


Figure 2. IgG membrane tail does not alter tyrosine phosphorylation of CD22 or ERK phosphorylation. Splenocytes from IgM and IgMG transgenic mice were stimulated with 50 μg/ml anti-IgM $F(ab')_2$. Total cellular proteins (A) or immunoprecipitated CD22 (B) were fractionated by SDS-PAGE and Western blotted with antiphosphotyrosine antibody (A and B, top) or anti-CD22 antibody (B, bottom). The ratios of phosphorylated CD22 to total CD22 are indicated. (C and D) Mean fluorescence intensity (MFI) of permeabilized $B220^{+}CD21^{medium}CD23^{+}$ follicular B cells stained by flow cytometry for phosphorylated ERK either (C) at the indicated times after stimulation with 50 μg/ml anti-IgM $F(ab')_2$ or (D) in unstimulated (−) versus stimulated (+) cells after 2 min in the presence of MEK inhibitors PD98059, U0126, or DMSO as diluent controls. Data are representative of two experiments.


A
IgMG
13
114
151
IgM
B
IgMG
46
40
168
IgM
C
Signal (Arbitrary Unit)
Ptpn8 PEP
Fos
Cebpb
Egr1
Zfp36 TIS11
Zfp36l1 TIS11b
Dusp2 PAC-1
Ly78 RP105
Evi2
Evi2
Slc3a2 4F2
IL1r2
IL1r2
Nab2
Mlp MacMARCKS
Bhlhb2 Stra13
Egr2
Myc
Ahr
Gem
Irf4 LSIRF
Plaur uPAR
Mafk
Nr4a1 Nur77
Gfi1
Ddit3 Gadd153
MyD116
Snk
Ifrd1 TIS7
Atf3 LRG-21
Ccl3 MIP1a
Ccl4 MIP1b
Dusp1 MKP1
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Bcl2a1d
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Klf4 GKLF/EZF
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BCR signaling in primary HEL-specific IgM/IgD B cells caused by clonal anergy or by pharmacological inhibition of calcium, calcineurin, or MEK/ERK (22). To eliminate any contribution from the serum response genes observed previously when we compared profiles of fresh ex vivo B cells with 1-h cultured cells (23), IgM and IgMG B cells were stimulated in vivo by intraperitoneal injection of a dose of HEL antigen that—like the concentrations of HEL used in vitro—rapidly saturated antigen receptors and gave robust, synchronous CD69 and CD86 induction on Ig-transgenic B cells (unpublished data). Spleen cell suspensions were prepared 1 h later, follicular B cells were sorted as $CD21^{medium}$ and $CD23^{+}$, and mRNA was purified immediately. Multiple independent sorting experiments were performed from stimulated and unstimulated control IgM and IgMG mice, and the mRNA from independent experiments was combined into one or the other of two independent pools for each of the four conditions. Labeled cRNA from each of the eight independent pools was analyzed on Affymetrix Murine Genome Array U74Av2 chips.

We initially focused our analysis on the set of pharmacologically profiled BCR response genes previously defined in naive IgM/IgD B cells stimulated by HEL for 1 h in vitro. This set comprised 35 HEL-induced and 21 HEL-repressed genes that are also tiled on the U74Av2 chip used in this study. After 1 h of in vivo HEL stimulation of IgM B cells, 34 out of the 35 HEL-induced genes were reproducibly increased compared with unstimulated IgM B cells, and 15 out of the 21 HEL-repressed genes were decreased (Fig. 3 C and Fig. S2, available at htttp://www.jem.org/cgi/content/full/jem.20061923/DC1). Examining the response of IgM and IgMG B cells, only 3 out of the 35 HEL-induced genes (*Fos*, *Ptpn8*, and *Cebpb*) and 0 out of the 21 HEL-repressed genes (Fig. S2 D) showed any evidence of increased signaling by the IgG tail, and these effects were subtle (Fig. 3 C). 19 out of the 34 HEL-induced genes in IgM B cells were either equally increased by HEL signaling through IgMG (*Egr1*, *Tis11*, *Tis11b*, *Dusp2*, *Rp105*, *Evi2*, *4F2*, *IL1r2*, and *Dusp1*) or were slightly less well induced in IgMG B cells (*Nab2*, *MacMARCKS*, *Stra13*, *Egr2*, *Myc*, *AhR*, *Gem*, *Bcl2a1b/A1*, *Fosb*, and *Cd72*). These included established reporters for the ERK pathway (*Egr1*, *Nab2*, and *Dusp2/Pac1*), the calcium–nuclear factor of activated T cells pathway (*Egr2* and *CD72*), and the NF-κB pathway (*c-myc* and *A1/Bcl2a1b*). All 15 of the HEL-repressed genes in IgM cells were equally decreased in IgMG cells (Fig. S2 D). These data confirm that the IgM and IgMG cells had been equally stimulated by antigen, and that the IgG tail does not cause a global increase in BCR signaling.

In contrast, 12 out of the 34 IgM HEL response genes were poorly induced in IgMG cells, attaining levels that were only 10–50% of those in IgM cells (*Irf4*, *uPAR*, *Mafk*, *Nur77*, *Gfi1*, *Gadd153*, *Myd116*, *Snk*, *Tis7*, *Atf3*, *Ccl3*, and *Ccl4*). The poorly induced genes did not correspond to any particular BCR signaling pathway, nor did they correspond to genes that are poorly induced in anergic B cells. For example, although *Ccl3*, *Ccl4*, and *Myd116* were previously shown to be completely calcium/calcineurin dependent and not induced in anergic B cells (22), other genes with this profile were induced in IgMG B cells (e.g., *Tis11b*, *Evi2*, *Ptpn8*, *MacMARCKS*, and *A1/Bcl2A1b*). Similarly, *mafK* was poorly induced in IgMG cells and corresponds to a subset that is MEK dependent but blocked in anergic B cells, implying a requirement for ERK signaling plus one of the blocked signaling pathways in anergic cells, such as NF-κB or c-Jun N-terminal kinase. However, other genes in this category are induced normally (*Tis11b* and *MacMARCKS*). Based on these data, we conclude that a chief effect of the IgG tail is to diminish BCR signaling for a selected subset of the normal response, but this qualitative change does not correspond to any known pharmacological and functional BCR signaling profile.

Because this analysis was limited to response genes defined previously in IgM/IgD B cells, we next extended it globally to include all of the genes on the U74 microarray and identified those showing a reproducible HEL response in either IgM or IgMG B cells. HEL stimulation increased the expression of 265 genes in IgM B cells, but of these only 114 were substantially increased in IgMG B cells, thus extending the observations made with the smaller set (Fig. 3, A and D). Among the additional genes that were strongly induced in IgM B cells but either not induced or only very poorly in IgMG B cells were genes encoding chemokine receptors *Ccr9* and *Ccr7*, the cell surface lectin *Cd83*, the NF-κB signaling regulator *IκBβ*, and the transcription factor *Atf4*. Also consistent with this evidence, only 13 genes showed evidence of greater induction in IgMG B cells compared with IgM cells,

Figure 3. The IgG tail does not confer an enhanced gene expression response but blunts a subset of the IgM response. IgM and IgMG follicular B cells were stimulated (S) with 5 mg HEL for 1 h in vivo or were unstimulated (U) and then flow sorted based on CD21 and CD23 staining. Purified RNA from multiple experiments was combined into two independent pools for each stimulation condition and analyzed on Affymetrix U74A microarrays. Increased and decreased genes were defined as those with more than twofold induction or suppression, respectively, and present in both stimulated samples or in both unstimulated samples, respectively. (A and B) Schematic comparing the total number and overlap of genes with increased (A) or decreased (B) expression upon antigen stimulation. The sizes of squares indicate the number of genes. (C) Expression of genes previously defined as induced by HEL stimulation of naive IgM/IgD B cells (references 22, 23). The left end of the line represents the signal of gene expression in unstimulated cells; the right end represents the same in cells stimulated with HEL for 1 h. Each line represents one experiment. Blue line (crosses), IgM B cells; red line (circles), IgMG B cells. (D and E) Hierarchical clustering on all induced (D) or suppressed (E) genes. Red denotes the increased expression relative to the average of all samples, whereas green denotes decreased expression relative to the average. Black denotes values near the average of all samples. Note that approximately half of the response genes in stimulated IgM B cells are induced to lower levels in IgMG B cells.

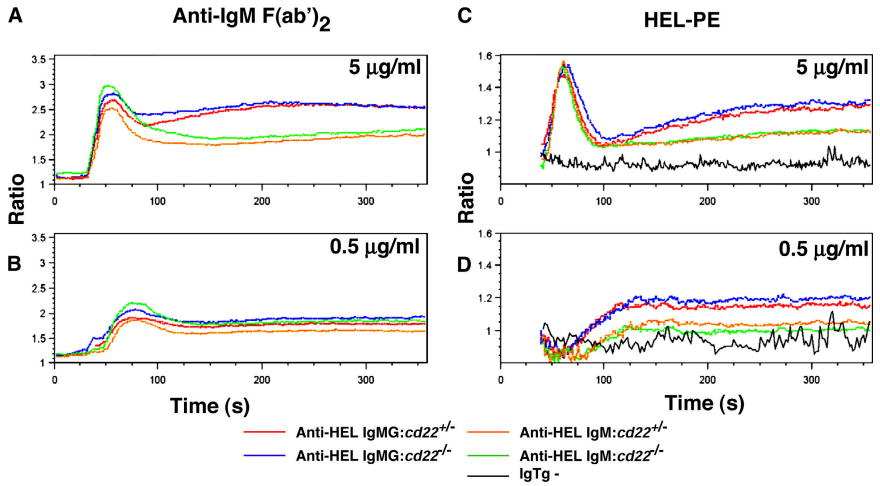

Figure 4. IgG membrane tail evokes an augmented Ca^{2+} response independent of CD22. RBC-depleted splenocytes from mice of the indicated genotypes were labeled with 1 μM Indo-1 for 30 min at 37°C. (A and B) The cells were counterstained with antibodies against B220 and CD21 for the final 10 min of Indo-1 loading. The stained cells were at first acquired for 30 s and then stimulated with the indicated concentrations of anti-IgM $F(ab')^2$ antibody (A, 5 μg/ml; and B, 0.5 μg/ml). Lines show the mean Indo-1 ratio in $B220^+CD21^{medium}$ cells. Data from one out of three independent experiments are shown. (C and D) Indo-1–loaded cells stained with antibody against B220 were simulated with the indicated concentrations of HEL conjugated to PE (HEL-PE; C, 5 μg/ml; and D, 0.5 μg/ml), and the Indo-1 ratio was measured on gated HEL^+ $B220^+$ cells.

and these effects were marginal for most, except *Akap9*, *Ddx6*, and *4930517K11Rik*, where the IgM response appeared to be in the opposite direction (Fig. S2 B). These results indicate that the IgG tail does not increase BCR signaling for gene expression, and establish that its predominant effect is a graded decrease affecting a substantial subset of response genes that do not match currently established BCR signaling pathways in any straightforward manner.

IgG membrane tail increases calcium flux and antibody production independent of CD22

We next crossed CD22-deficient mice with IgM or IgMG transgenic mice to test the possibility that differences in CD22 activity might explain the heightened calcium response to antigen in B cells with IgG tail BCRs or the heightened T cell–dependent antibody response in vivo. CD22 deficiency caused a slight decrease in BCR density on follicular B cells of IgM and IgMG transgenic mice (unpublished data). Consistent with published data in nontransgenic mice (17–20), intracellular Ca^{2+} elevation was slightly but consistently increased in CD22-deficient IgM and IgMG B cells compared with CD22-expressing counterparts over a range of anti-IgM $F(ab')_2$ antibody concentrations (Fig. 4, A and B). The effect of CD22 deficiency was subtle compared with the effect of the IgG tail, but this subtle enhancement of calcium response was also caused by CD22 deficiency in IgMG B cells, indicating that CD22 has a comparable inhibitory effect on signaling by BCRs with the IgG tail. Moreover, when IgM and IgMG B cells were stimulated with HEL antigen, there was no exaggeration of intracellular calcium response caused by CD22 deficiency, whereas the IgG tail again markedly enhanced calcium responses (Fig. 4, C and D). The absence of any effect of CD22 deficiency on Ca^{2+} response to HEL antigen was also observed in IgMD B cells (i.e., B cells coexpressing IgM and IgD) (24) (Fig. S3, available at htttp://www.jem.org/cgi/content/full/jem.20061923/DC1). Thus, the IgG tail enhances BCR-induced calcium responses via a mechanism that does not involve differences in the activity of CD22.

Because the most dramatic effect of the IgG tail is observed in T cell–dependent antibody responses in vivo, where it increases B cell differentiation into plasma cells approximately 50-fold (6), we tested whether or not this was explained by differential action of CD22. Equal numbers of HEL-binding IgM or IgMG B cells, expressing or lacking CD22, were adoptively transferred into nonirradiated C57BL/6 mice, and the recipient mice were immunized with lysozyme in adjuvant. The concentration of HEL-reactive IgM^a in the serum was measured 10 d after immunization (Fig. 5). As previously observed (6), $CD22^+$ IgMG B cells produced 10–50 times more antibody than $CD22^+$ IgM B cells. CD22 deficiency did not increase antibody secretion by IgM or IgMG B cells, and there was an equally large difference between responses from IgM and IgMG B cells on the CD22-deficient background, establishing that the IgG tail increases antibody responses by a mechanism that is independent of CD22.

CD22 deficiency does not suppress marginal zone B cell differentiation when the BCR carries the IgG tail

Differentiation of splenic marginal zone B cells has been suggested to require a lower level of BCR signaling compared with differentiation of follicular B cells (25), although other studies indicate that greater BCR signaling correlates with marginal zone cell differentiation (26, 27). Enhancement of BCR signaling caused by CD22 deficiency thus represents one explanation for the suppressed formation of marginal

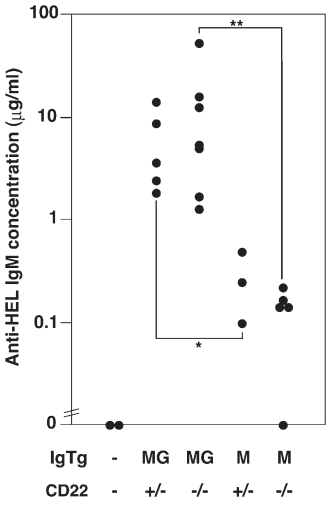

Figure 5. IgG membrane tail increases antibody production independent of CD22. 5×10^5 HEL-binding splenic B cells from IgMG or IgM transgenic donors of the indicated CD22 genotypes were adoptively transferred into nonirradiated C57BL/6 mice, and the recipient mice were immunized with HEL in CFA. The concentration of anti-HEL IgM^a antibody 10 d after immunization was measured in the serum of individual recipient mice (circles) by ELISA. Data are representative of three separate experiments. Significant differences were determined by the Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$.

zone but not follicular B cells in CD22-deficient mice (20, 28). Thus, we examined differentiation of HEL-specific B cells into these two subsets in $CD22^{+/-}$ and CD22-deficient IgM and IgMG mice by staining for CD21/CD23 or CD21/CD1d (Fig. 6). In $CD22^{+/-}$ IgM transgenic mice, 4.69 ± 0.97% of HEL-binding B cells were marginal zone B cells. This population was decreased 10-fold (0.48 ± 032%) in CD22-deficient IgM transgenic mice, consistent with published data in mice with a polyclonal BCR repertoire. As noted previously in IgG and IgMG Ig^{HEL} transgenic mice (6, 21), exchange of the IgM tail for the IgG tail dramatically increased the formation of marginal zone B cells to account for 39.4 ± 7.4% of HEL-binding B cells in $CD22^{+/-}$ IgMG animals. Remarkably, CD22 deficiency had no effect on IgMG B cell differentiation into marginal zone cells, as these still accounted for 36.4 ± 7.3% of HEL-binding B cells in $CD22^{-/-}$ IgMG animals. As discussed in the next section, the enhanced formation of marginal zone B cells even in the absence of CD22 is consistent with lower signaling by BCRs with the IgG tail.

DISCUSSION

The experiments described in this paper reveal striking qualitative differences in BCR signaling and responses to antigen that are conferred solely by exchanging the transmembrane region and short cytoplasmic tail of IgM for the corresponding regions of IgG1, which contain a longer cytoplasmic tail that is highly conserved between IgG subtypes and species. It has previously been shown that the IgG tail segment is necessary for high-titer IgG secretion in a polyclonal repertoire (4, 5) and sufficient to confer the dramatic enhancement in plasma cell formation and T cell–dependent antibody secretion that is observed when the entire constant region is switched from IgM to IgG1 (6). Logically it might be expected that the IgG tail enhances antibody responses by increasing BCR signaling to proimmunity pathways, either by recruiting additional signaling components or by avoiding inhibitory countersignals such as CD22 and SHP-1. The data in this study show that the IgG tail does enhance intracellular calcium responses (Fig. 1), but this is not because of any difference in activity of the inhibitory receptor CD22 (Fig. 2 and Fig. 4), nor is it reflected in a general increase in protein tyrosine phosphorylation, ERK signaling, or induction of gene expression in the nucleus (Fig. 2 and Fig. 3). At the level of gene expression responses induced by antigen, the chief effect of the IgG tail is to selectively decrease a large subset of the response (Fig. 3). This qualitatively diminished signaling for gene expression is paralleled by the effects of the IgG tail on differentiation into marginal zone B cells, which is enhanced by the tail and no longer blocked when BCR signaling is exaggerated by CD22 deficiency. These findings, together with complementary data from studies of IgG knock-in mice by Waisman et al. (see Waisman et al. [29] on p. 747 of this issue), raise an alternative hypothesis for the action of the tail, namely that it qualitatively alters BCR signaling to diminish gene expression responses that normally oppose marginal zone differentiation, extrafollicular B cell proliferation, and differentiation into plasma cells.

In this study, we found that the heightened antibody production in memory B cells is not caused by a general increase in BCR signaling secondary to sequestration of the inhibitory receptor CD22 from BCRs with the IgG tail. Consistent with published data in lymphoma cell lines (7) and with the accompanying study of primary B cells in IgG knock-in mice (29), the IgG tail increased the intracellular Ca^{2+} response of primary follicular or immature B cells to antireceptor antibodies or to antigen (Fig. 1 and Fig. 4). However, based on the data from crossing CD22-deficient mice to IgMG or IgM transgenic mice (Fig. 4), we conclude, as do Waisman et al. (29), that in primary B cells the IgG tail enhances intracellular calcium responses through a route that is unrelated to CD22. Equally, the data in Fig. 5 showed that the dramatic enhancement of T cell–dependent antibody responses conferred by the IgG tail was unrelated to CD22. These results are consistent with the findings of three groups out of four that show normal thymus- dependent antibody responses in CD22-deficient mice (17, 19, 20), although one group has reported augmented thymus-dependent response (18). It is worth noting that our previous sorting experiments established that the enhanced antibody responses by IgMG and IgG B cells are derived from follicular cells and not from the expanded subset of marginal zone B cells (6).

What has brought about the discrepancy between Wakabayashi et al. (7) and the findings in this study and Waisman et al. (29)? Wakabayashi et al. studied an IgG2a/λ

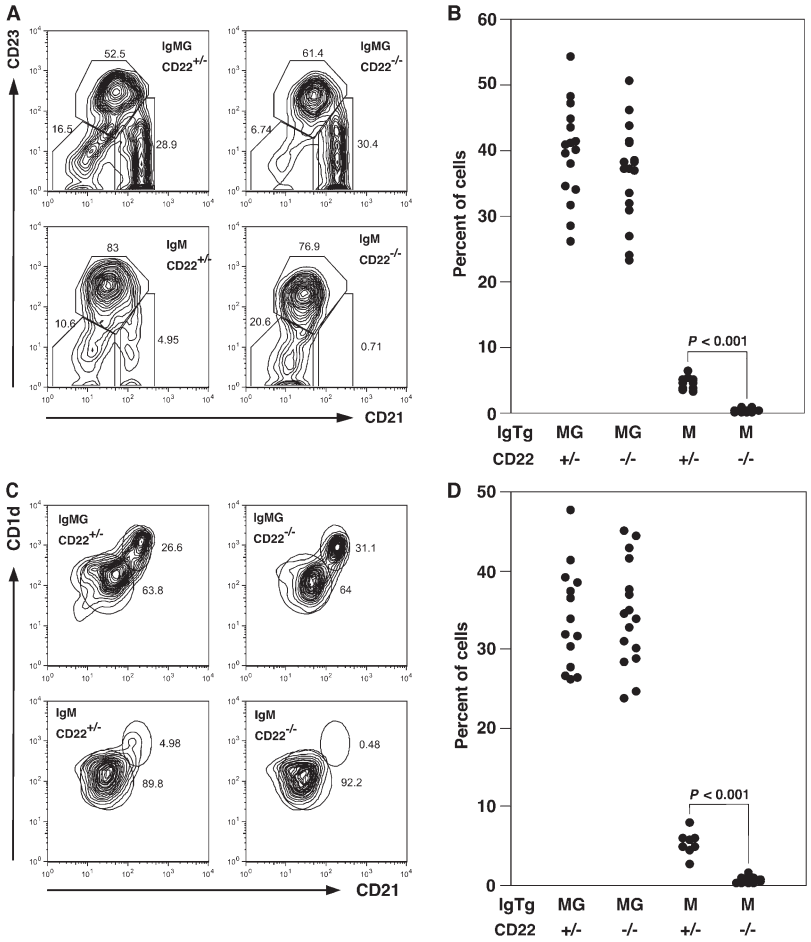

Figure 6. CD22 deficiency suppresses IgM but not IgMG marginal zone B cell differentiation. Splenocytes were stained with HEL and combinations of antibodies against CD21/CD23/HyHEL9/B220 (A and B) or CD21/CD1d/HyHEL9/B220 (C and D). (A and C) The displayed profiles are gated on $B220^+$ HEL-binding cells, and the percentage of $B220^+$ HEL-binding cells in each window is shown. (B and D) Percentages of HEL-binding B cells in the marginal zone subset in individual mice (circles). Significant differences, as determined by the Student's *t* test, are indicated.

BCR specific for the hapten NP expressed mostly in the B lymphoma line K46 and stimulated with NP-BSA antigen. It is unlikely that this discrepancy can be explained by the difference in isotypes, because the cytoplasmic regions of IgG2a and IgG1 have high sequence similarity (3). In terms of BCR stimulation, we used two different types of stimulants, anti-IgM antibody or the antigen HEL for the CD22 tyrosine phosphorylation experiment, resulting in similar results. They used other B cell lines, such as WEHI-279, BAL17, and A20 and even LPS-stimulated primary B cells, as well as K46. However, we speculate that it can be most likely caused by the difference between cell lines and primary cells or a specific characteristic of the NP-reactive BCR.

How does the IgG tail enhance the antigen-induced calcium response, and how might this relate to the enhanced formation of plasma cells? A recent report showed that CD22 attenuated calcium signaling by physical association with plasma membrane calcium–ATPase in a BCR stimulation–dependent way (30). However, the comparison of CD22-deficient and wild-type B cells in this paper establish that the exaggerated calcium response conferred by the IgG tail cannot be caused by an effect of CD22 on plasma membrane calcium–ATPase. It is conceivable that in primary B cells, the IgG tail might enable the BCR to escape calcium-attenuating effects of coreceptors other than CD22, or it may enhance the open state of other Ca^{2+} channels, such as calcium release–activated Ca^{2+} (CRAC) channels, voltage-gated L-type Ca^{2+} channels, or transient receptor potential channels. The enhanced calcium response caused by the IgG tail is nevertheless not reflected in the profile of genes induced by antigen. For example, the HEL-induced genes *Egr2*, *Cd72*, *Tis11b*, *Ccl4*, *Ccl5*, *Ptpn8*, *MacMARCKS*, *Myd116*, *Ebi2*, *A1*, and *Evi2* are all calcium/calcineurin dependent (22), yet only one of these genes (*Ptpn8*) shows marginal evidence of increased induction in IgMG cells, whereas the rest are either induced equally or much less. Instead of enhancing the induction of calcium response genes, it is possible that the main effect of the enhanced calcium is to activate inhibitory signaling circuits.

We also found almost no difference in ERK phosphorylation between control and $CD22^{-/-}$ IgM/IgD B cells

(unpublished data), which is in line with reports using $CD22^{-/-}$ mice that showed normal ERK activation (31, 32). Analysis of BCR signaling to the MAP kinase ERK and the global pattern of antigen-induced genes are inconsistent with the hypothesis that the IgG tail causes a generalized enhancement of BCR signaling. In primary B cells with IgM or IgMG BCRs, engagement of these receptors induced comparable ERK phosphorylation (Fig. 2) and comparable induction of the *Egr1* and *Nab2* genes, which are targets of ERK signaling in the nucleus (22). Examining the global pattern of genes that are induced by HEL also clearly indicates that the IgG tail enhances very few gene responses, whereas it diminishes induction of almost 50% of the response genes.

The diminished induction of BCR response genes caused by the IgG tail may explain the dramatic increase in marginal zone B cells in IgMG and IgG mice in this paper and in IgG knock-in mice in the study by Waisman et al. (29). Several lines of evidence indicate that marginal zone B cell differentiation is favored by genetic alterations that diminish BCR signaling strength compared with follicular B cell differentiation (25), although evidence to the contrary also exists (26, 27). Consistent with this idea, CD22 deficiency selectively eliminates marginal zone B cells with a polyclonal BCR repertoire (20, 28) or with HEL-specific IgM BCRs (Fig. 6). The 900% increase in marginal zone B cells caused by the IgG tail, as well as the lack of any reduction when combined with CD22 deficiency despite heightened calcium signaling, may reflect poor induction by the IgG tail of BCR response genes that oppose marginal zone differentiation. In this respect, it is noteworthy that the chemokine receptor CCR7 failed to be induced by antigen in IgMG cells (Fig. 3 D). Induction of CCR7 by BCR engagement attracts IgM/IgD B cells away from the marginal zone and into the T-zone, whereas B cells that lack CCR7 are attracted toward the marginal zone (33, 34). Because marginal zone differentiation also depends on Notch2 signaling, it is also possible that the qualitative changes in BCR signaling caused by the IgG tail may directly or indirectly enhance Notch signaling, although this will need to be explored in future studies.

The studies by Waisman et al. (29) on IgG knock-in mice are also consistent with diminished BCR signaling for immature B cell differentiation compared with IgM. They find that IgG-expressing cells progress inefficiently from pro– to pre–B cell/immature B cell stages, which could reflect too little or too much BCR signaling. Under competitive conditions, a low proportion of IgG-expressing B cells develop in the bone marrow compared with IgM-expressing cells, but this is reversed in the presence of an ITAM deletion in the Igα/CD79a cytoplasmic tail (29). Because the CD79a deletion dramatically enhances BCR signaling (35), the fact that it improves maturation of IgG-bearing cells while diminishing maturation of IgM-bearing cells indicates that IgG signaling for immature B cell maturation is normally suboptimal.

These findings suggest a novel "less-is-more" hypothesis to explain the heightened antibody production conferred by the IgG tail, whereby decreased signaling to a subset of BCR response genes increases plasma cell formation. Like the inhibitory effect of CCR7 induction on migration toward the marginal zone, some of the poorly induced genes in IgMG B cells may be inhibitory for B lymphoblast survival or differentiation into plasma cells. For example, the poorly induced chemokine ligand CCL4 (also know as macrophage inflammatory protein–1B) attracts $CD4^+CD25^+$ regulatory T cells to IgM-activated B cells (36), and these cells are potent inhibitors of B cell antibody production (37, 38). The qualitative changes in IgG tail BCR signaling defined here—in particular the evidence that it diminishes a subset of the cellular response rather than enhancing it, as had been predicted—may not only change views of how isotype switching to IgG enhances memory responses but also may have ramifications for the control of self-reactive B cells.

MATERIALS AND METHODS

Mice. C57BL/6 transgenic mouse strains expressing the IgM or IgMG BCR-recognizing HEL MM4 (39) or MG2 (21), respectively, were used in this paper. They were backcrossed on the B10.BR:$RAG1^{-/-}$ background and used for biochemical experiments. $cd22^{-/-}$ mice (provided by E.A. Clark, University of Washington, Seattle, WA) were previously described elsewhere (19) and were crossed with C57BL/6 MM4 and MG2 strains to generate IgM or IgMG Ig transgenic mice in a $CD22^{-/-}$ background. All mice were maintained and treated in accordance with principles outlined by the Australian National University Animal Experimentation Ethics Committee and the current Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Flow cytometric analysis and intracellular staining. Single-cell suspensions (10^6 cells) were made from spleens and blocked with FACS-staining buffer (PBS containing 2% FCS and 0.05% sodium azide) and anti-CD16/CD32 antibody (clone 2.4G2; BD Biosciences). After washing, the cells were stained with combinations of antibodies. Data were acquired with a FACSCalibur (Becton Dickinson) and analyzed with CellQuest (Becton Dickinson) or FlowJo (TreeStar Inc.) software. For the staining for lysozyme binding, splenocytes were first treated with 1 g/ml HEL and then stained with Tri-Color-conjugated HyHEL9. The following antibodies were used: FITC-conjugated anti-CD21 (7G6), anti-B220 (RA3-6B2), PE-conjugated anti-IgMa (DS-1), anti-CD1d (1D1), anti-CD23 (B3B4), and allophycocyanin-conjugated anti-B220 (RA3-6B2; all obtained from BD Biosciences).

For the intracellular staining of phospho-ERK, unstimulated or stimulated splenocytes were fixed with 2% paraformaldehyde at room temperature for 15 min (40, 41). Cells were centrifugated, resuspended in 100 μl PBS, and permeabilized with 900 μl of ice-cold methanol. After washing with the FACS staining buffer, the cells were stained with anti–phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (Cell Signaling Technology), followed by Cy5-conjugated AffiniPure F(ab')2 fragment donkey anti–rabbit IgG (H+L; Jackson ImmunoResearch Laboratories). MEK inhibitors PD98059 and U0126 (both obtained from Calbiochem) were used for checking the specificity of the phospho–MAP kinase antibody at a concentration of 10 μM.

Calcium measurement. Splenocytes were incubated with 1μM Indo-1 AM (Invitrogen) in RPMI 1640 containing 10 mM Hepes and 2% FCS at 37°C for 30 min. After washing the loaded cells, they were stained with FITC-conjugated anti-CD21 and PE-conjugated anti-B220 antibodies or FITC-conjugated anti-B220 antibody for HEL-PE stimulation at room temperature for 10 min. HEL concentrations >0.5 μg/ml were used in vitro, because these rapidly achieved receptor saturation and gave robust, synchronous calcium responses by Ig transgenic cells. The fluorescence intensity of

intracellular Indo-1 was monitored using a FACS LCR system (Becton Dickinson) and analyzed by FlowJo software.

Microarray analysis. Mice bearing IgM or IgMG BCR were left unstimulated or stimulated with an intraperitoneal injection of 5 mg of HEL. This dose of soluble HEL (which has a short plasma half-life) was chosen because it results in receptor-saturating concentrations of circulating HEL for several hours, and in systematic dose–response studies it gave robust, synchronous CD69 and CD86 responses by Ig transgenic cells in vivo. Comparable receptor saturation and CD69 and CD86 responses were induced by 0.5 μg/ml HEL or greater in vitro, which is the concentration used in the biochemical experiments and our previous microarray experiments (22). It is worth noting that CD69 is itself an ERK response gene (42, 43). 1 h later, spleen cell suspensions were prepared on ice and stained with anti-CD21–FITC and anti-CD23–PE, the follicular B cell fraction was purified on a FACS DIVA sorter and held on ice during sorting, and an aliquot was reanalyzed to confirm purity >90% and centrifuged and lysed in TRIzol (Invitrogen). Total RNAs were purified, quantified, and pooled from multiple sorting experiments (unstimulated IgM 1, two mice; unstimulated IgM 2, four mice; stimulated IgM 1, four mice; unstimulated IgM 2, two mice; unstimulated IgMG 1, five mice; unstimulated IgMG 2, six mice; stimulated IgMG 1, eight mice; and unstimulated IgMG 2, six mice). cRNA was generated according to the Affymetrix protocol. Fragmented cRNA were hybridized with mouse U74Av2 chip sets. The data were analyzed after normalization using Microarray Suite 5.0 (Affymetrix). Clusterings of gene expression were performed with Genes@Work (IBM Research) (44). Microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus under accession no. GSE7218 and sample nos. GSM173561–GSM173569.

Immunization and ELISA. The same number of HEL-binding B cells ($0.5–1 \times 10^6$ cells) were adoptively transferred to unirradiated C57BL/6 recipient mice. The recipient mice were intraperitoneally immunized with 100 μg HEL in CFA. This dose was used because it induces optimal antibody responses in vivo. The sera were obtained 10 d after immunization, and the titers were measured by HEL-specific ELISA. Lysozyme-binding IgM were captured by HEL-coated plates and treated with biotinylated allo-specific anti-IgMa (clone DS1; BD Biosciences), followed by streptavidin-peroxidase (Sigma-Aldrich).

Western blotting and immunoprecipitation. Total splenocytes or purified splenic B cells were stimulated with anti-IgM $F(ab')_2$ or HEL for the times indicated in the figures. The stimulated cells were lysed with TNE buffer (1% [wt/vol] nonident P-40, 20 mM Tris-HCl [pH 8], 0.1 mM sodium orthovanadate with complete protease inhibitor; Roche). The lysates were centrifugated to remove insoluble debris. After an addition of 3× sampling buffer, NP-40–soluble total proteins were subjected to SDS-PAGE. For immunoprecipitation of CD22, the lysates were precleared with protein G–sepharose (GE Healthcare), and CD22 was immunoprecipitated with anti-CD22 antibody (clone Cy34.1, BD Bioscience; or clone 2D6, Chemicon) and protein G–sepharose. After extensive washing, the immune complexes were fractionated by SDS-PAGE. The membranes were stained with antiphosphotyrosine (clone 4G10; Upstate Biotechnology), followed by horseradish peroxidase–conjugated anti–mouse IgG (MP Biomedicals). The antibody-reacted bands were visualized with Western Lightning Chemiluminescence Reagent (PerkinElmer). After stripping, the membranes were stained with goat anti–mouse Siglec-2 (CD22) antibody (R&D Systems), followed by horseradish peroxidase–conjugated anti–goat antibody (Jackson ImmunoResearch Laboratories). The band intensities were measured by ImageJ software (National Institutes of Health).

Online supplemental material. Fig. S1 shows tyrosine phosphorylation of CD22 in response to anti-IgM $F(ab')_2$ or HEL stimulation in IgM versus IgMG B cells. Fig. S2 shows additional expression patterns of gene induction or decrease from the microarray experiments. Fig. S3 shows intracellular calcium responses in CD22-deficient or wild-type IgMD B cells. Online supplemental material is available at htttp://www.jem.org/cgi/content/full/jem.20061923/DC1.

We are grateful to Dr. E.A. Clark for CD22-deficient mice. We thank Dr. L. Tze for collaboration and suggestions and the members of the laboratory for encouragement. We are also grateful to the staff of the Australian Phenomics Facility and the John Curtin School of Medical Research Animal Services Division for expert animal care and genotyping.

This project was supported by National Health and Medical Research Council project grants 224264 and 366771.

The authors have no conflicting financial interests.

Submitted: 6 September 2006
Accepted: 19 March 2007

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