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High-Affinity B Cell Receptor Ligation by Cognate Antigen Induces Cytokine-Independent Isotype Switching

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The selection of an appropriate Ig isotype is critical for an effective immune response against pathogens. Isotype regulation is sensitive to external signals, particularly cytokines secreted by Th cells. For example, IL-4 induces isotype switching to IgG1 via a STAT6-dependent signaling pathway. In this study, we show that BCR ligation also induces IgG1 switching in mouse B cells. The extent of switch induction by Ag is affinity-dependent, and high-affinity Ag binding leads to IgG1 switching levels comparable to those induced by saturating IL-4. However, the Ag-induced IgG1 switch does not require additional cytokine signals and occurs in a STAT6-independent manner. Thus, BCR ligation represents a novel pathway for direct isotype switching leading to IgG1 secretion. ***The Journal of Immunology*****, 2010, 184: 6592–6599.**

The development of a humoral immune response relies on the proliferation of Ag-specific B cells and their differentiation to Ab-secreting cells (ASCs). The choice of Ab isotype is also crucial, because different Ig H chains have differing functions in fighting pathogens (reviewed in Ref. 1). Because B cells can only secrete Ab of one isotype at a time, control of isotype switching by B cells is an important feature of an effective immune response. Isotype switching is known to be regulated by soluble factors secreted by T cells and other cells during an immune response. IL-4 was the first T cell-derived "switch factor" identified (2). When added to cultures of stimulated mouse B cells, IL-4 promotes cell surface expression of IgG1 and secretion of IgG1 Ab (3–5). IL-4 also induces switching to IgE (2, 3, 6). Numerous other secreted factors have been identified that direct isotype switching, including IFN-γ, which induces switching to IgG2a (7) and IgG3 (8), and TGF-β, which induces switching to IgG2b (9) and IgA (10, 11).

Further studies have revealed a B cell-intrinsic mechanism of isotype switching regulation, in which the progression of B cells through successive cell divisions determines the likelihood of isotype switching and ASC differentiation (12–14). Cytokines act to alter the rates with which these differentiation events occur within each division cycle (12, 14, 15). Thus, cytokines and division progression appear to work in conjunction to regulate the proportion of B cells expressing the various isotypes.

BCR ligation by Ag strongly influences B cell proliferation and differentiation outcomes. For example, the Ag hen egg lysozyme (HEL) enhances the in vitro proliferation of HEL-specific B cells from MD4 mice in response to LPS (16). In contrast, ASC differentiation is profoundly inhibited when stimulation with LPS is combined with Ag or anti-Ig reagents as BCR cross-linking surrogates (17–19). One study investigating the influence of BCR signals on isotype switching found that stimulation with anti-Ig resulted in reduced switching rates per cell division during T-dependent B cell responses (20). However, the role of BCR ligation in regulating isotype switching has not been thoroughly characterized.

In this study, we used isotype switching-competent Ag-specific B cells from SW_{HEL} mice (21, 22) to investigate the effect of cognate Ag stimulation on isotype switching. HEL-specific B cells from SW_{HEL} mice have the same BCR specificity as MD4 mice, but the rearranged V_HDJ_H gene is targeted to the physiologically appropriate location of the germline IgH gene, allowing normal isotype switching in B cells from these mice. B cells from SW_{HEL} mice develop normally and proliferate, differentiate to ASCs, and switch isotypes in vitro in response to polyclonal T-dependent or T-independent activating signals (22). Using these mice, we found that Ag stimulation induces B cell switching to IgG1 in a manner dependent on both the concentration and affinity of the Ag. Ag-induced IgG1 switching mimics that seen following IL-4 stimulation but occurs without the requirement for additional cytokine signals. These results identify Ag signals as a novel third factor, in addition to cytokines and cell division progression, that influences isotype switching outcomes.

Materials and Methods

Mice

B cells were obtained from 6- to 12-wk-old mice bred and maintained at the Walter and Eliza Hall Institute animal facilities in accordance with Walter and Eliza Hall Institute animal ethics committee regulations. All of the mice were of C57BL/6 background. SW_{HEL} mice (21, 22) were bred with Blimp-$1^{GFP/+}$/$RAG2^{-/-}$ (23) mice kindly provided by Stephen Nutt (Walter and Eliza Hall Institute). SW_{HEL} $RAG2^{-/-}$ mice were also bred with $STAT6^{-/-}$ mice (24).

B cell isolation and cell culture

Naive follicular B cells were prepared from mouse spleens. Cell suspensions were RBC-depleted and separated on 50/65/80% Percoll density gradients. Cells from the 65/80% interface were purified using magnetic bead B cell isolation kits (Miltenyi Biotec, Auburn, CA). Cell populations at the start of culture were ≥97% B220- and CD19-positive and did not contain IgG1-positive cells, as assessed by flow cytometry. Where specified, purified B cells were labeled with CFSE (Molecular Probes, Eugene, OR) according to the published method (25). B cells were cultured at 37°C in RPMI

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Abbreviations used in this paper: AID, activation-induced cytosine deaminase; ASC, Ab-secreting cell; CT, cycle threshold; HEL, hen egg lysozyme; ND, not detected; WT, wild type.

1640 medium (Life Technologies, Carlsbad, CA) with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 10 mM HEPES (pH 7.4), 100 μg/ml streptomycin, 100 U/ml penicillin, 50 μM 2-ME (all supplements from Sigma-Aldrich, St. Louis, MO), and 10% heat-inactivated FCS (CSL, Parkville, Victoria, Australia).

The following reagents were included in cultures as indicated: *Salmonella typhosa* LPS (Sigma-Aldrich), recombinant mouse IL-4 (kindly provided by Robert Kastelein, DNAX Research Institute, Palo Alto, CA), anti-CD40 mAb 1C10 prepared from the hybridoma cell line (kindly provided by Maureen Howard, DNAX Research Institute), oligonucleotide CpG 1668 (sequence TCCATGACGTTCCTGATGCT, fully phosphothioated) (Geneworks, Hindmarsh, South Australia, Australia), and imidazoquinoline compound R848 (Invitrogen). Commercially available HEL (Sigma-Aldrich) was used for all of the experiments except where defined as HEL^{WT}, HEL^{2X}, or HEL^{3X}. The generation of the HEL affinity variants has been previously described (26)

Flow cytometry

Cells to be analyzed by flow cytometry were fixed with 2% paraformaldehyde in PBS for 30 min at 4°C and then permeabilized with 0.1% Tween 20 in PBS at 4°C overnight. Staining Abs used were PE-conjugated anti-IgM, anti-CD45.1, and anti-B220, allophycocyanin-conjugated anti-IgG1, and FITC-conjugated anti-CD19 Abs (BD Pharmingen, San Diego, CA) and PE-conjugated anti-IgG2b and anti-IgG3 Abs (Southern Biotechnology Associates, Birmingham, AL) and a biotin-conjugated anti-IgG2c Ab (Southern Biotechnology Associates) with PE-conjugated streptavidin (BD Pharmingen) for detection. Data were collected on FACScalibur or FACScan flow cytometers and analyzed with FlowJo software (Tree Star, Ashland, OR).

ELISA

IgG1 titer in cell culture supernatants were determined by sandwich ELISA. ELISA plates (Costar, Corning Glass, Corning NY) were coated with unlabeled mouse anti-IgG1 (Southern Biotechnology Associates) at a concentration of 2 μg/ml overnight at 4°C. Plates were blocked with 2% FCS prior to incubation with serial dilutions of cell culture supernatants, followed by a biotinylated anti-IgG1 Ab (Southern Biotechnology Associates) and subsequent streptavidin coupled to HRP. Ab binding was detected with substrate solution containing 1 mg/ml ABTS, 0.03% H_2O_2, 0.1 M citric acid, and 0.1 M trisodium citrate. Absorbance at 492 nm was measured using a Softmax microplate reader (Molecular Devices, Sunnyvale, CA). Ig concentrations were determined from a standard curve calculated from IgG1 standards (values and mean of three replicate cultures are shown).

Quantitative real-time PCR

Total cellular RNA was purified using RNeasy Mini kits (Qiagen, Valencia, CA), and cDNA was synthesized using the SuperScript III First-Strand Synthesis system (Invitrogen). Quantitative real-time PCR reactions were set up using a QuantiTect SYBR Green RT-PCR kit (Qiagen). PCR was performed on an ABI PRISM 7900H cycler (Applied Biosystems, Foster City, CA). Expression of the hydroxymethylbilane synthase housekeeping gene was used for normalization of each gene tested (27). Cycle threshold (CT) values for each of three replicates were normalized to mean hydroxymethylbilane synthase gene CT values determined from three replicates, and these normalized values were used for statistical analysis. The normalized CT values were transformed to mean expression values by calculating $2^{(\text{mean normalized CT value})}$; error bars represent $2^{(\text{mean normalized CT value} \pm \text{SEM})}$. Mean LPS expression values were set to 1, and other expression values (means and errors) were transformed to be relative to this.

Primer sequences used were: activation-induced cytosine deaminase (AID), forward, 5′-CCGGCACGTGGCTGAGTTT-3′, reverse, 5′-GATGCGCCGAAGTTGTCTGGTTAG-3′; *Cμ* (total cellular IgM), Cμ common, 5′-CCCACAGCATCCTGACTGTGACAGAGGAG-3′, Cμ membrane, 5′-GATGAAGGTGGAGGCAGTGGTCCACAGG-3′; germline transcript γ1 (28), Ig1 5′-GGCCCTTCCAGATCTTTGAG-3′, Cg1R; postswitch transcript γ1 (28), ImF, 5′-CTCTGGCCCTGCTTATTGTTG-3′, Cg1R, 5′-GGATCCAGAGTTCCAGGTCACT-3′; *hmbs*, forward, 5′-GACCTGGTTGTTCACTCCCTGAAG-3′, reverse, 5′-GACAACAGCATCACAAGGGTTTTC-3′.

Results

High-affinity Ag induces switching to IgG1 during B cell responses to LPS

Because BCR ligation has previously been shown to have profound effects on ASC differentiation during B cell responses to LPS (17–19), we first used this system to test the effect of Ag on isotype switching. Splenic B cells were prepared from SW_{HEL} $RAG^{-/-}$ Blimp-$1^{GFP/+}$ mice. The SW_{HEL} $RAG^{-/-}$ mice provide pure populations of HEL-specific B cells, and the Blimp-$1^{GFP/+}$ allele allows identification of Blimp-1–expressing ASCs using the GFP marker (23). Purified B cells were stimulated in vitro with LPS and varying concentrations of HEL. Cells were assayed after 4 d of culture for ASC differentiation and for expression of IgM or switched Ig isotypes. As expected, the proportion and number of Blimp-1–expressing ASCs (GFP-positive cells) decreased with increasing HEL concentrations (Fig. 1*A*). In addition, we observed that the addition of HEL induced a significant reduction in the proportion of cells expressing IgM (Fig. 1*B*), also in an Ag concentration-dependent manner. This unexpected finding suggested that the presence of Ag had increased the proportion of cells that had undergone isotype switching. Our supposition was supported by detection of increased levels of AID, an essential regulator of isotype switching (28), by quantitative PCR using cDNA prepared from B cells stimulated with LPS alone or together with HEL (Fig. 1*C*).

B cells proliferating in response to LPS as the sole stimulatory factor typically switch to isotypes IgG2b or IgG3 (29). Low concentrations of HEL induced weak increases in the proportions of cells switching to IgG2b and IgG3 (Fig. 1*D*). However, the proportions of cells expressing these isotypes were not affected by high concentrations of HEL, despite the substantial reduction of IgM-expressing cells induced by these concentrations. This suggested that another isotype was being induced. Upon examination of other Ig isotypes, no switching to IgE or IgA was observed with any concentration of HEL up to 1000 ng/ml, and only very weak switching to IgG2c [the isotype expressed by C57BL/6 mice instead of IgG2a (30, 31)] was observed (Fig. 1*E*). In contrast, we surprisingly detected a strong induction of isotype switching to IgG1 (Fig. 1*F*).

Ag-induced switching to IgG1 is not due to B cell-derived soluble factors

IgG1 switching is known to be induced by IL-4 stimulation (4, 5, 7). The proportion of switched cells that we observed in HEL-stimulated cultures was very similar to that observed when IL-4 was used as the IgG1-inducing factor (Fig. 1*F*). These observations could result from autocrine IL-4 secretion by SW_{HEL} B cells stimulated with LPS and HEL. However, supernatants of cell cultures stimulated with HEL and LPS did not contain detectable levels of IL-4 when analyzed by ELISA (data not shown). Furthermore, wild-type C57BL/6 B cells were not induced to switch isotype to IgG1 when cocultured with SW_{HEL} B cells in LPS and HEL (Fig. 1*G–J*), indicating that the switching observed occurs directly in response to the BCR signal and not through autocrine influence.

Ag-induced isotype switching can lead to IgG1 secretion

ASC differentiation and isotype switching are division-linked processes, and the frequency with which these differentiation events occur typically increases as cells move through division cycles (12–14). Thus, changes in differentiation outcomes in the population may occur through directly modulating the rate of differentiation or as a derivative effect of affecting the proliferation dynamics, or a combination of both. Although multiple aspects of SW_{HEL} B cell proliferation are altered by Ag (M.L. Turner and P.D. Hodgkin, manuscript in preparation), the division progression of cells responding to LPS stimulation alone or in combination with IL-4 or HEL was comparable (Fig. 2*A–C*). Furthermore, HEL acted to induce a similar pattern of division-linked isotype

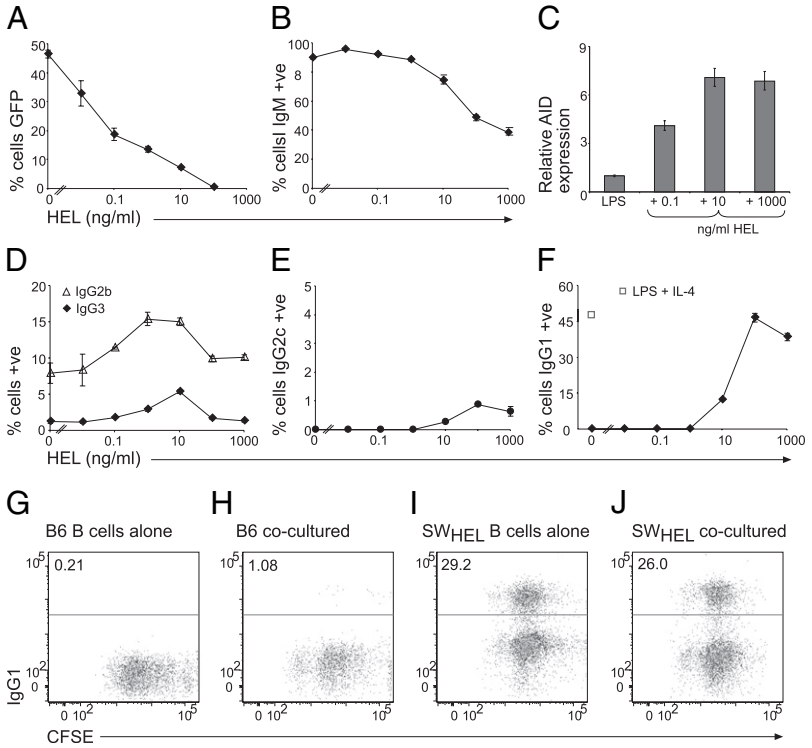

FIGURE 1. BCR ligation by HEL induces isotype switching in SW_{HEL} B cells. Splenic B cells from SW_{HEL} $RAG2^{-/-}$ Blimp-$1^{GFP/+}$ mice were stimulated in vitro with 15 μg/ml LPS either alone, with 500 U/ml IL-4 (indicated in red), or with various concentrations of HEL. Cells were harvested, fixed and permeabilized on day 4, and stained for analysis by flow cytometry. Percentages of cells expressing GFP (Blimp-1) (*A*), IgM (*B*) IgG2b (*D*), IgG3 (*D*), IgG2c (*E*), or IgG1 (*F*) were determined (mean and SEM of three replicate cultures shown). In another experiment, SW_{HEL} B cells were stimulated in vitro with 15 μg/ml LPS alone and with various concentrations of HEL for 4 d prior to RNA and cDNA preparation for use in quantitative PCR for AID (*C*). In another experiment, splenic B cells from SW_{HEL} $RAG2^{-/-}$ (CD45.2) or C57BL/6 (CD45.1) mice were stimulated in vitro with 15 μg/ml LPS and 100 ng/ml HEL alone or as a coculture of cells in a 1:1 ratio. Cells were fixed and permeabilized on day 3 and stained for flow cytometry. Representative dot plots from three replicate cultures are shown for C57BL/6 B cells alone (*G*) and as the CD45.1-positive population in the cocultures (*H*) and SW_{HEL} B cells alone (*I*) and as the CD45.1-negative population in the cocultures (*J*). *A*, *B*, and *D–F* contain representative results from one of three independent experiments; *C* and *G–J* contain representative results from one of two independent experiments.

switching to that observed in cultures containing IL-4 (Fig. 2*D*). Thus, it appears that Ag–BCR binding inhibits ASC differentiation and induces isotype switching directly rather than as a simple consequence of changes in cell division progression.

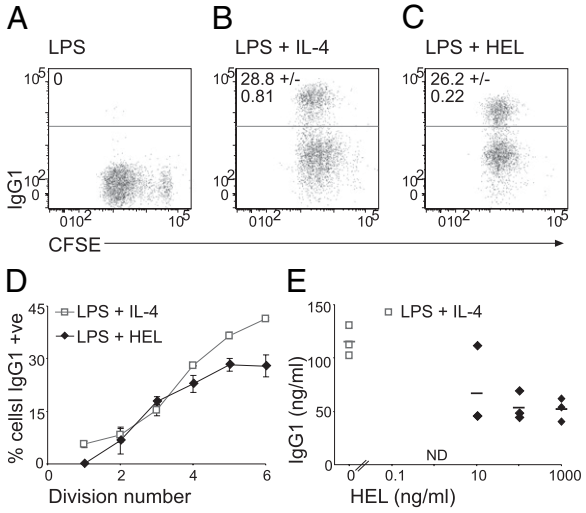

FIGURE 2. HEL induces SW_{HEL} B cells to undergo division-linked isotype switching and to secrete IgG1 Ab. Splenic B cells from SW_{HEL} $RAG2^{-/-}$ mice were labeled with CFSE prior to stimulation in vitro with 15 μg/ml LPS alone and with 500 U/ml IL-4 (red) or 100 ng/ml HEL (black). Cells were harvested, fixed and permeabilized on day 3, and stained for flow cytometry (*A–C*; representative dot plots from one of three replicate cultures are shown). The percentage of IgG1-positive cells per division (based on CFSE profiles) was determined for three replicate cultures in each stimulation condition (*D*). In another experiment, splenic B cells from SW_{HEL} $RAG2^{-/-}$ Blimp-$1^{GFP/+}$ mice were stimulated in vitro with 15 μg/ml LPS alone and with 500 U/ml IL-4 or various concentrations of HEL for 4 d. Supernatants from these cultures were analyzed for IgG1 Ig concentration by ELISA (*E*). Not detected (ND) indicates secretion level <30 ng/ml. *A–E* contain representative results from one of three independent experiments.

To ascertain whether the IgG1-expressing cells seen in cultures stimulated with combined LPS and HEL can secrete IgG1 Ab, culture supernatants were analyzed by ELISA. Cells stimulated with 10 ng/ml HEL secreted the highest concentration of IgG1, although this Ab concentration was lower than that obtained from cultures stimulated with combined LPS and IL-4 (Fig. 2*E*). These results reflect the concentration-dependent action of Ag on inhibiting ASC differentiation but indicate that an intermediate dose of high-affinity Ag can induce substantial IgG1 switching without completely inhibiting the capacity for this IgG1 to be secreted.

IgG1 switching induced by Ag occurs independently of STAT6

Isotype switching to IgG1 and IgE in response to IL-4 is dependent on signaling through the transcriptional regulator STAT6 (24, 32). IL-13, which also induces IgE switching, also signals through STAT6 (33). However, although IgE Abs are not detectable in serum of naive or immunized mice lacking either STAT6 or IL-4, IgG1 Abs are detectable in these mice, albeit at levels approximately one third of those found in wild-type mice (24, 34, 35). Furthermore, the concentration of IgG1 increases in these mice following immunization (24, 34, 35). These data suggest that an alternative, STAT6-independent pathway to IgG1 switching may exist.

To assess whether HEL activates a STAT6 signaling pathway to induce isotype switching to IgG1 in SW_{HEL} B cells, SW_{HEL} $RAG2^{-/-}$ mice were bred with mice lacking the STAT6 gene ($STAT6^{-/-}$ mice) so that STAT6 signaling is ablated and IL-4 responses are impaired (24, 32). Cellular expression of germline transcript γ1 and postswitch γ1 was compared in B cells from SW_{HEL} or SW_{HEL} $STAT6^{-/-}$ mice. As expected, SW_{HEL} $STAT6^{-/-}$ B cells stimulated with LPS and IL-4 did not express IgG1 (Fig. 3*F*), unlike SW_{HEL} B cells with functional STAT6 (Fig. 3*B*). However, stimulation with LPS and HEL induced switching to IgG1 in an Ag dose-dependent manner in both cell types (Fig. 3*C*, 3*D*, 3*G*, 3*H*). These results indicate that the IgG1 switching induced by HEL during LPS stimulation is not dependent on STAT6 signaling. These results also indicate that the switching to IgG1 observed under these stimulation

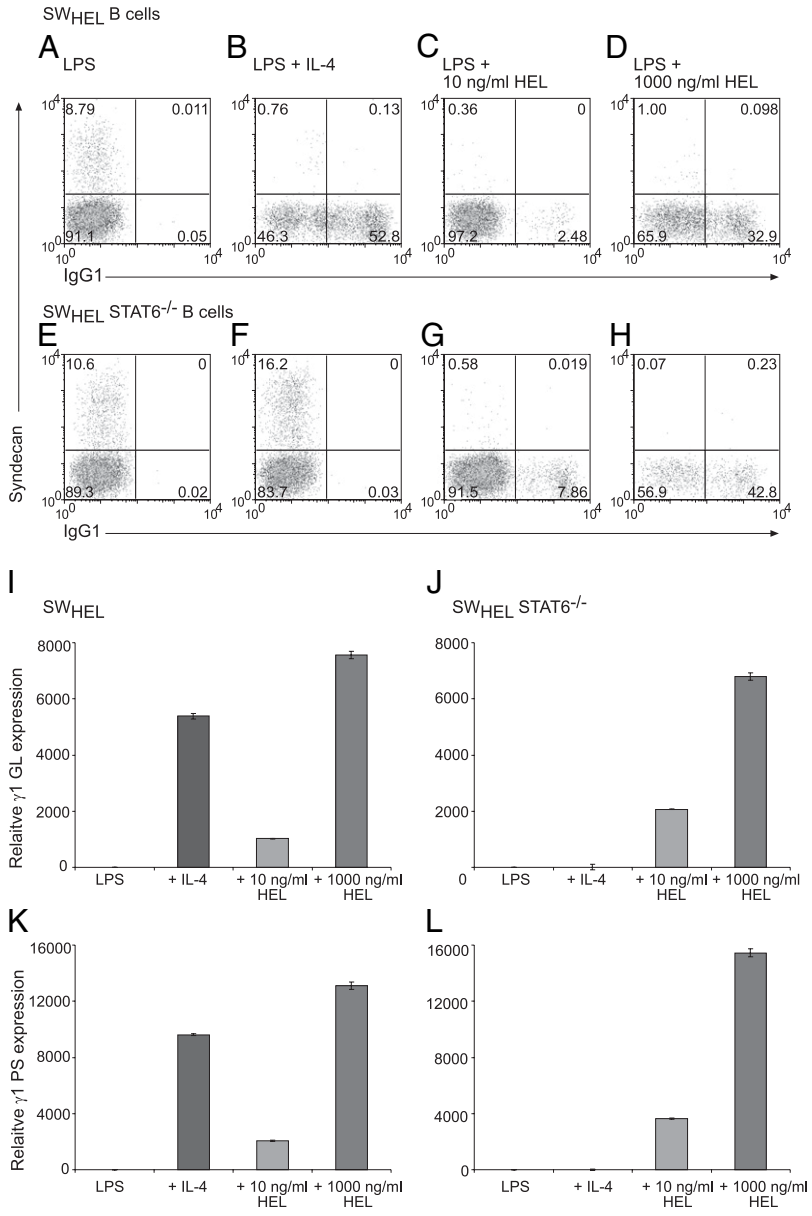

FIGURE 3. HEL, and not IL-4, induces SW_{HEL} $STAT6^{-/-}$ B cells to switch to IgG1 during LPS stimulation. Splenic B cells from SW_{HEL} $RAG2^{-/-}$ and SW_{HEL} $RAG2^{-/-}$ $STAT6^{-/-}$ mice were prepared and stimulated in vitro with 15 μg/ml LPS alone and with 500 U/ml IL-4, 10 ng/ml HEL, or 1000 ng/ml HEL. Cells were harvested, fixed and permeabilized on day 4, and stained for flow cytometry (*A–H*). In another experiment, SW_{HEL} B cells were stimulated in vitro with 15 μg/ml LPS alone and with 500 U/ml IL-4 or 10 ng/ml HEL or 1000 ng/ml HEL or 500 U/ml IL-4 together with 1000 ng/ml HEL. SW_{HEL} $STAT6^{-/-}$ B cells were prepared separately and stimulated in the same manner. Cells were harvested on day 3 and used for preparation of RNA and cDNA for quantitative PCR for germline transcript γ1 (*I*, *J*) and post-switch γ1 (*K*, *L*). *A–J* contain representative results from one of three independent experiments.

conditions occurs via the conventional isotype switching pathway of germline transcript induction.

The lack of a requirement for STAT6 in the IgG1 switching response to HEL was also tested by quantitative real-time PCR using cDNA prepared from B cells from SW_{HEL} or SW_{HEL} $STAT6^{-/-}$ mice that had been stimulated with LPS alone and with IL-4 or HEL. SW_{HEL} B cells expressed significantly greater levels of IgG1 mRNA following stimulation with LPS and IL-4 compared with those with LPS stimulation alone (Fig. 3*I*). In contrast, IgG1 transcript levels in B cells from SW_{HEL} $STAT6^{-/-}$ mice that had been stimulated with LPS and IL-4 were not higher than those with LPS stimulation alone (Fig. 3*J*), again confirming that isotype switching in response to IL-4 requires STAT6 signaling. However, B cells from both SW_{HEL} and SW_{HEL} $STAT6^{-/-}$ mice expressed significantly higher levels of IgG1 mRNA following stimulation with HEL, and this was dependent on the dose of HEL used (Fig. 3*I*, 3*J*).

Affinity regulates the degree of isotype switching induced by BCR ligation

Having observed that the degree of isotype switching induced depends on the dose of Ag (Fig. 1), we sought to assess whether Ag affinity could additionally regulate the switching outcome. We used HEL mutants that are recognized by the SW_{HEL} BCR with varying affinities: HEL^{WT} (2×10^{10} M^{-1}), HEL^{2X} (8×10^{7} M^{-1}), and HEL^{3X} (1.5×10^{6} M^{-1}) (26) in conjunction with LPS stimulation. Although both lower-affinity HEL proteins (HEL^{2X} and HEL^{3X}) were capable of inducing IgG1 switching in LPS-stimulated SW_{HEL} B cells, higher concentrations were needed to produce a similar degree of switching to that obtained using high-affinity HEL (HEL^{WT}). Importantly, the maximum level of isotype switching obtained decreased with decreasing Ag affinity (Fig. 4*A*). This effect was also observed on a per division basis (Fig. 4*B*), indicating that Ag affinity directly regulates the rate of isotype switch induction by Ag.

We also assessed the effect of BCR cross-linking by anti-Ig Abs, which are often used as surrogates for Ab. We tested a panel of seven anti-Ig Abs (Table I) at concentrations of 100 μg/ml in conjunction with LPS stimulation of wild-type C57BL/6 B cells. These Abs gave only very weak, if any, induction of switching to IgG1 (Fig. 4*D–J*), revealing a qualitative difference in the signal provided to B cells by cognate Ag compared with those provided by BCR cross-linking Abs.

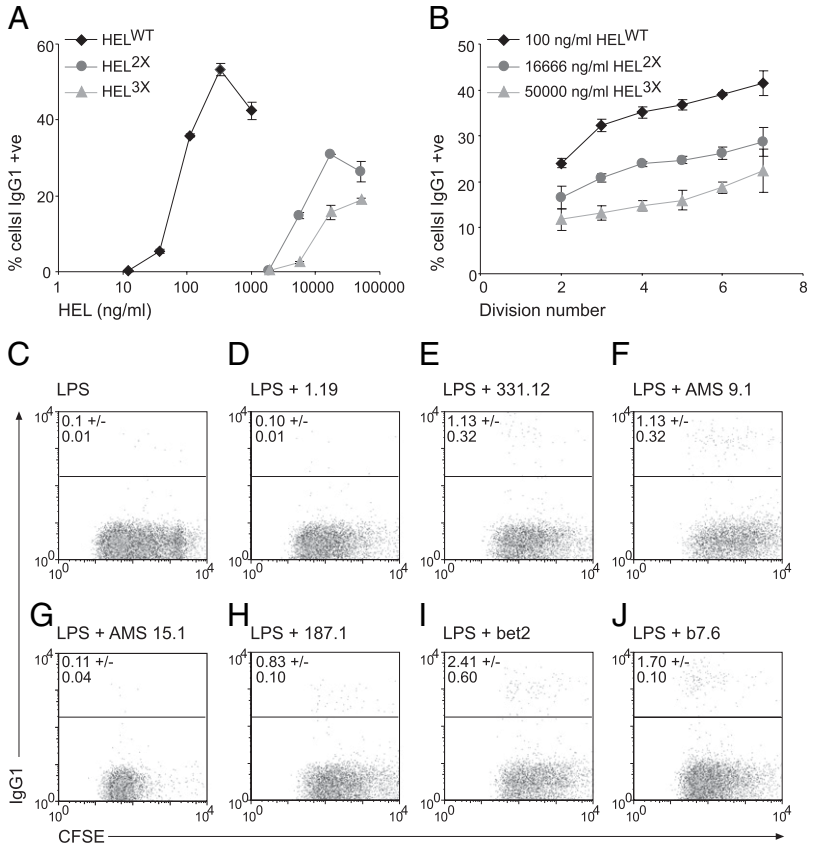

FIGURE 4. Affinity of BCR ligation regulates the extent of IgG1 isotype switching. Splenic B cells from SW_{HEL} $RAG2^{-/-}$ mice were labeled with CFSE prior to stimulation in vitro with 15 μg/ml LPS alone and with various concentrations of HEL^{WT} (high affinity; black), HEL^{2X} (lower affinity; gray), or HEL^{3X} (lowest affinity; light gray). Cells were harvested, fixed and permeabilized on days 3 and 4, and stained for flow cytometry. The total number of IgG1-positive cells in the population on day 4 (*A*) and percentage of IgG1-positive cells per division (based on CFSE profiles) (*B*) are shown (mean and SEM of three replicate cultures). In another experiment, CFSE-labeled splenic B cells from SW_{HEL} $RAG2^{-/-}$ mice were stimulated in vitro with 15 μg/ml LPS alone and with 100 μg/ml of each of seven anti-Ig Abs (Table I). Cells were harvested, fixed and permeabilized on day 4, and stained for flow cytometry. Representative dot plots are shown with mean and SEM of three replicate cultures indicated as inset) (*C–J*). *A–J* contain representative results from one of three independent experiments.

Ag induces IgG1 switching following stimulation with other B cell agonists

We next sought to ascertain whether the IgG1 isotype switching observed in SW_{HEL} B cells in response to combined LPS and HEL stimulation was unique to this system or whether the Ag signal can also act to induce IgG1 switching during responses to other stimuli. B cells are activated to proliferate in vitro by stimulation with the TLR7 agonist R848 and the TLR9 agonist CpG DNA. In both systems, IgG1 isotype switching was not detected in response to the TLR stimuli alone (Fig. 5*A*, 5*C*), but IgG1-expressing cells were detected when HEL was added to both cultures (Fig. 5*B*, 5*D*). No further increases in proportions of cells staining for IgG1 were seen at HEL concentrations >100 ng/ml. However, the levels of isotype switching measured were considerably below those achieved when the cells were exposed to combined LPS and HEL stimulation (Fig. 1*F*).

Assessing the effect of HEL stimulation on IgG1 switching in an in vitro model of a T-dependent B cell response is complicated by the requirement for IL-4 (a known IgG1 switch factor, as described above) in B cell cultures stimulated with anti-CD40 for proliferation to occur. Furthermore, proliferation in response to an anti-CD40 Ab and IL-4 is retarded by high concentrations of HEL (M.L. Turner et al., manuscript in preparation). In light of this, we assessed IgG1 switching in these conditions on a per-division basis. During optimal conditions for T-dependent B cell proliferation, using a high concentration of IL-4, very little effect of additional HEL on IgG1 switching was observed (Fig. 5*E*). However, when the concentration of IL-4 was lowered to a level that promoted proliferation but did not provide a saturating switching signal, a strong dose-dependent increase in IgG1 switching induced by Ag was observed.

Discussion

Previous studies of B cell differentiation in response to BCR binding have focused on the effects of this signal on Ab production (17–19). The data presented here add to these studies by assessing another important effector outcome of B cell differentiation: isotype switching. We have shown that potent induction of B cell isotype switching to IgG1 occurs in response to BCR ligation by high-affinity cognate Ag during conditions mimicking both T-dependent and T-independent B cell responses. The proportion of cells that switch to IgG1 during combined stimulation with LPS

Table I. *Anti-Ig Abs used for testing the effect of BCR cross-linking on B cell isotype switching during concurrent LPS stimulation (Fig. 4)*

Ab Name	Source	Isotype	Specificity	Reference
1.19	Rat	IgG2a	Anti-mouse δ	(49)
331.12	Rat	IgG2b	Anti-mouse Cμ1 (IgM H chain)	(50)
AMS 9.1	Mouse	IgG2a	Anti-mouse Ig5.4 (Fab fragment of IgD)	(51)
AMS 15.1	Mouse	IgG2a	Anti-mouse Ig5.1 (Fab fragment of IgD)	(51)
187.1	Rat	IgG1	Anti-mouse κ	(52)
bet-2	Rat	IgG1	Anti-mouse Cμ2 (IgM H chain)	(50)
b7.6	Rat	IgG1	Anti-mouse Cμ2 (IgM H chain)	(53)

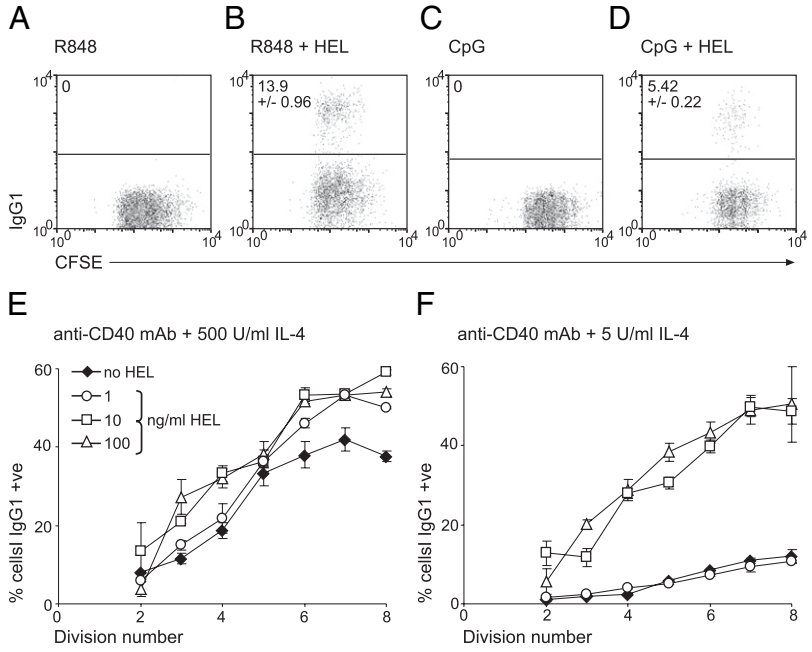

FIGURE 5. HEL also induces IgG1 switching in SW_{HEL} B cells during proliferative responses to TLR9 and TLR7 agonists and to T-dependent activation signals. Splenic B cells from SW_{HEL} $RAG2^{-/-}$ were CFSE-labeled and stimulated in vitro with 3 μM CpG DNA or 1 μg/ml R848 alone and with various concentrations of HEL. Cells were harvested, fixed and permeabilized on day 3, and stained for flow cytometry (*A–D*). Representative dot plots are shown at 100 ng/ml HEL (*B*, *D*), at which the greatest induction of IgG1 switching was observed (mean and SEM of three replicate cultures indicated as inset). Alternatively, CFSE-labeled SW_{HEL} B cells were stimulated in vitro with 20 μg/ml anti-CD40 mAb and various concentrations of HEL in the presence of either 500 or 5 U/ml IL-4. Cells were harvested, fixed and permeabilized on day 4, and stained for flow cytometry. The percentage of IgG1-positive cells per division (based on CFSE profiles) was determined for three replicate cultures in each stimulation condition (*E*, *F*). *A–F* contain representative results from one of three independent experiments.

and Ag is equivalent to the proportion of cells expressing IgG1 following combined LPS and IL-4 stimulation, but the switching response to Ag occurs via a STAT6–independent pathway. These data suggest that BCR engagement could represent the IL-4–independent control of IgG1 switching hypothesized by Shimoda et al. (34) upon the observation of significant concentrations of IgG1 Ab in mice deficient for either IL-4 or STAT6 (24, 34, 35). We have termed the Ag-induced switching that we observe "cytokine-independent" isotype switching, because experiments in which B cells sensitive to the specific Ag signal (SW_{HEL} B cells) were activated in high-density cocultures with non-BCR-sensitive cells (wild type [WT] B cells) revealed no induction of IgG1 switching in the WT cells (Fig. 1). Although it is not possible to definitively exclude the role of an extremely short-range and labile soluble factor in inducing this switching behavior, we have used this terminology to highlight the important distinction between this BCR-induced switching and previously known switch induction pathways.

Our data demonstrate that an Ag signal induces two effects relating to isotype switching: expression of AID is upregulated and switching is directed to the IgG1 isotype. Induction of increased AID expression begins at a concentration of 0.1 ng/ml HEL, although no IgG1 switching is seen in response to this HEL concentration (Fig. 1). Therefore, we believe that these two signals operate across different ranges of Ag signal strength. At intermediate Ag concentrations (0.1–10 ng/ml HEL), we see an enhancement in AID expression that results in significant, but relatively minor, increases in the levels of IgG2b and IgG3 expression, the isotypes that typically appear in response to LPS stimulation. A higher Ag concentrations (10–1000 ng/ml), no further increase in AID expression is observed. However, induction of γ1 germline transcript and the proportion of IgG1-expressing cells increases substantially across this higher concentration range. Thus, it appears that high concentrations of Ag induce IgG1 switching both by allowing transcription at the γ1 locus and by increasing the amount of AID available to target this locus.

The effect of high-dose Ag in inducing IgG1 switching mimics the effect of IL-4, but we have shown that Ag-induced switching occurs in a STAT6-independent manner (Fig. 3) and thus must result from a divergent pathway to that downstream of the IL-4R. Other cytokines known to contribute to IgG1 switching in the mouse include IL-13 and IL-21 (36). IL-4 and IL-21 exert opposing effects on cell surface markers CD23 and CD44 in mouse B cells, suggesting that IL-21 might function in a different manner than IL-4 and IL-13 (36). However, the signal provided by IL-21 does not resemble that seen here for Ag activation, because IL-21 signaling inhibits proliferative responses of follicular B cells to both LPS and CpG DNA in vitro (36), whereas the addition of Ag did not inhibit proliferation in our studies. It has also been shown that B cells stimulated in vitro with the cytokine-like molecules BAFF (B cell activating factor belonging to the TNF family) or APRIL (a proliferation-inducing ligand) secrete IgG1 Ab, and this level is enhanced by the presence of IL-4 (37, 38). However, levels of IgG1 secretion and the proportion of IgG1-expressing cells were found to be significantly lower following BAFF and APRIL stimulation (with or without IL-4) than those in response to LPS and IL-4 stimulation (37, 38). Although it is likely that these cytokines and costimulatory molecules, or others, may act to additionally influence the behavior of B cells in response to Ag and polyclonal activation, no previously reported costimulatory action seems to correspond directly to the SW_{HEL} B cell responses to LPS and HEL observed here. Instead, it appears that BCR ligation leads to direct downstream signaling events that induce IgG1 switching.

Our finding that both γ1 germline transcript and postswitch γ1 are induced by Ag ligation indicate that assessing the relative activities of transcription factor binding sites in the γ1 promoter (other than STAT6) may help to identify signaling intermediates that lead to this switching. Sites for binding of the transcription factors C enhancer binding protein β and C enhancer binding protein γ overlap with the STAT6 site in a region of the γ1 promoter that has been termed the basic leucine zipper domain element, a region that is activated following CD40 stimulation of B cells (39, 40). The γ1 promoter also contains three NF-κB binding sites. It has been shown previously that B cell surface CD40 ligation leads to binding of NF-κB to the γ1 promoter and induces γ1 transcription in a cytokine-independent manner (41), and it is known that HEL stimulation of naive HEL-specific B cells activates the NF-κB pathway (42). Thus, signals activating any of these molecules to interact with the γ1 promoter could potentially lead

to IgG1 switching in response to Ag. The fact that high concentrations of Ag induced very low but repeatedly evident levels of IgG2c (Fig. 1) may also be a useful indicator for further studies investigating signaling pathways downstream of the BCR.

We have also shown that IgG1 switching requires high-affinity BCR ligation and that this outcome is not seen following BCR aggregation by anti-Ig Abs alone. This is an important distinction that invites new consideration of existing findings. In contrast to our observation that high-affinity Ag induces upregulation of AID expression, it has previously been shown that AID mRNA expression is delayed and reaches a lower maximum at later days in in vitro murine B cell cultures stimulated with anti-IgM plus IL-4 compared with that in cultures stimulated with LPS and IL-4 (43). In fact, the addition of anti-IgM to cell cultures stimulated with LPS and IL-4 or anti-CD40 and IL-4 was shown to reduce the proportion of class-switched cells in these cultures. Furthermore, these authors reported that B cells stimulated with anti-IgM and IL-4 did not switch to either IgG1 or IgG3 compared with cells stimulated with LPS alone (IgG3 switched) or with LPS and IL-4 (IgG1 switched) (43). However, no difference was found in the levels of germline γ1 transcripts of cells stimulated with IL-4 and LPS or anti-IgM, indicating that the inhibitory effect of anti-IgM on switching occurs at a stage subsequent to the formation of the germline transcript. In similar findings, Jabara et al. (44) demonstrated that inclusion of an anti-IgM Ab in B cell cultures stimulated with LPS and IL-4 inhibits IgG1 expression and secretion and that this inhibition is not due to either suppressed or delayed proliferation or increased apoptosis. In another example of this phenomenon, Hauser et al. (45) found that addition of an anti-IgM Ab decreased AID protein levels by 73% 1 h after addition to a culture of B cells that had been previously stimulated for 48 h with LPS and IL-4. This reduction in protein was due to inhibition of AID mRNA transcription rather than degradation of existing AID protein. By combination of our results with these previous reports it becomes clear that the signals induced by BCR binding by Ag and BCR cross-linking with anti-Ig Abs lead to quantitatively different outcomes in terms of AID expression and isotype switching. These distinctions are important for two reasons. First, they demonstrate that care must be taken when correlating the effect of an anti-Ig reagent with that of the signal provided by cognate Ag. Secondly, they may provide a useful readout for subsequent studies designed to investigate the molecular mechanisms by which these distinct outcomes arise.

Our results also demonstrate that BCR binding affinity plays a crucial role in isotype switching decisions. An indication that affinity can regulate switching rates has been previously seen in vivo, where fewer SW_{HEL} B cells were observed to switch to IgG1 in response to immunization with sheep RBCs conjugated with low-affinity HEL (HEL^{3X}) than those in response to sheep RBCs conjugated with high-affinity HEL (HEL^{WT}) on day 5 following immunization (21). However, this immunization protocol induces a T-dependent immune response, and the effect of affinity on IgG1 switching may be small in comparison with the strong IgG1 switch signals provided by IL-4 in such a setting. Using our T-independent stimulation system, we were able to study the effect of an Ag signal directly and without the confounding presence of cytokines. Our results indicate that not only are greater concentrations of lower-affinity Ag required to induce isotype switching at rates comparable to those seen during stimulation with high-affinity Ag but also that the maximum degree of switching that can occur in response to a BCR signal is itself a function of the affinity of the signal.

When considering the implications of our results for understanding immune responses, it is important to consider the functional outcome of Ab secretion rather than simply the isotype-expressing properties of responding cells. The paradoxical result that inhibition of ASC differentiation occurs upon the addition of an anti-Igμ Ab to cultures of mouse spleen cells stimulated with LPS, despite an accompanying increase in proliferation of responding cells, was first observed >30 y ago (17, 18, 46). The inhibitory effect of anti-Ig has been reported again more recently, together with the finding that concurrent stimulation of HEL-specific BCR transgenic (MD4) B cells with LPS and HEL also leads to the same outcome (19). The fact that both specific Ag and BCR cross-linking inhibit ASC differentiation during LPS stimulation suggests that this phenomenon is regulated in a different manner than the IgG1 switching we describe here, which is strongly induced in response to Ag but not to anti-Ig reagents. However, the findings combine to reveal a functional range of BCR ligation strength that determines B cell differentiation. High-dose, high-affinity stimulation induces strong IgG1 switching but inhibits Ab secretion to such an extent that little IgG1 appears in cell culture supernatants. Low-dose, low-affinity stimulation does not inhibit ASC differentiation but also has little effect on isotype switching. Thus, most Ab produced following these activation conditions will be low-affinity IgM Ab, as previously postulated (47, 48). Although we remain unsure of the physiological relevance of these observations, it is possible that isotype-switched, high-affinity B cells are preferentially directed to a memory pool rather than to plasma cell differentiation. However, we also show that stimulation with intermediate concentrations of high-affinity Ag induces a balance of isotype switching and ASC differentiation that leads to significant IgG1 secretion.

Our findings demonstrate that BCR binding by Ag does not simply serve to control the specificity of B cell responses but also plays an active role in determining the functional outcome of the response. The mammalian immune system has evolved to produce multiple different isotypes with different functional activities. If particular isotypes are known to function more effectively or efficiently to clear different classes of infectious agents, then it would be desirable to target vaccination strategies to generate memory B cell pools containing a preferential abundance of that particular isotype. Our findings on the role of Ag affinity and dosage in regulating the level of isotype switching (via AID expression) and the isotype outcome thus provide important fundamental information for future progress in vaccine design.

Disclosures

The authors have no financial conflicts of interest.

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