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ORIGINAL ARTICLE

Naïve and memory B cells exhibit distinct biochemical responses following BCR engagement

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Immunological memory is characterized by the rapid reactivation of memory B cells that produce large quantities of high-affinity antigen-specific antibodies. This contrasts the response of naïve B cells, and the primary immune response, which is much slower and of lower affinity. Memory responses are critical for protection against infectious diseases and form the basis of most currently available vaccines. Although we have known about the phenomenon of long-lived memory for centuries, the biochemical differences underlying these diverse responses of naïve and memory B cells is incompletely resolved. Here we investigated the nature of B-cell receptor (BCR) signaling in human splenic naïve, IgM$^+$ memory and isotype-switched memory B cells following multivalent BCR crosslinking. We observed comparable rapid and transient phosphorylation kinetics for proximal (phosphotyrosine and spleen tyrosine kinase) and propagation (B-cell linker, phospholipase Cγ2) signaling components in these different B-cell subsets. However, the magnitude of activation of downstream components of the BCR signaling pathway were greater in memory compared with naïve cells. Although no differences were observed in the magnitude of Ca^{2+} mobilization between subsets, IgM$^+$ memory B cells exhibited a more rapid Ca^{2+} mobilization and a greater depletion of the Ca^{2+} endoplasmic reticulum stores, while IgG$^+$ memory B cells had a prolonged Ca^{2+} uptake. Collectively, our findings show that intrinsic signaling features of B-cell subsets contribute to the robust response of human memory B cells over naïve B cells. This has implications for our understanding of memory B-cell responses and provides a framework to modulate these responses in the setting of vaccination and immunopathologies, such as immunodeficiency and autoimmunity.
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Long-lived serological memory is critical for protection against human infectious diseases and is the basis for the success of most currently available human vaccines.[1,2] Antibody (Ab) memory is mediated by two distinct but complementary populations of differentiated B cells—memory cells and plasma cells—which are generated during the primary response from naïve B cells following their initial encounter with foreign antigens (Ag).[2–4] In contrast to the primary Ab response elicited from naïve B cells, the secondary response is characterized by the rapid activation of memory B cells that produce large quantities of high-affinity Ag-specific Ab upon challenge with the same Ag.[1–4]

There are numerous features of memory B cells that enable them to respond rapidly following re-exposure to a pathogen or immunizing Ag. These include a greater frequency of high-affinity Ag-specific memory B cells compared with naïve B cells[1,5]; the localization of memory B cells to sites of Ag drainage (splenic marginal zone (MZ), mucosal epithelium of the tonsil), providing greater access to foreign Ag[6–9]; and the constitutive expression by memory B cells of co-stimulatory molecules (CD80, CD86),[8–14] allowing them to activate CD4$^+$ T cells and solicit help for their subsequent differentiation.[8,11] Memory B cells also express higher levels of key pro-survival and proliferative genes than naïve cells and reduced levels of negative regulators of the cell cycle, indicating the ability of naïve B cells to enter cell cycle and differentiate into plasma cells is more stringently regulated than memory cells.[11,15–20] These findings are consistent with memory B cells having greater turnover *in vivo* than naïve B cells,[21] as well as with memory B cells entering their first division earlier and undergoing greater proliferation and differentiation *in vitro* than naïve B cells.[22–30] Collectively, these features of memory B cells contribute to the rapidity and efficacy of memory Ab responses.

Although previous studies have focused on comparing responses of naïve and memory B cells induced by engaging CD40 and cytokine receptors,[11,26–30] a key determinant of the B-cell response to Ag is the quality and quantity of the signal delivered through the B-cell receptor (BCR).[4,31,32] Naïve and memory B cells can differ with respect to the nature of their BCR isotype, with naïve cells expressing immunoglobulin M (IgM) and IgD, and memory B cells expressing either IgM/IgD, IgM only or the switched isotypes IgG, IgA or IgE.[2,4,33] BCR signaling is a complex multistep cascade, involving tyrosine, serine-threonine and lipid kinases, numerous adaptor molecules and transcription factors that regulate the expression of target genes[4,31–33] (see Figure 1). Igα/β chains link the BCR to the downstream signaling machinery through cytoplasmic immunoreceptor tyrosine-based activating motifs, which are phosphorylated by Lyn following BCR ligation. This activates Syk resulting in the recruitment and activation

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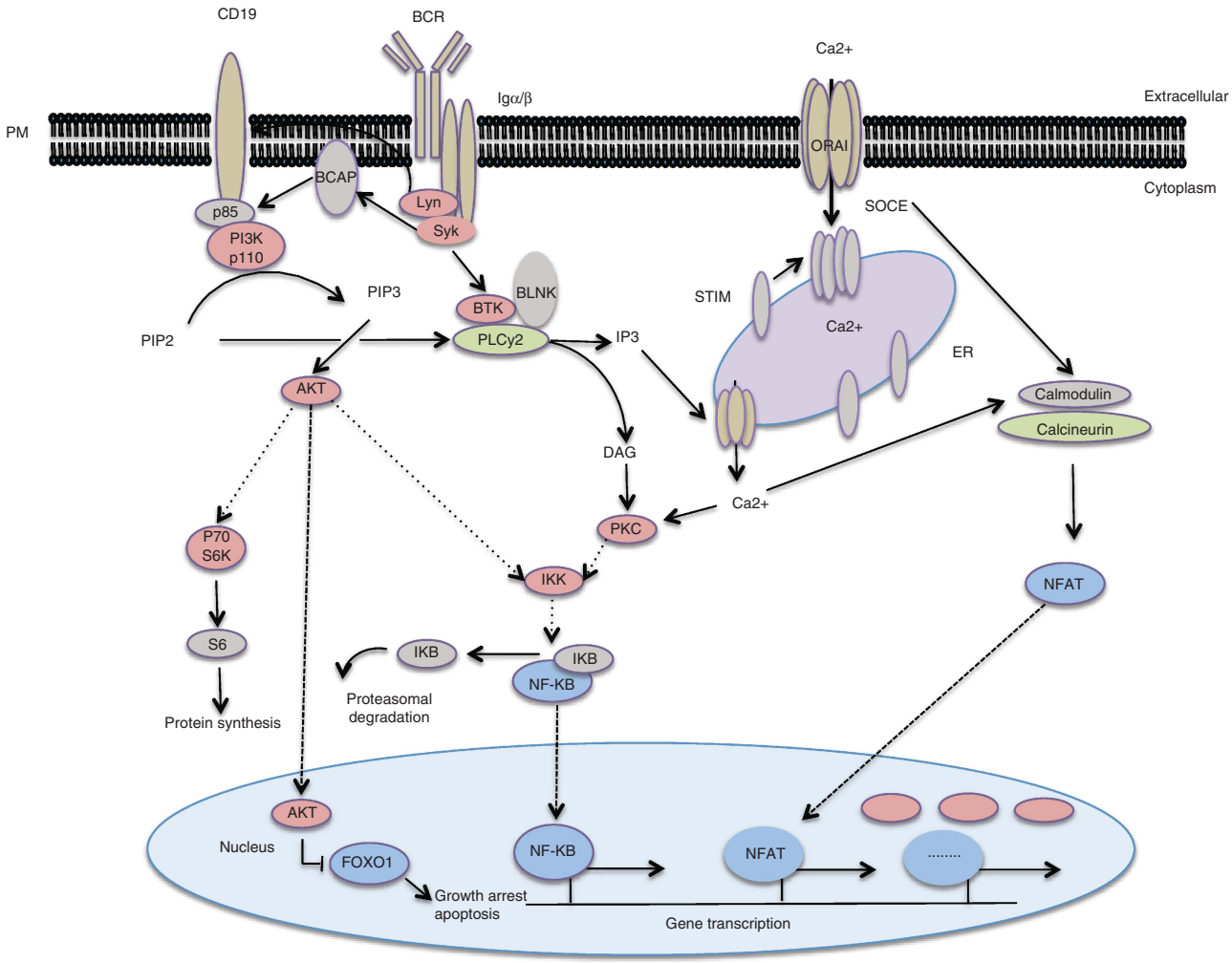


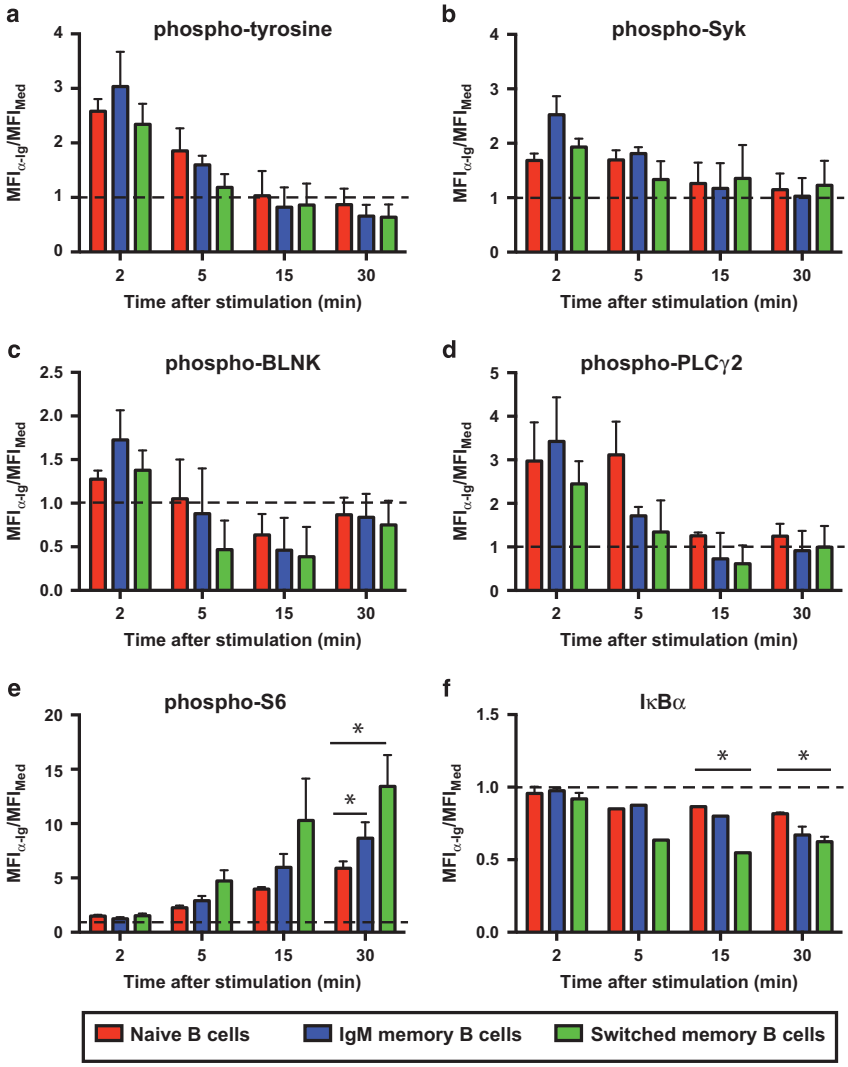
Figure 1 BCR signaling pathway. Simplified schematic presentation of the BCR signaling pathway. This shows the major proximal, propagation and distal pathways activated following ligation of the B-cell Ag receptor and the regulation of Ca^{2+} flux in response to BCR stimulation. BCAP, B-cell adaptor for PI3K; DAG, diacylglycerol; PM, plasma membrane; IKK, inhibitor of kappa B kinase; IkB, inhibitor of kappa B kinase beta subunit; IP3R, IP3 receptor; NFAT, nuclear factor of activated T cells; PKC, protein kinase C; S6K, ribosomal protein S6 kinase; SOCE, store-operated Ca^{2+} entry.

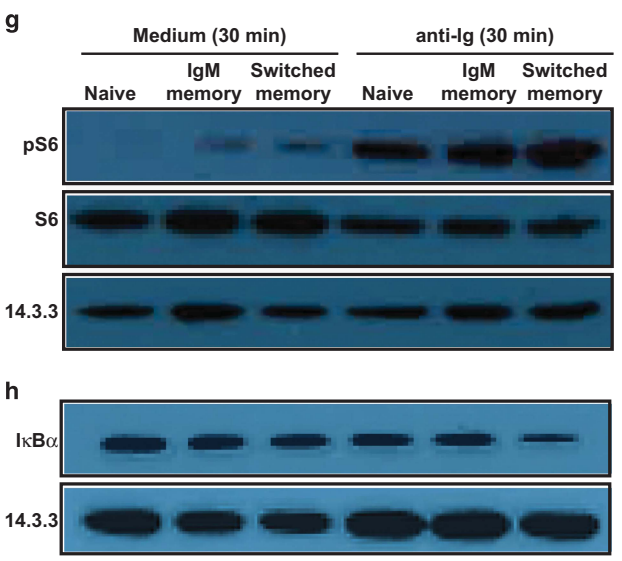
of Bruton's tyrosine kinase (Btk), phospholipase Cγ2 (PLCγ2) and phosphatidylinositol 3-kinase (PI3K). Activated PLCγ2 cleaves phosphatidylinositol-4,5-biphosphate (PIP2) to generate diacylglycerol and inositol 1, 4, 5-triphosphate (IP3), while activated PI3K generates PIP3 from PIP2. Subsequent downstream BCR signaling cascades result in activation and translocation of nuclear factor (NF)-κB, IP3 binding its receptor on the endoplasmic reticulum (ER) inducing the prompt release of Ca^{2+} from ER stores, while stromal interaction molecules (STIMs) sense changes in the levels of Ca^{2+} stored within the ER lumen to regulate Ca^{2+}-release activated Ca^{2+} channel in the plasma membrane, a process referred to as store-operated Ca^{2+} entry.[31,33,34] Additional outcomes of BCR engagement include PLCγ2-mediated activation of Ras resulting in mitogen-activated protein kinase activation, Ca^{2+}-dependent activation of NF-AT and PI3K/PIP3-mediated recruitment of Akt to the plasma membrane where it is phosphorylated and activated.[4,31–34] Akt has key roles in BCR signaling by activating mechanistic target of rapamycin, p70S6K and ribosomal protein S6 (S6) to regulate protein synthesis and, by phosphorylating and inactivating FoxO proteins, induce clonal expansion.[35]

The different Ig isotypes expressed by naïve and memory B cells have distinct structures: IgM and IgD have very short cytoplasmic domains (three amino acids), whereas those of IgG, IgA and IgE are longer (25–30 amino acids).[4,32,33] Thus enhanced signaling by switched isotypes is one mechanism that promotes the rapid response of memory B cells. Indeed, the IgG1 cytoplasmic domain increased the response *in vivo* of IgG1-expressing murine B cells, compared with those expressing IgM, in response to T-dependent Ag.[36–38] Detailed analyses of primary murine and human B cells indicated that IgG BCRs are more motile and exhibit greater spontaneous oligomerization and cluster formation than IgM BCRs.[39–41] IgG, but not IgM, associates with signaling molecules such as Grb2[42–44] and SAP97.[45] Collectively, these attributes may in part explain the increased activation of BCR-associated signaling pathways in IgG^+ versus IgM^+ B cells, which may underlie rapid and enhanced responses typical of IgG^+ memory B cells.[38,40,42–47]

Despite several studies highlighting that a switched Ig isotype is the determinant underlying accelerated responses of memory B cells, there are several caveats that need consideration. First, the memory compartment in humans and mice is heterogeneous, comprising populations that are IgM^+, as well as isotype-switched (that is, IgG^+)

cells.[2,4,9,48,49] Thus *in vitro* studies of human B cells that defined naïve and memory cells according to IgM or IgG, respectively, expression overlooked the contribution that IgM$^+$ memory B cells would make to the response of the 'naïve' population.[40,47] Second, *in vitro* studies of human B-cell subsets indicated that the kinetics and magnitude of responses of both IgM$^+$ memory and switched memory B cells substantially exceeded that of corresponding naïve B cells, although the response of switched memory B cells tends to be greater than that





of IgM$^+$ memory cells.[11,16,26,29,30,50] Thus the cytoplasmic domain of switched Ig isotypes alone does not explain the robust response of human IgM$^+$ memory B cells. This is supported by an elegant study of Kurosaki and colleagues, who found that naïve B cells engineered from embryonic stem cells to express IgG1 behaved more like naïve IgM$^+$ B cells when exposed to cognate Ag for the first time than IgG1$^+$ memory B cells that were generated *in vivo* following encounter with specific Ag.[51] Third, SAP97-deficient mice exhibited no defect in mounting a secondary humoral immune response,[52] while Grb2-deficient B cells exhibit both enhanced and impaired responses to BCR stimulation,[53,54] questioning whether unique associations between IgG and Grb2[42–44] or SAP97[45] in murine B cells are the only mechanisms underlying the enhanced responses of memory B cells *in vivo*. Thus factors beyond Ig isotype alone underlie the ability of memory B cells to exhibit greater responses than naïve B cells. To gain further insight into the unique response of naïve and memory B cells, we have now investigated phosphorylation kinetics of BCR signaling proteins, degradation of IκBα and Ca^{2+} mobilization in human splenic naïve, IgM$^+$ memory B cells and isotype-switched memory B cells.

RESULTS

Phosphorylation kinetics of signaling proteins following BCR stimulation of human B-cell subsets

The changes in phosphorylation status of different BCR signaling proteins were measured as the ratio of mean fluorescence intensity of cells stimulated with F(ab)'$_2$ fragments of goat anti-human Ig (specific for IgM, IgG, IgA) at a specific time point over the mean fluorescence intensity of non-stimulated cells at the same time point to exclude non-specific changes in phosphorylation status based on sample handling. When we assessed the activation kinetics of proximal (phospho-tyrosine, spleen tyrosine kinase (SYK)) and propagation (B-cell linker (BLNK), PLCγ2) elements of the BCR signaling pathway, phosphorylation was maximal after 2 min of BCR stimulation; after this time, these signaling molecules underwent dephosphorylation, returning to basal levels within 30 min (Figures 2a–d). No significant differences were observed in the phosphorylation kinetics of tyrosine, SYK, BLNK or PLCγ2 in naïve, IgM memory or switched memory B-cell subsets after BCR stimulation (Figures 2a–d).

In contrast to the proximal and propagation components, phosphorylation of distal BCR signaling proteins (represented by S6) increased over time in naïve, IgM memory and switched memory B-cell subsets, reaching the greatest levels 30 min after BCR engagement (Figure 2e). Furthermore, the magnitude of S6 phosphorylation at this time was significantly higher in both IgM and switched memory B cells compared with naïve B cells (Figure 2e). Greater levels of phospho-S6 was also observed in switched memory B cells over IgM memory B cells 30 min after BCR stimulation (Figure 2e). These observations were confirmed by western blotting, with heightened levels of pS6 being detected in memory versus naïve B cells (Figure 2g).

Degradation of IκBα after BCR stimulation in human B-cell subsets

Phosphorylation of IκBα leads to its degradation, resulting in nuclear translocation of NF-κB dimers, where they induce gene transcription. To evaluate another distal event in the BCR signaling pathway, we measured IκBα degradation. IκBα degradation was first detected within 5 min of stimulation and continued for up to 30 min. Significantly increased degradation of IκBα was observed in switched memory B cells compared with naïve B cells 15 and 30 min after BCR stimulation (Figure 2f). No differences were detected between IgM or switched memory B-cell subsets (Figure 2f). Similar results were also made when IκBα degradation was assessed by western blotting (Figure 2h). Thus switched memory B cells exhibit accelerated kinetics and a greater response following BCR ligation than IgM memory and naïve B cells, with the response of IgM memory B cells being intermediate. Importantly, although IgD is expressed at a higher level on human naïve B cells than on IgM$^+$ memory B cells,[7,9,22,49] as the F(ab')$_2$ anti-Ig reagent used here lacked specificity for IgD, the differential responses of naïve and IgM$^+$ memory B cells did not reflect differential involvement of IgD-mediated signaling in these cells.

Ca^{2+} mobilization after BCR stimulation in human B-cell subsets

We next measured Ca^{2+} mobilization as a readout of BCR signaling downstream of SYK, BLNK and PLCγ2 in different human B-cell subsets (Figure 3). Naïve, IgM$^+$ memory, IgG$^+$ memory and IgA$^+$ memory B cells were stimulated with F(ab)$_2$ fragments of goat anti-human Ig (IgG/A/M) and Ca^{2+} mobilization was recorded over time (10 min). On average, IgM$^+$, IgG$^+$ and IgA$^+$ cells comprised 49.8 ± 10.3% (range: 23.2-77.0%), 26.9 ± 7.8% (range: 9.7-50.2%) and 21.0 ± 2.7% (range: 11.4-28%) of the memory B-cell population (mean ± sem; $n = 5$), respectively. In our hands, IgE$^+$ memory B cells are largely undetectable in the human spleens (not shown). Ca^{2+} mobilization kinetics of human B-cell subsets is presented in Figure 3a. During the last 2 min of the assay, B-cell subsets were treated with ionomycin; no differences in their maximum Ca^{2+} capacity uptake were observed. No differences were observed for the extent of maximum Ca^{2+} mobilization between the four B-cell subsets after BCR stimulation, as measured by the ratio of the peak Ca^{2+} level over the level at baseline (Figures 3a and b). However, IgM memory B cells showed more rapid Ca^{2+} mobilization than naïve B cells as measured by the slope of the reaction during the first minute following BCR stimulation (Figures 3a and c). Naïve B cells showed a prompt decline to baseline Ca^{2+} levels that was significantly quicker than memory B-cell subsets after BCR stimulation (Figure 3a and d). In contrast, Ca^{2+} levels in BCR-stimulated IgM$^+$ and IgA$^+$ memory B cells remain relatively constant, whereas IgG$^+$ memory B cells displayed prolonged Ca^{2+} uptake over time (Figure 3a and d). These observations were confirmed when we determined the time taken to reach maximal Ca^{2+} level in the B-cell subsets, with that of IgG$^+$ memory B cells being substantially greater than naïve and IgM$^+$ memory B cells (Figure 3a and e).

Figure 2 BCR signaling events in response to BCR crosslinking in different B-cell subsets. Sort-purified human splenic naïve, IgM memory and switched memory B cells were stimulated with 15 μg ml^{-1} F(ab')$_2$ fragments of goat anti-human Ig (IgG+IgA+IgM) for 2, 5, 15 and 30 min. After this time, induction of phosphorylation of (**a**) total tyrosine (pTY), (**b**) SYK, (**c**) BLNK, (**d**) PLCγ2 and (**e**) S6 was measured as the ratio of the mean fluorescence intensity (MFI) of stimulated over MFI of unstimulated cells at the same specific time point by flow cytometry. (**f**) Degradation of IκBα after BCR stimulation over time was measured by flow cytometry. The values in panels (**a**–**f**) represent the mean ± s.e.m. ($n = 3$–6 (except for 5- and 15-min time points for IκBα, $n = 1$)). *$P < 0.05$; multiple *t*-tests with correction for multiple comparisons (Prism). (**g**) Phosphorylation of S6 and the levels of total S6 and (**h**) degradation of IκBα was also determined in sorted and BCR-stimulated B-cell subsets by western blotting. Expression of 14.3.3. protein was used as the sample loading control (representative of two independent experiments).

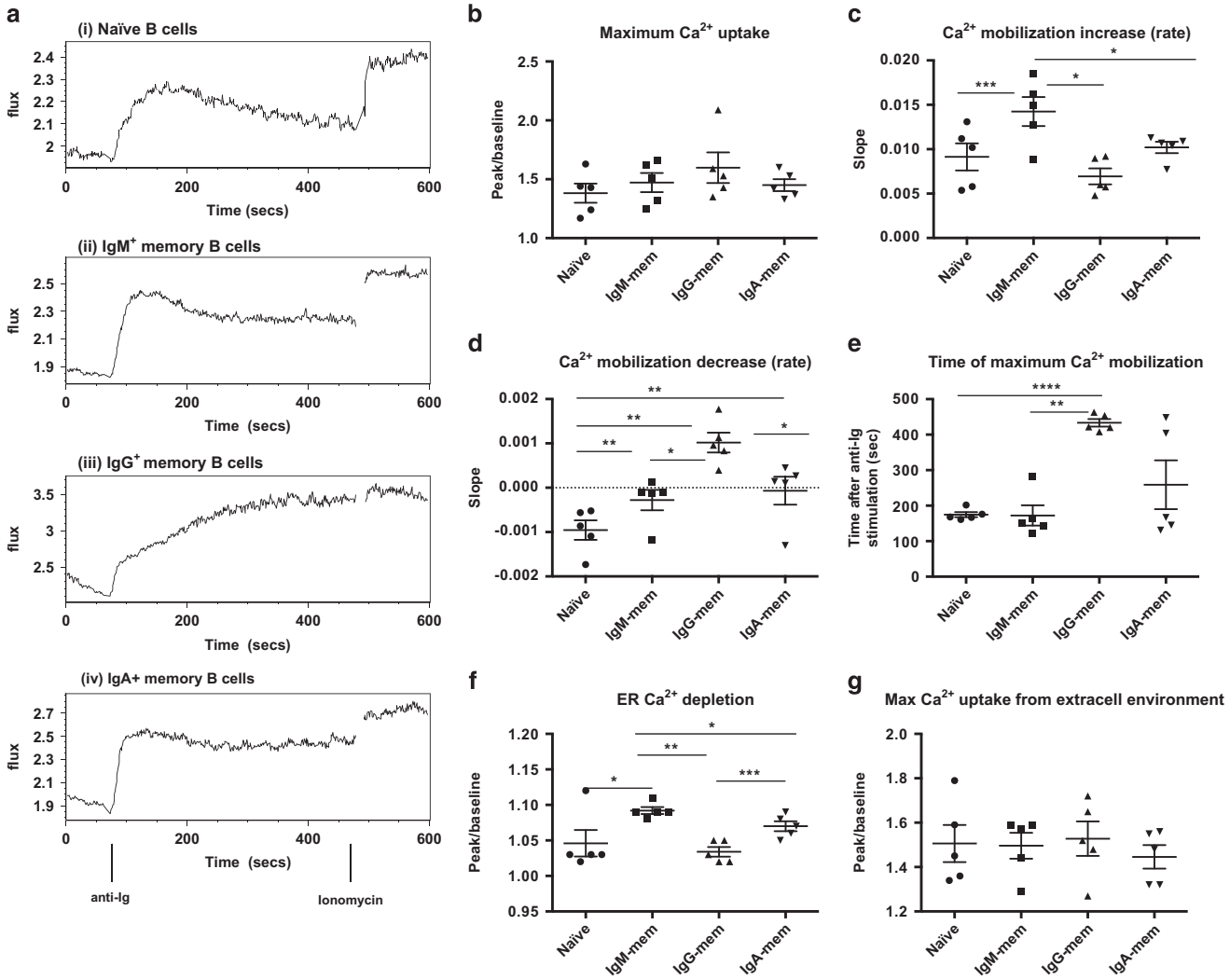


Figure 3 Ca^{2+} mobilization after BCR crosslinking in different B-cell subsets. Human splenic naïve ($CD20^+CD27^-IgG/E/A^-$), IgM^+ memory ($CD20^+CD27^+IgG/E/A^-$; IgM-mem), IgG^+ memory ($CD20^+CD27^+IgM/D/E/A^-$; IgG mem) and IgA^+ memory ($CD20^+CD27^+IgM/D/E/G^-$, IgA mem) labeled B cells were loaded with Indo 1-AM dye and then stimulated with 70 μg ml^{-1} $F(ab')_2$ fragments of goat anti-human Ig (IgG/A/M; anti-Ig) and Ca^{2+} mobilization was recorded over the next 10 min. After 7 min of anti-Ig stimulation, the cells were then stimulated with ionomycin (2 μg ml^{-1}). (**a**) Kinetics of Ca^{2+} mobilization of the different B-cell subsets, shown as geometric mean and Gaussian smoothing by FlowJo (representative of five independent experiments). (**b**) Maximum Ca^{2+} uptake presented as Ca^{2+} level peak/baseline Ca^{2+} level. (**c**) Rate of increase of Ca^{2+} mobilization, calculated as the slope of the curve during the first minute following anti-Ig stimulation. (**d**) Rate of decline of Ca^{2+} mobilization, calculated as the slope of the curve during the second phase (between 3 and 8 min) after anti-Ig stimulation. (**e**) Time of maximal Ca^{2+} mobilization following anti-Ig stimulation. (**f**, **g**) Depletion of ER Ca^{2+} storage (**f**) and maximum uptake of Ca^{2+} from the extracellular environment (**g**) were determined by performing the experiments in medium supplemented with 0.5 mM EGTA; medium was reconstituted with 4 mM $CaCl_2$ 4 min after anti-Ig stimulation. $^*P<0.05$, $^{**}P<0.01$; $^{***}P<0.005$ one-way analysis of variance followed by correction for multiple comparisons (Holm–Sidak method) (Prism; GraphPad Software).

Ca^{2+} mobilization consists of two phases: (1) depletion of intracellular Ca^{2+} stores from the ER and (2) uptake of Ca^{2+} from the extracellular environment.[33] For this reason, Ca^{2+} mobilization in the different B- cell subsets was also measured after depletion of Ca^{2+} from medium with EGTA (ethylene glycol tetraacetic acid) just prior to BCR stimulation. IgM^+ and IgA^+ memory B cells were found to have a higher depletion of ER-stored Ca^{2+} compared with IgG^+ memory B cells (Figure 3f). In contrast, uptake of Ca^{2+} from the extracellular environment was comparable for the different B-cell subsets examined (Figure 3g). Overall, memory B-cell subsets exhibit unique patterns of Ca^{2+} mobilization that could underlie their greater response to BCR stimulation than naïve B cells.

Differential expression of intracellular signaling proteins by human splenic B-cell subsets

Memory B cells are 20–30% larger than naïve B cells (Figure 4a).[8–10,13,14] Therefore, memory B cells have a greater protein content than naïve B cells. Thus it was important to determine whether differences in the proximal phase of BCR signaling (Figure 2) or Ca^{2+} mobilization (Figure 3) by naïve and memory B cells reflected differences in the expression levels of some of the key proteins involved in these processes in these B-cell subsets. For this reason, we assessed the protein levels of PLCγ2, IP3R, STIM1 and ORAI1 in lysates prepared from sort-purified naïve, IgM memory and switched memory B cells. IgM memory and switched memory B cells contained significantly higher levels of PLCγ2 than corresponding naïve B cells (Figure 4b).

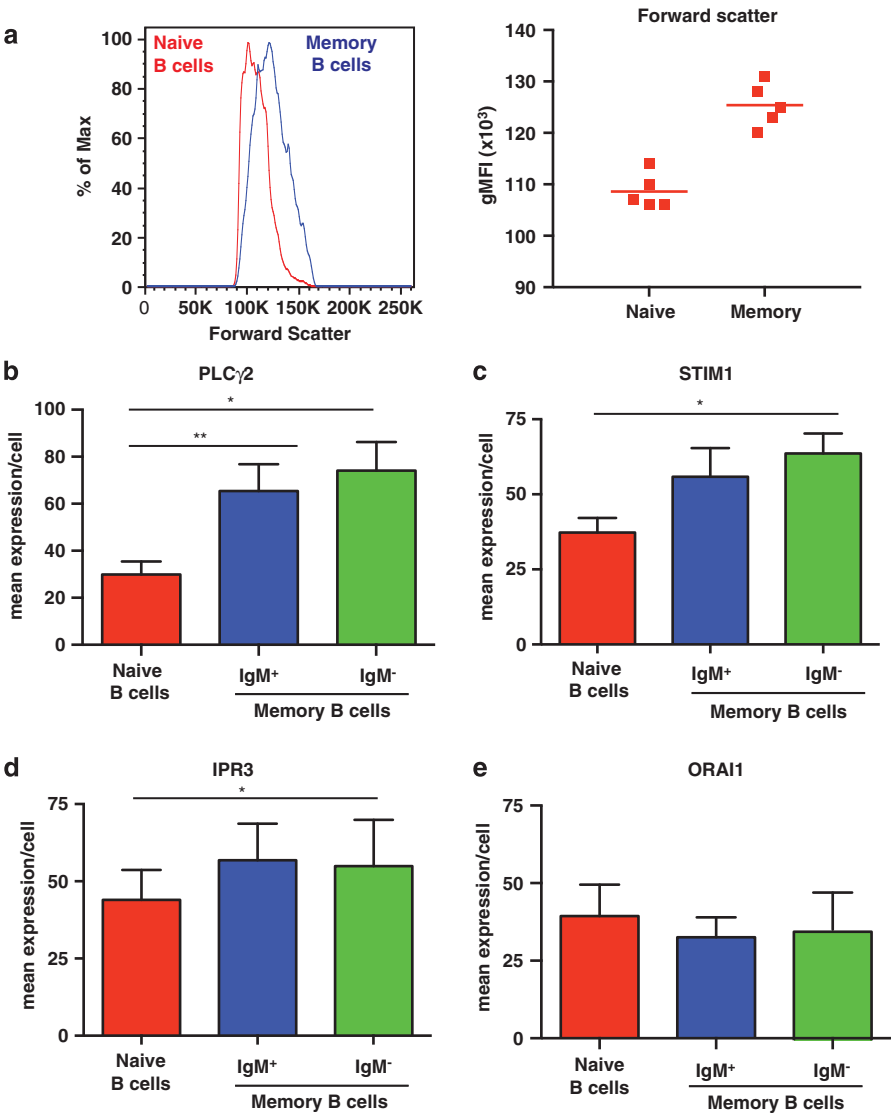


Figure 4 Memory B cells express elevated levels of PLCγ2 and STIM1 but not other downstream signaling components. (**a**) Forward scatter of naïve (red histogram) and memory (blue histogram) B cells was determined by flow cytometry. The summary graph depicts the mean forward scatter (determined by geometric mean fluorescence intensity (gMFI)) of naïve and memory B cells isolated from five different donor spleens. (**b**–**e**) Naïve ($CD20^+CD27^-IgM/D^+$), IgM memory ($CD20^+CD27^+IgM/D^+$) and switched memory ($CD20^+CD27^+IgG/E/A^+$) B cells were isolated from human spleens. Whole-cell lysates were then prepared and the expression levels of PLCγ2 (**b**), STIM1 (**c**), IP3R (**d**) and ORAI1 (**e**) were determined by western blotting. Data are presented as mean area under the curve/cell ± s.e.m. corrected with the loading control 14.3.3 ($n=4$–6). $^*P<0.05$; $^{**}P<0.01$; one-way analysis of variance correction for multiple comparisons (Holm–Sidak method, Prism; GraphPad Software).

Switched memory B cells also have significantly higher levels of STIM1 and IRP3 per cell than naïve cells (Figures 4c and d). In contrast, the expression of ORAI1 (Figure 4e) by naïve, IgM memory and switched memory B cells was similar on a per cell basis. Similarly, the expression levels of STAT3 (signal transducer and activator of transcription factor 3) and ERK2 (extracellular signal–regulated kinase 2) are similar in naïve and memory B-cell subsets.[14] Thus memory B cells can express increased levels of some but not all key signaling intermediates than naïve B cells.

Dose-dependent induction of Ca^{2+} mobilization reveals a lower activation threshold in IgM memory B cells compared with naïve B cells

IgM^+ memory B cells express approximately twice as many BCRs on their surface than naïve B cells (Figure 5a).[12,13,26,55] To test the possibility that the significant differences in Ca^{2+} mobilization between naïve B cells and IgM^+ memory B cells (Figures 3c,d and f) resulted from differences in the amounts of surface IgM, naïve and IgM^+ memory B cells were stimulated with different concentrations of $F(ab')_2$ anti-Igμ and Ca^{2+} mobilization was then tracked. The level of Ca^{2+} mobilization increased in a dose-dependent manner in both naïve and IgM^+ memory B cells. However, initial mobilization of Ca^{2+} —as measured by the slope of the curve during the first minute after stimulation—was quicker in IgM^+ memory B cells than in naïve B cells for all concentrations of $F(ab')_2$ anti-Ig tested (Figure 5b). Furthermore, the concentration of $F(ab')_2$ anti-Ig required to induce a response in all IgM^+ memory B cells was approximately 10-fold less than that required to yield a comparable response from naïve B cells (3.8 versus 30 μg ml^{-1}). Thus it is likely that the heightened response of IgM^+ memory B cells results from both engagement of a higher

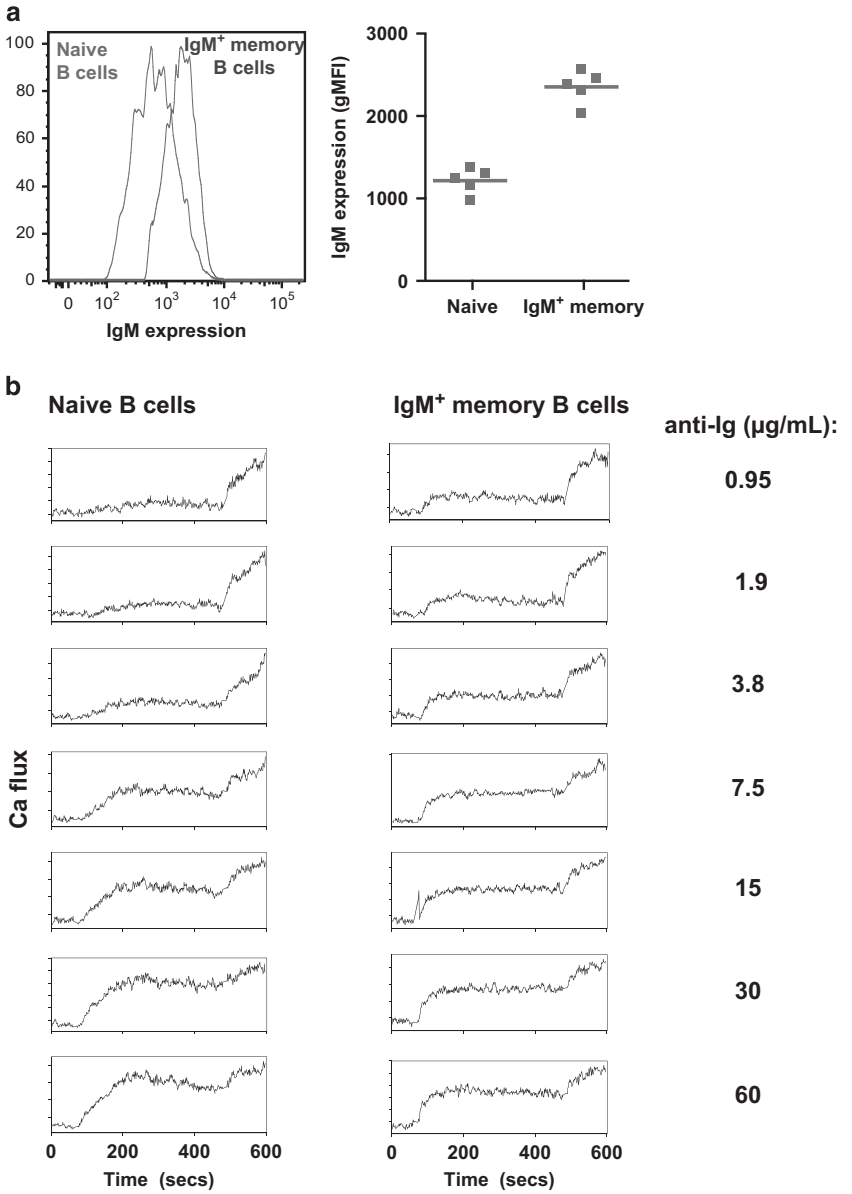
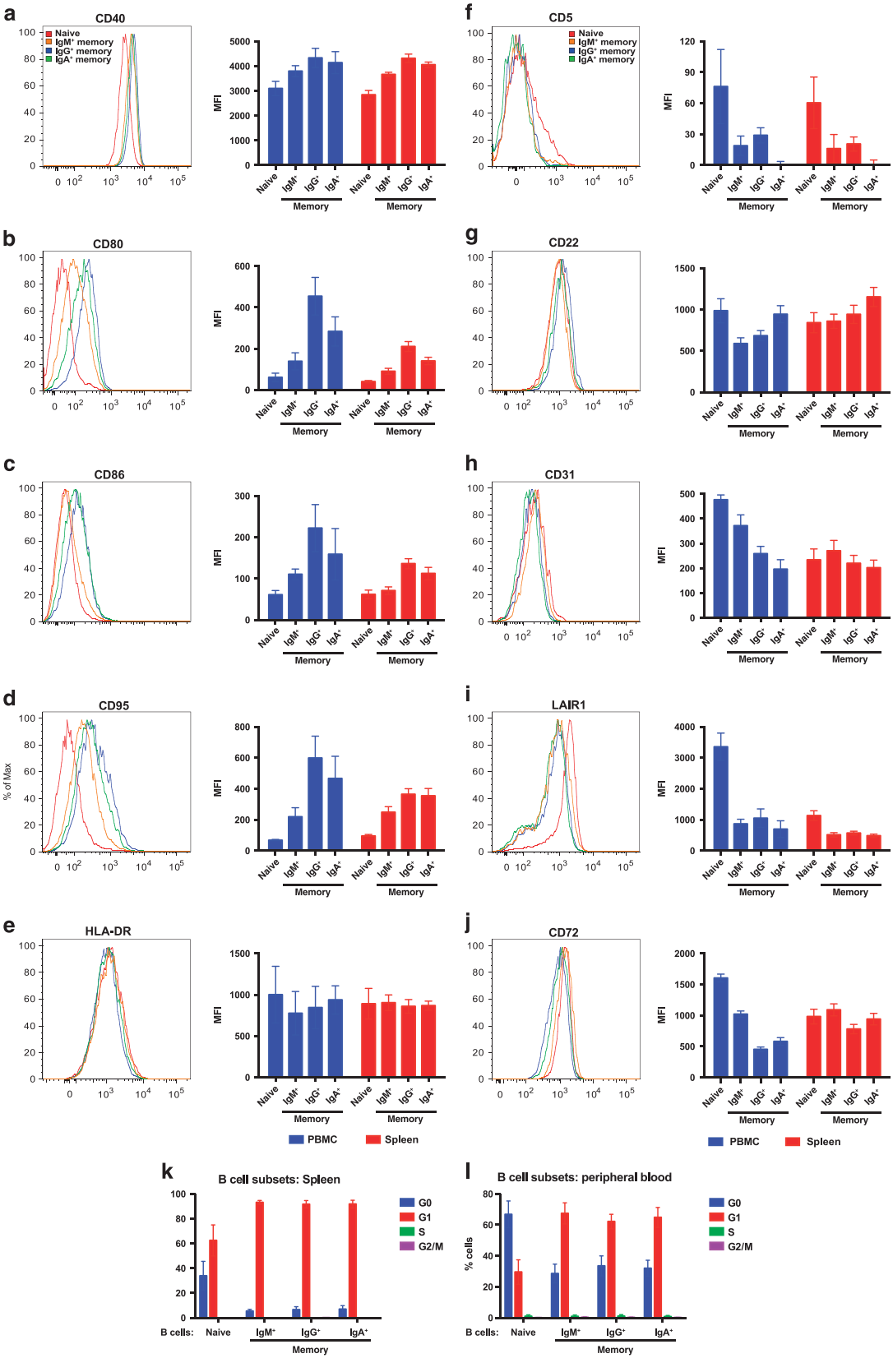


Figure 5 IgM memory B cells exhibit exaggerated dose-dependent Ca^{2+} mobilization compared with naïve B cells. (**a**) Splenic mononuclear cells were labeled with anti-CD20, anti-CD27 and anti-IgM mAbs. Naïve B cells and memory B cells were identified as $CD20^+CD27^-$ and $CD20^+CD27^+$, respectively, and the mean fluorescence intensity (MFI) of IgM expression on each B-cell subset was then determined. A representative histogram plot of IgM expression on naive and IgM^+ memory B cells is depicted. The summary graph depicts the mean IgM expression (determined by geometric MFI (gMFI)) of naïve and memory B cells isolated from five different donor spleens. (**b**) Human splenic naïve ($CD20^+CD27^-IgG/E/A^-$) and IgM memory ($CD20^+CD27^+IgG/E/A^-$) B cells were stimulated with the indicated concentrations of $F(ab')_2$ fragments of goat anti-human IgM, and Ca^{2+} mobilization was recorded, as detailed for Figure 3. These data are indicative of three different experiments. A full color version of this figure is available at the *Immunology and Cell Biology* journal online.

Figure 6 Distinct phenotype and cell cycle distribution of naïve, IgM^+ memory and class switched memory B cell subsets. (**a**–**j**) Human peripheral blood (red columns) or splenic (blue columns) mononuclear cells were labeled with anti-CD20, CD27, IgM, IgG and IgA mAbs and either mAbs specific for (**a**) CD40, (**b**) CD80, (**c**) CD86, (**d**) CD95, (**e**) HLA-DR, (**f**) CD5, (**g**) CD22, (**h**) CD31, (**i**) LAIR-1 or (**j**) CD72. Naïve (red histogram; $CD20^+CD27^-IgM^+IgG^-IgA^-$), IgM^+ memory (orange histogram; $CD20^+CD27^+IgM^+IgG^-IgA^-$), IgG^+ memory (green histogram; $CD20^+CD27^+IgM^-IgG^+IgA^-$) or IgA^+ memory (blue histogram; $CD20^+CD27^+IgM^-IgG^-IgA^+$) B cells were identified and the expression of the indicated surface receptor was determined. The histograms are representative of the expression of the indicated surface receptor on the different populations of splenic B cells; the summary graphs represent the mean geometric mean fluorescence intensity (gMFI) ± s.e.m. from experiments using B cells from 5 different spleens of 2–4 different blood donors. (**k**, **l**) Human splenic (**k**) or peripheral blood (**l**) mononuclear cells were labeled with anti-CD20, CD27, IgM, IgG and IgA mAbs, fixed and permeabilized followed by labeling with anti-Ki67 and DAPI. The distribution of naïve and IgM^+, IgG^+ or IgA^+ memory B cells in the indicated phases of the cell cycle was then determined. The summary graphs represent the mean % ± s.e.m. from experiments using three different spleens of four different blood donors.


a
CD40
Naive
IgM+ memory
IgG+ memory
IgA+ memory
MFI
Naive
IgM+
IgG+
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Memory
b
CD80
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CD86
d
CD95
% of Max
e
HLA-DR
f
CD5
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CD22
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B cell subsets: Spleen
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number of BCRs and a lower threshold of activation of these cells relative to corresponding naïve B cells.

Phenotypic differences between naïve and memory B-cell subsets may also contribute to enhanced responses of memory B cells

Naïve and memory B cells can be distinguished from each other by expression of a unique phenotype. In addition to differences in the expression of CD27,[9,49] memory B cells express increased levels of CD80, CD86 and CD95,[8–14] while naïve B cells express low levels of CD5 which is further downregulated on memory B cells.[11,12] Thus we next examined human B-cell subsets for the expression of activation and inhibitory receptors to determine whether differences in expression of these molecules may also contribute to the enhanced signaling and response characteristics of memory over naïve B cells. Specifically, we determined the expression of CD40, CD80, CD86, CD95, HLA-DR, CD5, CD22, CD31, LAIR-1 and CD72 on naïve B cells as well as memory B cells that expressed IgM, IgG or IgA. To extend our study beyond splenic B-cell subsets, this analysis was performed not only on B-cell subsets present in the spleen but also on those in peripheral blood from different donors. The expression of CD40 increased as naïve B cells differentiated into memory B cells and underwent Ig class switching (Figure 6a). Consistent with previous studies from our laboratory,[9–12] as well as those from other groups,[8] that studied naïve B cells and either total memory B cells or IgM-expressing and class switched memory B cells, the expression of CD80, CD86 and CD95 was substantially increased on IgG^+ and IgA^+ memory B cells compared with naïve B cells, while expression on IgM^+ memory B cells was intermediate to naïve and switched memory B cells (Figures 6b–d). In contrast to these receptors, HLA-DR was expressed comparably across all B-cell subsets (Figure 6e). This pattern of expression of activation molecules was observed for both splenic and circulating B-cell subsets (Figures 6a–e). When we assessed inhibitory receptors, CD5 and LAIR-1 were expressed highest on naïve B cells in both the spleen and peripheral blood (Figures 6f and i). Both of these receptors were markedly downregulated on all memory B cells, with CD5 being largely undetectable on splenic and blood IgA^+ memory B cells (Figures 6f and i). Peripheral blood naïve B cells expressed the highest levels of CD31 and CD72, and these receptors were reduced on IgM^+ memory B cells and further downregulated on class switched memory B cells (Figures 6h and j). In contrast to circulating memory B cells, CD31 and CD72 were expressed at similar levels on all B-cell subsets present in the human spleen (Figures 6h and j). Finally, and similar to HLA-DR, CD22 was expressed at comparable levels on all B-cell subsets in both the spleen and peripheral blood (Figure 6g). Thus notable differences in the expression of activating and inhibitory receptors by naïve and memory B cells could contribute to greater responses of memory over naïve B cells.

Distinct distributions of naïve and memory B-cell subsets in the different phases of the cell cycle

Following activation with diverse stimuli, such as agonists of CD40, the BCR, TLRs or cytokine receptors, memory B cells enter the cell cycle significantly earlier than naïve B cells.[11,16,26,28] This is consistent with earlier studies demonstrating that memory B cells exhibit greater proliferative responses *in vitro* than naïve B cells.[15,22,24] Coupled with the current observations of enhanced BCR-induced Ca^{2+} signaling in memory over naïve B cells (Figures 2 and 3), we sought to determine the distribution of resting naïve and memory B cells throughout the different stages of the cell cycle. This was achieved by simultaneously staining cells with 4,6-diamidino-2-phenylindole (DAPI) to determine DNA content as well as with anti-Ki67 monoclonal Ab (mAb) to determine the proportion of dividing cells.[56] Thus the distribution of cells across the G_0 ($DAPI^-Ki67^-$), G_1 ($DAPI^+Ki67^+$), S ($DAPI^{++}Ki67^+$) and G_2/M ($DAPI^{hi}Ki67^+$) phases of the cell cycle, where the different staining for DAPI corresponds to different DNA content in the cells, could be quantified. Analysis was performed on B-cell subsets from both the spleens and peripheral blood. For both anatomical sites, it was found that the vast majority (~95%) of naïve and memory B cells are in the G_0/G_1 phases of the cell cycle (Figures 6k and l). This is reminiscent of a previous study that examined peripheral blood naïve and memory B cells defined as $IgD^+G^-A^-$ and $IgD^-G^+A^+$ B cells, respectively.[15] However, when the G_0 and G_1 phases were examined individually, striking differences were observed. In the spleen, most memory B cells—>90%, irrespective of Ig isotype—were in G_1, while naïve B cells were split between G_0 (33.9%) and G_1 (62.4%) ($n=5$; Figure 6k). This indicates that the population of naïve B cells contains a large subset of quiescent (that is, G_0) cells, but almost all memory B cells are poised to enter division. A similar result was obtained from analysis of peripheral blood, that is, >95% of naïve and memory B cells were in G_0/G_1 (Figure 6l). However, there were notable differences between naïve and memory B cells in the blood and spleen. Although substantially more memory B cells were in G_1 than naïve B cells, a much larger fraction of peripheral blood naïve (~65%) and of all peripheral blood memory B-cell subsets (~30%) were in G_0 compared with corresponding subsets in the spleen (Figures 6k and l). This indicates that more naïve and all memory B cell subsets are in a resting/quiescent state in the peripheral blood compared with that in spleen, which is consistent with the likelihood of greater exposure of splenic B cells to immune activation compared with those in the peripheral circulation. Thus the heightened propensity of memory B cells to enter cell division would contribute to the enhanced responses of these cells to diverse stimuli compared with corresponding naïve B cells. These results shed new light on dynamic differences between naïve and memory B cells in humans, with the finding that the majority of memory B cells are positioned in G_1 is a further explanation for these cells being able to enter cell division more rapidly than naïve B cells.

DISCUSSION

Over the past few decades, the biochemical and molecular events involved in BCR signaling have been studied in detail.[4,31–33,35,41] Many studies have utilized immortalized B-cell lines from different species,[4,31,39] primary B cells from transgenic mice[37,46,51] or syngeneic or heterologous overexpression systems.[39,57] More recently, some studies have addressed BCR signaling events in primary human B cells.[40,47,55] These have revealed the complexities of BCR signal transduction and documented the influence of the Ig isotype on these processes with respect to the kinetics and magnitude of the response.[4,32,33,41] The latter findings are significant, because identifying molecular determinants of enhanced responses of IgG-expressing memory B cells over IgM-expressing naïve B cells could reveal mechanisms that underpin long-lived humoral immune responses that are mediated by memory cells and provide the host with protective immunity in the face of repeated pathogen exposure.[1–3] Indeed, the unique ability of the IgG cytoplasmic domain to couple to Grb2 and SAP97 has been proposed to mediate enhanced signaling through IgG versus IgM BCRs.[42–45] However, while differences in signaling between these BCRs, and by extension between naïve and memory B cells, have been described,[32,36–43,46,47] the fact that IgM^+ B cells are a component of humoral memory in mice and humans[2,4,30,48,49] and that the magnitude of responses of IgM^+

memory cells exceeds those of naïve cells[11,16,26,28,29,50] cannot be overlooked. Thus mechanisms beyond unique signaling characteristics of IgG BCRs must exist that contribute to the accelerated responses of memory B cells.

We have now analyzed BCR signaling in primary human splenic B-cell subsets (naïve, IgM^+ memory, switched memory) after multivalent BCR crosslinking. We observed comparable rapid and transient phosphorylation kinetics for proximal (phosphotyrosine, SYK) and propagation (BLNK, PLCγ2) signaling components in these different B-cell populations, with responses peaking within 2–5 min for all subsets and returning to baseline levels within 30 min. Investigation of signaling components further downstream of the BCR revealed slower phosphorylation kinetics, with phosphorylation of S6 and degradation of IκBα being detected in the three different B-cell subsets after 5 min of BCR engagement and continuing to increase at all subsequent time points analyzed. Importantly, at later times, the magnitude of S6 phosphorylation and IκBα degradation was greater in memory compared with naïve B cells. PI3K activates Akt, which subsequently phosphorylates S6.[35] Thus enhanced pS6 in BCR-activated memory B cells is consistent with greater accumulation of phosphorylated PI3K at the plasma membrane of human IgG^+ B cells,[40] and increased phosphorylation of Akt in IgM memory B cells,[55] compared with naïve B cells.

Early BCR signaling events converge to result in Ca^{2+} flux, which is important for activating NF-κB and ERK pathways.[31,34] The importance of calcium signaling in lymphocytes is evidenced by perturbed B-cell development, or the development of immune deficiencies or autoimmunity, in mice with mutations in components of the BCR signaling complex (for example, Btk, BLNK, PLCγ2, CD19, SHP1, SHIP, CD22, FcγRIIb, ITPKB, Grb2, STIM1, ORAI) that regulate calcium mobilization.[34] Until now, Ca^{2+} mobilization in naïve or IgM^+, IgG^+ or IgA^+ memory B cells following BCR engagement had not been investigated. We did not detect differences in the magnitude of Ca^{2+} mobilization between these different human B-cell subsets after BCR stimulation. However, we did observe important differences in the kinetics of Ca^{2+} mobilization. First, IgM^+ memory B cells exhibited more rapid Ca^{2+} mobilization than naïve and switched memory B cells. An extension of this was that IgM^+ memory B cells had greater depletion of Ca^{2+} from ER stores than other B-cell subsets. Second, IgG^+ memory B cells had a prolonged uptake of Ca^{2+}; this contrasts naïve B cells, where the Ca^{2+} level quickly returns to baseline, and IgM^+ and IgA^+ memory B cells, where the Ca^{2+} level remains constant, after BCR stimulation. Interestingly, Irish *et al.*[47] found that naïve B cells—defined as IgM^+ B cells—exhibit transient phosphorylation of ERK1/2 following BCR engagement, while BCR-induced ERK1/2 phosphorylation is sustained for up to 2 h in IgG^+ B cells. In light of our findings, and the involvement of Ca^{2+} mobilization in regulating ERK signaling,[34] it is likely that sustained Ca^{2+} responses in IgG^+ memory B cells underlies prolonged activation of ERK1/2 in these cells.

Our studies of signaling pathways in distinct human B-cell subsets offers parallels to previous studies, inasmuch that the kinetics and magnitude of memory B-cell responses exceeded those of naïve cells.[40,47,55] Together with data from transgenic systems in mice using IgM- or IgG-expressing B cells,[36–39,42–46] it is likely that our findings of heightened S6 phosphorylation and IκBα degradation and sustained Ca^{2+} flux in IgG^+ memory B cells compared with naïve and IgM^+ memory B cells can be attributed to unique signaling features of the IgG BCR.[4,32] However, the nature of the biochemical response in IgM^+ memory B cells was also distinct from naïve B cells, indicating that mechanisms beyond BCR Ig isotype contribute to the robust response of memory B cells over naïve B cells. Indeed, this is implicit from previous studies reporting that IgM^+ memory B cells exhibit greater responses than naïve B cells when stimulated with a diverse array of agonists, such as BCR engagement,[15,26] but more importantly pathways that do not involve the BCR such as CD40 ligand, TLR ligands or cytokines.[11,16,26,28,50]

One mechanism underlying differences in responses of naïve and IgM^+ memory B cells is the distinct activation threshold of these cells. Indeed, naïve B cells required 5–10-fold greater concentrations of anti-Igμ than IgM^+ memory B cells to initiate Ca^{2+} mobilization. This is consistent with our finding that the dependency of memory B cells on the levels of functional STAT3 to respond to interleukin (IL)-10 and IL-21 is dramatically less than that of naïve B cells.[14] It is likely that greater expression of IgM on memory compared with naïve B cells also provides them with a stimulatory advantage when responding to limiting amounts of BCR agonists. Thus, while the IgG cytoplasmic domain substantially contributes to the robust response of IgG^+ B cells to BCR stimulation over IgM^+ B cells, this is not the sole determinant underpinning heightened responses of all human memory B cells. Rather, the process of differentiation of a naïve B cell into a memory cell rewires the cell such that they can integrate signals provided not only by cognate Ag but also other factors in a manner distinct from or more efficient than naïve B cells, endowing them with the ability to respond more rapidly and robustly than their naïve precursors. This was recently demonstrated for naïve, IgM^+ and IgG^+ memory B cells in mice.[51] Additional factors that could contribute to enhanced responses of memory over naïve B cells that are independent of the Ig isotype expressed by the B cells include differential expression of activating and inhibitory receptors. Here we found that the expression of CD40 increases, while that of CD5 and LAIR-1 decreases, as naïve B cells differentiate into memory B cells and undergo Ig class switching. Interestingly, the expression of CD22—one of the best-characterized inhibitory receptors expressed by B cells—was comparable on all subsets of B cells present in the human blood and spleen, suggesting that CD22 does not have a unique function in restraining the response of only naïve B cells, as previously postulated.[58] Indeed, a lack of a role for CD22 in differentially regulating responses of IgM- versus IgG-expressing B cells has also been demonstrated in *in vivo* models using transgenic murine B cells.[38,46] Strikingly, expression of the inhibitory receptor CD31 and CD72 by peripheral blood B cells follows the same pattern as CD5 and LAIR-1 (that is, highest on naïve; downregulated on memory), suggesting that inhibitory receptors may function to restrain responses of naïve B cells more so than memory B cells following activation by specific Ags. Interestingly, CD5 expression is actually the greatest on human transitional B cells, and these cells exhibit a lessened response at least *in vitro* to polyclonal stimuli compared with naïve B cells.[12,20] These findings collectively parallel those from our previous studies which reported that negative regulators of the cell cycle are expressed at greater levels in naïve B cells compared with IgM^+ and class switched memory B cells.[16] Indeed, findings in this current study substantially extend these observations by demonstrating that a much greater fraction of all subsets of memory B cells compared with naïve B cells are in the G_1 phase of the cell cycle, thereby indicating the 'poised state' of memory B cells to rapidly undergo clonal expansion upon recognition of specific Ag. This mirrors the previous finding of greater turnover *in vivo* of human memory B cells compared with naïve B cells.[21] The fact that there are mechanisms allowing all memory B cells—irrespective of Ig isotype—to respond more rapidly than naïve B cells makes sense teleologically. It would be counterproductive, not to mention inefficient and potentially deleterious to the host, to be able

to generate memory B cells with diverse functions owing to the expression of distinct Ig isotypes, yet only those cells that acquired IgG would be able to respond vigorously to Ag.

In the human spleens, naïve B cells localize to the follicle while memory B cells reside in the MZ.[7,9,58] Indeed, human IgM$^+$ memory B cells largely correspond to MZ B cells.[7,9,59] An important distinction between humans and mice is that, while human MZ B cells are enriched for memory cells,[7,9] murine MZ B cells are considered to represent a lineage distinct from follicular B cells.[60] Despite these developmental differences, murine and human MZ B cells share many similarities. Thus MZ B cells from both species are larger, express higher levels of IgM, CD21, CD80, CD86 but lower IgD and CD23, differentiate more rapidly into plasmablasts and are more efficient at activating CD4$^+$ T cells than follicular naïve B cells.[9–11,26,27,29,61–64] Studies of BCR signaling in murine B cells demonstrated that MZ B cells exhibit increased phosphorylation and activation of downstream intermediates, including Syk and PLCγ2, and greater Ca^{2+} flux than follicular B cells, following engagement of IgM.[62,65] However, activation of other components of the BCR pathway—Vav, Btk, BLNK, PI3K p85—were comparable between follicular and MZ B cells.[65] Furthermore, the weaker responses of BCR-activated follicular B cells could be augmented to resemble MZ B cells when both IgM and IgD were engaged, indicating that a contributing factor to the greater response of MZ B cells is their higher level of expression of IgM relative to follicular B cells, together with increased sensitivity to BCR stimulation.[65] Interestingly, several of these findings resemble our observations of human splenic naïve and memory B cells. As murine follicular and MZ B cells both express IgM, this is further evidence that a 'rewiring' process—rather than only differences in the function of the IgM and IgG BCRs—occurs in these different populations during differentiation such that their behavior in response to specific stimuli are distinct, providing for a more rapid response by MZ over follicular B cells.

Although transcriptional analyses have identified differential gene expression patterns between human naïve, IgM$^+$ memory and switched memory B cells,[11,16,28,30] and our current study demonstrates heightened signaling capacity of IgM$^+$ memory over naïve B cells, the exact mechanism(s) underlying functional differences between these cell types remains incompletely resolved. However, previous studies, coupled with our current analysis of intracellular signaling, surface phenotype and cell cycle distribution, have provided a framework for future investigations to explore the molecular and cellular basis of differences in the behavior of naïve B cells and different classes of memory B cells. This will shed more light on the process of long-lived immunological memory and will provide opportunities to modulate this process in the settings of vaccination as well as in immunopathologies, such as autoimmunity and immunodeficiency where memory cells are dysfunctional.

MATERIALS AND METHODS

B-cell subsets and phenotyping

Spleens were obtained from cadaveric organ donors from Australian Red Cross Blood Service (Alexandria, NSW, Australia). Buffy coats were purchased from the Australian Red Cross Blood Service. Approval for this study was obtained from human research ethics committees of the St Vincent's Hospital, Darlinghurst, NSW, Australia and Sydney South West Area Health Service (Camperdown, NSW, Australia). Mononuclear cells were labeled with mAbs specific for CD20, CD27, IgM, IgG and IgA and then with anti-CD40, CD80, CD86, CD95, HLA-DR, CD5, CD31, CD72, CD22 or LAIR-1. The cells were gated to identify naïve (CD20$^+$CD27$^-$IgM$^+$IgG$^-$IgA$^-$), IgM memory (CD20$^+$CD27$^+$IgM$^+$IgG$^-$IgA$^-$), IgG memory (CD20$^+$CD27$^+$IgM$^-$IgG$^+$IgA$^-$) or IgA memory (CD20$^+$CD27$^+$IgM$^-$IgG$^-$IgA$^+$) B cells and the expression of these surface molecules on the different subsets was then determined. To perform cell cycle analysis, the mononuclear cells were first labeled with mAbs specific for CD20, CD27, IgM, IgG and IgA and then fixed and permeabilized. Thereafter the cells were labeled with anti-Ki67 and then incubated in DAPI-containing buffer, as described.[56] The B-cell susbets were identified as above, and the distribution of cells across the G_0 (DAPI$^-$Ki67$^-$), G_1 (DAPI$^+$Ki67$^+$), S (DAPI^{++}Ki67$^+$) and G_2/M (DAPI^{hi}Ki67$^+$) phases of the cell cycle, where the different staining for DAPI correspond to different DNA content in the cells, was determined.[56]

Isolation of human B-cell subsets

Enriched splenic B cells (Dynabeads Untouched Human B Cells Kit, Life Technologies, Carlsbad, CA, USA) were labeled with mAbs against CD20, CD27 and either IgG/E/A or IgM/D, and subsets of naïve (CD20$^+$CD27$^-$IgG/E/A$^-$), IgM memory (CD20$^+$CD27$^+$ IgG/E/A$^-$) and isotype-switched (CD20$^+$CD27$^-$IgM/D$^-$) memory B cells were collected (FACSAria, Becton Dickinson, San Jose, CA, USA).[11,26,29] The purity of the recovered populations was typically $>98\%$. B-cell subsets were rested for 3 h after sorting to minimize non-specific activation of BCR signaling pathway.

BCR in vitro stimulation and analysis of intracellular signaling

Naïve and memory splenic B cells were stimulated with medium alone or F(ab')$_2$ fragments of goat anti-human Ig heavy chains (IgM/G/A specific; Jackson ImmunoResearch Laboratories, Inc., Westgrove, PA, USA) for 2, 5, 15 and 30 min at 37 °C. Cells were then fixed, permeabilized, labeled with mAbs against IκBα (Cell Signaling, Danvers, MA, USA), phosphoS6 (pSer235/236; Cell Signaling), phosphoPLCγ2 (pY759; BD Phosflow, Becton Dickinson), phosphoBLNK (pY84, BD Phosflow), phosphotyrosine (BD Phosflow) and phosphoSYK (pY352, BD Phosflow) and analyzed by flow cytometry (FACS Canto II, Becton Dickinson). Alternatively, cells were lysed in RIPA buffer (500×10^3 cell equivalents) after stimulation and immuno-blotting was performed using anti-IκBα, anti-S6, anti-phosphoS6 (pSer235/236), anti-PLCγ2 (Cell Signaling), anti-phosphoPLCγ2 (pY753), anti-ORAI1, anti-STIM1, anti-IP3R I/II/III and 14.3.3 (Santa Cruz Biotechnologies Inc., Santa Cruz, CA, USA).

Measurement of Ca^{2+} mobilization

Enriched splenic B cells were washed twice in Hank's Balanced Salt Solution (Life Technologies; containing 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5% bovine serum albumin) and loaded with cell permeant Indo-1 AM dye (Life Technologies) at 0.2 μM containing 2% Pluronic F-127 (Life Technologies) for 30 min at 37 °C. Indo-1 AM-loaded B cells were washed and then labeled with mAb against CD20, CD27, IgG/E/A, IgM/D/E/A or IgM/D/E/G to identify naïve (CD20$^+$CD27$^-$IgG/A/E$^-$), IgM memory (CD20$^+$CD27$^+$IgG/A/E$^-$), IgG switched memory (CD20$^+$CD27$^+$IgM/D/E/A$^-$) and IgA switched (CD20$^+$CD27$^+$IgM/D/E/G$^-$) memory B cells. Measurements were performed using a BD LSRII flow cytometer. Readings were recorded for 60 s to establish a baseline measurement, and then 70 μg ml^{-1} F(ab')$_2$ fragments of goat anti-human Ig was added for BCR stimulation. Changes in intracellular Ca^{2+} concentrations were recorded for 10 min. During the last 2 min of the assay, B-cell subsets were stimulated with ionomycin (2 μg ml^{-1}) to establish the maximum Ca^{2+} response. To evaluate the origin of mobilized Ca^{2+}, extracellular Ca^{2+} was chelated with 0.5 mM EGTA, and 4 mM $CaCl_2$ was added to these cells after 5 min. Changes in the intracellular Ca^{2+} concentration were detected as a shift from an Indo 1-AM emission peak at 475 nm for unbound dye to an emission peak at 405 nm when the Indo 1-AM molecule was bound to Ca^{2+}. The intracellular Ca^{2+} concentration was quantified by calculating the ratio of Indo 1-AM emission at 405 nm to that at 475 nm (bound: unbound ratio).

Statistical analysis

Significant differences between data sets were determined using one-way analysis of variance followed by correction for multiple comparisons ($^*P<0.05$, Holm–Sidak method) (Prism; GraphPad Software, La Jolla, CA, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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