Silencing of B Cell Receptor Signals in Human Naive B Cells

Niklas Feldhahn,1,2 Ines Schwering,2 Sanggyu Lee,4 Maria Wartenberg,3 Florian Klein,1,2 Hui Wäng,1,2 Guolin Zhou,1 San Ming Wang,4 Janet D. Rowley,4 Jürgen Hescheler,3 Martin Krönke,1 Klaus Rajewsky,5 Ralf Küppers,2 and Markus Müschen1,2

1Institute for Medical Microbiology, Immunology and Hygiene, 2Institute for Genetics, and 3Institute for Neurophysiology, University of Cologne, 50931 Köln, Germany
4Department of Medicine, University of Chicago, Chicago, IL 60637
5Center for Blood Research, Harvard Medical School, Boston, MA 02115

Abstract
To identify changes in the regulation of B cell receptor (BCR) signals during the development of human B cells, we generated genome-wide gene expression profiles using the serial analysis of gene expression (SAGE) technique for CD34+/H11001 hematopoietic stem cells (HSCs), pre-B cells, naive, germinal center (GC), and memory B cells. Analysis of these SAGE profiles, genes encoding positive regulators of BCR signaling were expressed at consistently lower levels in naive B cells than in all other B cell subsets tested, and a large group of inhibitory signaling molecules, mostly belonging to the immunoglobulin superfamily (IgSF), were specifically or predominantly expressed in naive B cells. Quantitative differences observed by SAGE were corroborated by semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) and flow cytometry. In a functional assay, we show that down-regulation of inhibitory IgSF receptors and increased responsiveness to BCR stimulation in memory as compared with naive B cells at least partly results from interleukin (IL)-4 receptor signaling. Conversely, activation or impairment of the inhibitory IgSF receptor LIRB1 affected BCR-dependent Ca2+ mobilization only in naive but not memory B cells. LIRB1 and IL-4 may represent components of two nonoverlapping gene expression programs in naive and memory B cells, respectively: in naive B cells, a large group of inhibitory IgSF receptors can elevate the BCR signaling threshold to prevent these cells from premature activation and clonal expansion before GC-dependent affinity maturation. In memory B cells, facilitated responsiveness upon reencounter of the immunizing antigen may result from amplification of BCR signals at virtually all levels of signal transduction.

Key words: B cell receptor • IL-4 • ITIM • memory B cells • SAGE

Introduction
Signal transduction pathways initiated through the B cell receptor (BCR)* determine the fate of B cells within a context of BCR–affinity to antigen, expression levels of stimulatory or inhibitory coreceptors and the differentiation stage of B cells (1). Whereas BCR engagement by self-antigen in immature bone marrow B cells induces receptor editing, deletion, or inactivation (anergy), BCR cross-linking in mature B cells initiates a signaling cascade that ultimately confers positive selection, proliferation, and differentiation.

Early B cell differentiation is defined by a sequence of Ig gene rearrangements determining the configuration of the (pre)-BCR. The recombination machinery first targets DH and JH gene segments at the pro-B cell stage followed by V\textsubscript{H}-\textsubscript{D}\textsubscript{H}J\textsubscript{H} gene rearrangement in pre-B cells, which subsequently express a pre-BCR, composed of Ig heavy and surrogate light chains, on their cell surface. Expression of V\textsubscript{pre-B} and A-5 surrogate light chain genes precedes the expression of Vk-Jk or Va-Ja light chain gene rearrangements at the immature B cell stage, at which, for the first time, a BCR is expressed and the cells enter the peripheral
blood as naive B cells. The specificity and structure of the BCR is further modified by somatic hypermutation and class switch recombination during the affinity maturation process within germlinal centers (GCs), in which GC B cells are destined to die by apoptosis unless they are rescued through BCR-dependent survival signals upon antigen cross-linking. GC B cells expressing a BCR of high affinity to their cognate antigen subsequently differentiate into memory B cells or antibody-secreting plasma cells. Notably, signaling through the BCR not only determines the fate of a B cell at developmental checkpoints within the bone marrow and in GCs. Also mature B cells depend on the presence of a functional BCR, which continuously delivers a “maintenance” signal (2).

The BCR signaling cascade is initiated by rapid phosphorylation of tyrosine residues in the immunoreceptor tyrosine-based activation motifs (ITAMs) of the BCR coreceptors Igα and Igβ upon antigen cross-linking. The proximal signal transduction involving multiple different protein tyrosine kinase (PTK) activities including src-family PTKs, SYK, and BTK (3). In addition to kinases, several phosphatases (SHP1, SHP2, CD45, SHIP) and linker proteins (BLNK, GRB2, SHC, NCK) also regulate BCR signal transduction. BLNK was recently shown to act as a scaffolding protein, which mediates the interaction between SYK and the downstream signaling molecules VAV (4) and PLCγ. The latter can hydrolyze PIP2 to IP3 and diacylglycerol, which increases the levels of free intracellular Ca2+, and result in subsequent activation of Ca2+-mitogen-activated protein (MAP) kinases (5). These kinases ultimately initiate functional responses including proliferation, isotype switch, and Ig class switching. However, the downstream consequences of activation signals may be attenuated by engagement of one or more inhibitory receptor motifs (ITIMs). Many of the inhibitory receptors belong to the Ig superfamily (IgSF), which includes surface molecules such as CD5, CD22, and CD77 (6).

To identify changes in expression of BCR-dependent activation signals at checkpoints during normal human B cell development, we analyzed and compared genome-wide gene expression profiles from human bone marrow hematopoietic stem cell (HSC), bone marrow pre-B cells, naive B cells, GC B cells, and memory B cells. These gene expression profiles were generated using the serial analysis of gene expression (SAGE) technique, which allows for the genome-wide quantitative analysis of any expressed mRNA in a given cell population (6).

Materials and Methods

Isolation of Human Hematopoietic Stem Cells, Pre-B Cells, and Mature B Cell Subsets. HSCs and pre-B cells were purified from bone marrow and umbilical cord blood samples (Poietics) and from 28 umbilical cord blood samples (according to the principle of informed consent) by Ficoll density gradient centrifugation. T cells and myeloid cells were depleted using anti-CD3 and anti-CD15 immunomagnetic beads (Dynal). Among the remaining cells, immature CD10+CD19+CD20+ B cells and CD138+ plasma cells were depleted using an anti-CD20 IgG1 antibody (BD Biosciences) together with anti-IgG1 beads and anti-CD138 beads (Miltenyi Biotec), respectively (8). Thereafter, pre-B cells were enriched using anti-CD19 immunomagnetic multisort-beads (Miltenyi Biotec). The beads were released from the CD19+ cells enzymatically. The purified cells were subsequently labeled by a mouse anti–CD10 IgG1 antibody (CALLA; BD Biosciences) and separated using anti–mouse IgG1 beads (Miltenyi Biotec). IgD+CD19+CD27− naive B cells and CD19+CD27+ memory B cells were isolated from peripheral blood using anti-CD19 and anti-CD27 immunomagnetic beads (Miltenyi Biotec) as described (9) and from seven tonsillectomy specimens. For enrichment of tonsillar memory B cells, CD27+CD38+ GC B cells were depleted using an anti-CD38 PE antibody (BD Biosciences) together with anti–PE microbeads (Miltenyi Biotec). Tonsillar CD77+ GC B cells were isolated as described previously (9) using a rat anti–CD77 IgM Fab fragment (BD Biosciences) together with a mouse anti–rat IgG1 Fab fragment (Serotec) and an anti–mouse IgM IgG1 microbeads (Miltenyi Biotec). Only cell purifications of a purity >95% were considered for the SAGE analysis.

The identity of the isolated cell subsets was verified genotypically and phenotypically. The genotype of CD34+ pre-B cells and mature B cell subsets was assessed by PCR amplification of rearranged V genes from genomic DNA and subsequent cloning and sequencing of the PCR products as described (10). In addition, the phenotype of the purified HSC, pre-B, naive B cells, and memory B cells was assessed by quantitative RT-PCR at the mRNA level using pre-B-specific (IgLH Cγ1-, IgκC-, IgκCα-, VpreB1-, λ5-), and β2M-specific primers (Integrated DNA Technologies). To distinguish “mature” from germline Cγ1 transcripts, primers were chosen for a fragment between the Jγ and the hinge region of the constant region (11). The phenotype of purified pre-B cells was also verified by flow cytometry using CD10− and CD19-specific antibodies for pre-B cells, CD20−, and CD27− specific antibodies for naive and memory B cells, CD38− and CD77− specific antibodies for GC B cells, respectively (FITC− and PE-conjugated antibodies from BD Biosciences).

SAGE Analysis. cDNA-synthesis, SAGE analysis, cloning, and sequencing of SAGE concatemers was performed according to Velculescu et al. (6). The UniGene reference database (March 2001) was obtained at http://www.sagenet.org/SAGEDatabases/unigene.html. A total of 306,000 SAGE tags were collected for the five SAGE profiles. 106,000 tags were analyzed from the HSC library, 110,000 for pre-B cells, and each ~30,000 tags for naive, GC, and memory B cells. All SAGE libraries were normalized to 100,000 tags.

Controls for the Accuracy of SAGE Library Construction. RNA-degradation and incomplete digestion of 3′cDNAs by the so-called tagging enzyme NlaIII may interfere with the quantitative representation of expressed genes in SAGE libraries. RNA degradation within 3′ regions would result in the underrepresentation of genes, whose last NlaIII recognition site (CATG) is particularly far from the poly(dA) tail. The average distance between the last CATG site and the poly(dA) tail is ~250 bp (6). To search for a potential bias against SAGE tags derived from 5′ sequences, we selected 10 housekeeping genes, whose extreme 3′ CATG site
was more than 450 bp distant from the poly(dA) tail. We compared the tag counts for these genes with the tag counts in 55 published SAGE libraries (at http://www.ncbi.nlm.nih.gov/SAGE/index.cgi?db=tagesearch). In the SAGE libraries described here, tags for these genes were found at frequencies close to the average of 55 reference SAGE libraries, which argues against bias of quantitative representation introduced by RNA degradation.

Incomplete NlaIII digestion would result in the generation of SAGE tags that are aberrantly derived from upstream CATG sites instead of the extreme 3' SAGE tags that are aberrantly derived from upstream CATG sites. To address this issue, we amplified cDNA fragments of the GAPDH and EEF1 genes for the SAGE libraries for naive and memory B cells. Primers were chosen so that cleavage by NlaIII would result in the loss of the 5' primer binding site for GAPDH but not for EEF1. Consistent with high efficiency of NlaIII digestion, either no or only very small amounts of PCR product were obtained for GAPDH while amplification of EEF1 fragments yielded abundant amplification products (unpublished data).

**Selection of BCR-related Signaling Molecules.** In a comprehensive search for positive and negative BCR-related signaling molecules in PubMed, UniGene (http://www.ncbi.nlm.nih.gov/UniGene/) and OMIM (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM), we collected 211 genes, for which a role in positive (129) or negative (82) regulation of BCR-dependent signals was shown. Based on their UniGene-ID, 148 (97 positive and 51 inhibitory signaling molecules) of these genes could be retrieved from at least one of the five SAGE libraries.

**Verification of Quantitative Accuracy of SAGE Data.** To corroborate quantitative differences in gene expression among the five populations as determined by SAGE, semiquantitative RT-PCR analysis was performed for a set of 41 selected genes. The SAGE tag counts for these genes were compared with the tag counts in 55 published SAGE libraries (at http://www.ncbi.nlm.nih.gov/SAGE/index.cgi?db=tagesearch). In the SAGE libraries described here, unrelated cDNAs from HSC and pre-B cells purified from umbilical cord blood (see above) were used as template. To specifically address quantitative differences in mRNA content as compared with their resting precursors (12), PBMCs were cultured at a density of 5 × 10^4 g of a neutralizing anti–IL-4R antibody (Genzyme)/ml, plus anti-CD21 PE, anti-CD22 FITC, anti-CD5 PE, anti-CD40 PE, anti-CD66 FITC, anti-CD72 FITC, anti-CD74 PE, anti-CD80 PE, anti-CD86 FITC, anti-CD79a/ IgG1, anti-BCMA IgG and donkey anti–goat IgG FITC are from Santa Cruz Biotechnology, Inc. IL-4-dependent Regulation of BCR Signaling-associated Genes in Naive and Memory B Cells. Naive and memory B cells were purified from peripheral blood as described above and cultured at 37°C either in RPMI medium (including 10% fetal calf serum) alone, or with 1 ng/ml recombinant human IL-4 (Genzyme) and 50 μg of a neutralizing anti–IL-4Rα antibody (Genzyme)/ml, which was added after 8 h preincubation with IL-4. The cells were cultured at a density of 5 × 10^5 cells/100 μl/well in 96-well plates. After 48 h, the cells were subjected to RNA isolation and subsequent semiquantitative RT-PCR analysis for COX6, BLK, BTK, BLNK, SYK, LIRB1, LIRB2, LIRB3, SlgLe5, SlgLe6, CD66, CSK, SHIP, and SHP1 at 28 cycles and for LAIR1 at 32 cycles.

**Effect of LIRB1 Signaling on BCR-dependent Ca^{2+} Mobilization in Naive and Memory B Cells.** To address directly how LIRB1 (an inhibitory IgSF receptor found prominently expressed on naive B cells) can affect responsiveness of the BCR to antigen in naive and memory B cells, BCR-dependent Ca^{2+} mobilization was studied. To this end, peripheral blood naive and memory B cells were purified from four healthy donors as described above and cultured in medium, which had been conditioned for 24 h by PBMCs at a density of 10^6 PBMCs/ml. PBMCs were stimulated with 1 μg LPS/ml to induce secretion of soluble MHC class I molecules (13), which act as natural ligand of LIRB1 (14). To study the effect of LIRB1 on BCR signals, naive and memory B
cells were cultured for 24 h in the presence or absence of an antagonistic (clone HP-F1; reference 14) or agonistic (clone GV1/75; BD Biosciences; reference 15) LIRB1 antibody cross-linked by goat anti–mouse IgG serum (Jackson ImmunoResearch Laboratories). HP-F1 was a gift from Dr. Miguel López-Botet, Universitat Pompeu Fabra, Barcelona, Spain. After the preincubation, cells were washed and stained with fluo-3 dye (Calbiochem) for 30 min. Changes of cytosolic Ca\(^{2+}\) were measured by laser scans using confocal microscopy (16). After 30 s of measurement, anti-human IgM F(ab')\(_2\) and anti–human IgG F(ab')\(_2\) fragments (Jackson ImmunoResearch Laboratories) were added to naive and memory B cells, respectively. Cytosolic Ca\(^{2+}\) concentrations were calculated as described previously (17). As a negative control, purified B cell populations were also treated with an anti-CD3 antibody (BD Biosciences), which induces Ca\(^{2+}\) mobilization in T but not B cells. For statistical analysis, area under curve values were calculated and compared using Fisher’s exact test. \(P < 0.05\) was considered statistically significant.

**Effect of IL-4R Signaling on BCR-dependent Ca\(^{2+}\) Mobilization in Memory B Cells.** As treatment of naive B cells with IL-4 had no effect on the expression of genes related to BCR signaling (see above; see Fig. 5), modulation of BCR signals by IL-4 was studied in memory B cells only. Memory B cells from four healthy donors were purified and cultured in supernatant from LPS-stimulated PBMCs for 24 h in the presence or absence of human recombinant IL-4 or an inhibitory anti–IL-4R\(\alpha\) antibody (Genzyme). Changes of cytosolic Ca\(^{2+}\) concentrations upon BCR engagement were measured and analyzed as described above.

### Results and Discussion

**Verification of the Identity of Purified B Cell Subsets.** CD34\(^+\) HSCs (reference 7) and CD10\(^+\)CD19\(^+\) pre-B cells (Fig. 1 B) were purified from human bone marrow, CD19\(^+\)CD27\(^-\) naive and CD19\(^+\) CD27\(^+\) memory B cells from tonsils, and CD38\(^+\)CD77\(^-\) GC B cells from tonsils (Fig. 1 B). The identity of the purified subsets was further supported by the analysis of their genotype (Table I). As opposed to memory B cells, Ig\(V_H1\)-gene rearrangements amplified from pre-B cells and naive B cells were virtually devoid of somatic mutations, consistent with ongoing selection for the expression of a functional Ig heavy chain in bone marrow pre-B cells, nonproductive \(V_H1\)-gene rearrangements (either due to loss of reading frame or rearrangement of a pseudogene of the \(V_H1\) family) were over-represented in the isolated pre-B cell population (Table I). Prior to the SAGE analysis, specific fragments of the \(C_H\), \(C_Y1\), \(C_K\), and \(V_{preB}\) genes were amplified from pre-B

| \(V_H1\)-gene rearrangements | Pre-B cells | Naive B cells | Memory B cells |
|-----------------------------|------------|---------------|----------------|
| In-frame                    | 3          | 7             | 8              |
| Out-of-frame                | 7          | 3             | 2              |
| Pseudogene                  | 3          | 0             | 0              |
| Potentially functional      | 2          | 7             | 8              |
| Average mutation frequency (\(10^{-2}\) bp) | 0.4 | 0.6 | 7.7 |

Figure 1. Genotype and phenotype of purified B cell subsets. Bone marrow pre-B cells (CD10\(^+\) CD19\(^+\)), peripheral blood naive B cells (CD19\(^+\) CD27\(^-\)), tonsillar GC B cells (CD20\(^+\) CD77\(^+\)), and peripheral blood memory B cells (CD19\(^+\) CD27\(^+\)) were purified as described in Materials and Methods. The mRNA expression of \(C_H\), \(C_Y1\), \(C_K\), and \(V_{preB}\) was analyzed by semiquantitative RT-PCR analysis (A). The identity of the purified subsets was further verified by flow cytometry (B). FACS® plots for pre-B cells (CD10\(^+\) CD19\(^+\)), naive (CD20\(^+\) CD27\(^-\)), GC (CD20\(^+\) CD77\(^+\)), and memory B cells (CD20\(^+\) CD27\(^+\); from top to bottom) are given.
cells, naive, and memory B cells by RT-PCR, which was normalized for 2-microglobulin (Fig. 1 A). As expected, expression of VpreB is confined to pre-B cells, which, in turn, lack expression of Igκ light chains and IgG1 heavy chains.

Verification of Quantitative Accuracy of SAGE Profiles. In the analysis of SAGE profiles for CD34+ HSC, pre-B, naive, GC, and memory B cells, we identified a particular gene expression pattern, which involves positive and negative regulatory BCR signaling molecules (see references in Fig. 2). To corroborate the quantitative differences in the expression of BCR signaling molecules as observed by SAGE, semiquantitative RT-PCR was performed for 22 positive and 19 negative regulatory BCR signaling molecules (Fig. 3). For all 41 genes tested, the amounts of the amplification product mirrored the SAGE tag counts in the libraries for naive and memory B cells. Moreover, the expression of 10 co-stimulatory and 10 inhibitory surface molecules implicated in the propagation or attenuation of BCR-dependent signals was analyzed at the protein level by flow cytometry (Fig. 4). For all 20 surface molecules, the FACS® data correlated with the SAGE data, indicating that the large majority of the gene expression represented by alternative methods.

Opposing Regulation of BCR Signaling in Naive and Memory B Cells. In a comprehensive search throughout the five SAGE profiles and the UniGene database, we identified
148 molecules involved in positive (97 genes) and negative (51 genes) regulation of BCR signaling present in at least one SAGE library. Whereas most of the genes that positively regulate BCR-dependent activation signals are expressed at high levels in pre-B, GC, and memory B cells, this was not the case for naive B cells (Fig. 2, A and B, and Figs. 3 and 4). In many cases, expression of positive BCR signaling molecules was either missing in the SAGE library for naive B cells or reduced to expression levels as in CD34+ HSC. Conversely, inhibitory molecules were expressed either exclusively or predominantly in naive B cells (Figs. 2 C, 3, and 4).

Concomitant down-regulation of mediators of BCR-related activation together with increased expression of inhibitory molecules in naive B cells (Fig. 2, A–C) suggests that an elevated signaling threshold prevents naive B cells from being inappropriately activated upon antigen encounter. There are, however, some exceptions to this seemingly uniform picture: for instance, IKAROS, which appears to reduce BCR-dependent B cell activation (18) is expressed at higher levels in pre-B cells and memory B cells as compared with naive B cells. Also, SAGE tags matching the LYN gene, which is critical for ITIM-dependent negative signaling, and the IkBa gene, encoding an inhibitor of nuclear factor (NF)-κB, were found most frequently in pre-B cells, while expression levels in naive, GC, and memory B cells are similar. Many of the signaling molecules included in this study are also involved in signaling pathways that are not related to the BCR. This applies in particular to downstream kinases (e.g., p85α, JAK3, p110, LCK, FAK, IKK, AKT, HKP1, p115, PKCμ, PKCβ, PKA: Fig. 2) and receptors that are not specific for the B lineage, whose intracellular signals may converge with those of the BCR (SLAM, PDGFRα, CD38, CD27, FcR-H1, HHR1, CD86, NGFR, CD36, CD74, CD66, CD31, LAR1, CD5, CD33, LLIR, LIRb1, LIRb2, LIRb5, PIRb, Fig. 2).

However, mouse mutants for some of these genes exhibit a particular B cell phenotype, and many receptor molecules are involved in either ITAM- or ITIM-dependent signaling, which allows a prediction of their role in either amplification or attenuation of BCR-dependent activation signals. The five SAGE libraries also identified a number of components of the proximal BCR-signaling complex, which have an unambiguous function in (pre)-B cells. These “classical” BCR signaling molecules include BLK, BLNK, BTK, Igα, Igμ, and CD19, whose expression levels are consistently lower in naive B cells as compared with other B cell subsets (Fig. 2 A and B, and Figs. 3 and 4). To the contrary, well characterized inhibitors of BCR-induced activation including the protein tyrosine phosphatases (PTPs) SHP1 and SHIP (29), the PTK CSK, the NIK, the PTK-associated ubiquitin ligase CBL, and the ITIM-
bearing surface receptors CD22, CD32 and CD72 are expressed at high levels in naive B cells but not in pre-B, GC, or memory B cells (Figs. 2 C, 3, and 4).

Facilitated responsiveness of memory B cells to BCR engagement was suggested from earlier findings, namely the expression of the activating coreceptors CD21 (20) and CD27 (21) together with increased expression levels of the costimulatory molecules CD80 and CD86 (22) and in vitro experimentation, which demonstrated that memory cells have higher propensity to undergo activation-induced terminal differentiation than naive B cells (23).

To date, a genome-wide analysis of BCR signaling molecules in pre- and (post-) GC B cells is missing. As shown in Fig. 2, A and B, sensitization of memory B cells to BCR-dependent activation signals is not only related to upregulation of surface molecules such as CD21, CD27, CD80, and CD86 but involves virtually all levels of intracellular signal transduction including transmembrane receptors, kinases, linker molecules, phosphatases, Ca\(^{2+}\) channels and transcription factors.

In particular, it was not known that BCR signals in naive B cells can be specifically silenced by the prominent expression of ITIM-bearing molecules belonging to the emerging group of inhibitory immunoglobulin superfamily (IgSF) receptors (Figs. 2 C, 3, and 4). In the following, we describe the expression pattern of inhibitory and stimulatory BCR signaling molecules in naive and memory B cells more in detail, dividing these molecules into functional groups.

**Regulation of the Expression of Constituents of the BCR in Human B Cell Subsets.** In mature B cells, the BCR is composed of surface Ig, the coreceptors CD19, CD21, and CD81 and the Ig\(\mu\)/H9251- and Ig\(\lambda\)/H9252-signaling chains (1). In naive B cells, mRNA levels of BCR-related molecules are either moderately (Ig\(\lambda\)/H9252, CD19, CD81; Fig. 2 B) or substantially (Ig\(\mu\), CD21; Fig. 2 A) lower than in other B cell subsets.

Also, expression levels of Ig genes are lower in naive B cells as compared with other B cell subsets: in the SAGE library for naive B cells, we and 143 tags matching to the C\(\mu\) gene (pre-B cells: 512; GC B cells: 302; memory B cells: 329; memory B cells: 329).

In particular, it was not known that BCR signals in naive B cells can be specifically silenced by the prominent expression of ITIM-bearing molecules belonging to the emerging group of inhibitory immunoglobulin superfamily (IgSF) receptors (Figs. 2 C, 3, and 4). In the following, we describe the expression pattern of inhibitory and stimulatory BCR signaling molecules in naive and memory B cells more in detail, dividing these molecules into functional groups.

**Regulation of the Expression of Constituents of the BCR in Human B Cell Subsets.** In mature B cells, the BCR is composed of surface Ig, the coreceptors CD19, CD21, and CD81 and the Ig\(\mu\) and Ig\(\lambda\)-signaling chains (1). In naive B cells, mRNA levels of BCR-related molecules are either moderately (Ig\(\lambda\), CD19, CD81; Fig. 2 A) or substantially (Ig\(\mu\), CD21; Fig. 2 A) lower than in other B cell subsets.

Also, expression levels of Ig genes are lower in naive B cells as compared with other B cell subsets: in the SAGE library for naive B cells, we and 143 tags matching to the C\(\mu\) gene (pre-B cells: 512; GC B cells: 302; memory B cells: 329; memory B cells: 329).

**Figure 2.** Cluster analysis of activating and inhibitory B cell receptor signaling molecules. In a systematic survey of PubMed, UniGene and OMIM databases, 211 BCR-related genes were identified, 148 of which could be retrieved from at least one of the SAGE libraries for CD34\(^+\) HSC (HSC), pre-B cells (PBC), naive B cells (NBC), GC B cells (GCB), and memory B cells (MBC). In total, 97 activating (A and B) and 51 inhibitory (C) signaling molecules were identified the five SAGE libraries and listed with their gene names, SAGE tag counts for each library, UniGene ID, a brief description of their putative function including a reference. It should be noted that because of limited space in many cases only one functional aspect among others has been included. The SAGE data were sorted based on the ratio of tag counts in memory and naive B cells. For calculation of ratios, a tag count of 0 was set to 0.5. For graphic representation of SAGE data, tag counts have been transformed using the Cluster and Treeview softwares by M.B. Eisen, in which red denotes strong and black no or low expression.
B Cell Receptor Signaling in Naive B Cells

Many of the inhibitory receptors, which include members of the TNF receptor superfamily: in contrast to GC and memory B cells, CD40 (TNFRSF5), CD27 (TNFRSF7), and NGFR (TNFRSF16) are either missing or expressed only at low levels in naive B cells (Fig. 2, A and B). While CD40-engagement is known to cooperate with IL-4R- and BCR-dependent signals during the GC reaction (25), ligation of the “memory-specific” receptor CD27 (21) by CD70 increases responsiveness of memory B cells to BCR signals and induces plasma cell differentiation. Also, three recently identified members of the TNF receptor superfamily are differentially expressed in naive B cells compared with GC- and memory B cells: BAFF-R (TNFRSF13B) and BCMA (B cell maturation antigen; TNFRSF17) are receptors of the B cell activation factor BAFF and are expressed in GC and memory B but not naive B cells (Fig. 2, A and B, and Fig. 3). Unlike BCMA (26), BAFF-B (TNFRSF12) is not essential for the development of marginal zone (i.e., pre-B) progenitor cells and the T cell-dependent GC reaction (25). Although not essential for B cell activation, BCMA is highly expressed on GC B cells, which are said to induce NF-kB-activation (27). B cell activating receptor for BAFF (BASS), termed BAFF-R, is highly expressed on naive B cells (at low level, expressed at intermediate levels in memory B cells; Figs. 2, A and B, and Fig. 3) and acts as a negative regulator of B cell activation and class switch recombination (CSR; reference 30), is expressed in naive B cells but virtually missing in GC- and memory B cells (Figs. 2, C, 3, and 4). In this regard, it is notable that SWAP70, which promotes both B cell activation and CSR (31), is expressed reciprocally with CD30 ligand in naive and (post) GC B cells (Fig. 2 B).

**Specific Expression of Inhibitory Ig Superfamily Members in Naive B Cells.**

Many of the inhibitory receptors, which we find expressed at high levels in naive B cells belong to the Ig superfamily (IgSF). The inhibitory IgSF molecules are predominantly or exclusively expressed by naive B cells (Fig. 2 C) and typically carry one or more ITIMs within their cytoplasmic tail. Negative regulatory IgSF molecules specifically or predominantly expressed in naive B cells include SlgLex5, SlgLex6, and SlgLex8, members of the sialic acid binding Ig-like lectin-like family, and LIRB1, LIRB2, and LIRB5, which belong to the B group of leukocyte Ig-like receptors (collectively termed CD85, Figs. 3 and 4). Also, ITIM-bearing IgSF molecules, the lectin-like immunoreceptor LIRL, the paired Ig-like receptor PIRB, as well as CD22, CD31, CD32/FlkRγII, the biliary glycoprotein CD66 are expressed by naive B cells and, if at all, only at reduced levels in GC and memory B cells (Figs. 2 C, 3, and 4). This also applies to the newly identified IgSF molecules PD1 (32), G6B (33), and CMRF35H (34). Also, ITIM-bearing but a member of the C-type lectin family, the CD72 molecule is highly expressed in naive B cells (Figs. 2 C, 3, and 4). On the other hand, its antagonistic ligand
CD100 can relieve CD72-mediated inhibition of BCR-signals and is predominantly expressed in memory B cells (Figs. 2 B, 3, and 4). Within the IgSF, a group of Fc receptor homologues was recently identified, which comprises positive and negative regulatory coreceptors based on whether they harbor ITAMs or ITIMs within their cytoplasmic tail (35). Like many other inhibitory IgSF receptors, the ITIM-bearing FcRH2/SPAP1 (35) was only found in naive B cells (Fig. 2 C), whereas its ITAM-carrying homologue, FcRH1 was expressed in GC and memory but not naive B cells (Fig. 2 A).

Cytokine Receptor Signaling in Naive and Memory B Cells. The B cell–homing chemokine receptor BLR1, also termed CXCR5, can cooperate with the BCR by stimulation of Ca\(^{2+}\) influx (36) and is stronger expressed in GC and memory B cells as compared with their naive precursors (Fig. 2 B). Naive B cells also differ from GC B cells and memory B cells in that they lack expression of the signal transducer for IL-6 (CD130 or gp130) and the receptor for IL-4 (CD124). gp130 has been implicated in post-GC development of B cells, as mice expressing a dominant negative form of gp130 exhibit a marked reduction of Ig production (37). Engagement of IL-4R can augment activation signals through the BCR (for a review, see reference 38; see below).

Regulation of BCR-Downstream Linker Molecules and Kinases in Human B Cell Subsets. A large group of activating linker molecules and PTKs contributes to propagation of activation-stimuli within the distal BCR–signaling cascade. Without exception, activating linker molecules identified in the SAGE libraries, including BLNK, BAM32, GRB2, SOS1, SHC1, GAB1, GAB2, BRDG1, NCK, and BANK, were up-regulated in GC and memory as compared with naive B cells (Fig. 2, A and B). In contrast, inhibitory linker
molecules including p62DOK (39) and the SRC-like adapter proteins SLAP1 and SLAP2 (40) inhibit BCR downstream signals, predominantly in naive B cells (Fig. 2 C).

Activating kinase molecules, as far as identified in the SAGE libraries, are expressed at higher levels in (post) GC B cells as compared with their naive precursors (Fig. 2, A and B). PI-3 kinases p85α and p110 have in common that they may activate AKT/PKB (41) and BTK (42), while activated BTK can induce degradation of the NF-κB inhibitor IkBα by phosphorylation through IKK (43). Only moderately up-regulated in GC and memory B cells, the MAP kinase HPK1 (44) and the PTK PYK2 (45) can augment BCR signals, the former through interaction with BLNK, the latter with BRDG1 (Fig. 2 B). Interestingly, PYK2-deficient mice lack splenic marginal zones, which are thought to be mainly composed of memory B cells (45). Not specific for the B cell lineage, but expressed in GC and memory B cells (Fig. 2 A), the receptor of platelet derived growth factor PDGFα can act as a stimulatory PTK and promote proliferation (46).

Among the inhibitory kinases, the CD45-antagonist CSK (47) and the PKCs θ (48) and μ (49) acting as inhibitors of BTK and SYK, respectively, are expressed at higher levels in naive B cells as compared with GC and memory B cells (Fig. 2 C). However, expression levels of LYN, a key mediator of ITIM-dependent negative signaling, is expressed at similar levels throughout all B cell subsets. Specific inhibition of the tyrosine kinase BTK results in a lower level of BCR-signal attenuation, at which the activation-signaling pathways.

Regulation of PTPs during B Cell Development. The role of transcription factors in the regulation of BCR signaling is largely mediated by changes of gene expression occurring in GC B cells (57), which are expressed at high levels in pre-B and memory but not naive B cells (Fig. 2 B). In GC and memory B cells, OCT2 was found up-regulated together with its transcriptional target gene CD36 (Fig. 2 B). Stage-specific expression of CD36 could be meaningful because based on the dependence of CD36 expression on transcriptional activation by OCT2, it was speculated that OCT2 could regulate B cell differentiation through CD36 (58). The members of the ETS family of transcription factors, FLI-1 (59), PU.1, and SPL-B (60) have recently been demonstrated as critical components of BCR-dependent activation-signaling pathways. FLI-1, PU.1, and SPL-B are expressed at high or intermediate levels in GC and memory B cells but are not detected in the SAGE library for naive B cells (Fig. 2 A).

Relief of BCR-mediated apoptosis by Engagement of IL-4R in Post-GC B Cells. The data indicate that enhancement of BCR-mediated B cell activation by IL-4 at least in part results from the relief of inhibitory signals through regulatory tyrosine-based motifs in src-family PTKs (61).

Role of Transcription Factors in the Regulation of BCR Signals. The genes up-regulated in memory and GC B cells include classical transcriptional activators of Ig genes including OCT2, OBF1, NF-κB1, and NF-κB2 (Fig. 2, A and B). These genes are involved in autolytic loops initiated from the BCR through transcriptional activation of Igα (by OCT2; reference 55), synergism with BTK (by OBF1; reference 56), and amplification of BCR-dependent anti-apoptotic signals and proliferation-stimuli (by NF-κB; reference 43). BCR engagement and subsequent NF-κB activation are linked by the protooncogene BCL10 (57), which is expressed at high levels in pre-B and memory but not naive B cells (Fig. 2 B). In GC and memory B cells, OCT2 was found up-regulated together with its transcriptional target gene CD36 (Fig. 2 B). Stage-specific expression of CD36 could be meaningful because based on the dependence of CD36 expression on transcriptional activation by OCT2, it was speculated that OCT2 could regulate B cell differentiation through CD36 (58). The members of the ETS family of transcription factors, FLI-1 (59), PU.1, and SPL-B (60) have recently been demonstrated as critical components of BCR-dependent activation-signaling pathways. FLI-1, PU.1, and SPL-B are expressed at high or intermediate levels in GC and memory B cells but are not detected in the SAGE library for naive B cells (Fig. 2 A).

Thus, antigen-encounter during the GC reaction, up-regulation of IL-4R, and initiation of IL-4–dependent signals might result in a far-reaching phenotypic change, namely transcriptional silencing of a large group of inhibitory receptors that are abundantly expressed in naive B cells. Whether and to which extent the inverse regulation of responsiveness to BCR cross-linking in naive and memory B cells results from differential IL-4R signaling was tested in a cell culture experiment (Fig. 5). Naive and memory B cells were purified from peripheral blood and cultured either in medium alone, or with human recombinant IL-4. In another set of experiments, memory B cells were preincubated with IL-4 and subsequently treated with a neutralizing anti–IL-4R antibody. After 48 h, the cells were subjected to RNA isolation and subsequent semi-quantitative RT-PCR analysis for mRNA expression of the positive regulatory BCR signaling molecules BLK, BTK, BLNK, and SYK, their inhibitors CSK, SHIP, and SHP1 and negative regulatory IgSF receptors including LIRB1, LIRB2, LIRB5, SlgLeC5, SlgLeC8, LAIR1, and CD66 (Fig. 5).
In naive B cells, presence or absence of IL-4 did not affect the expression of positive or negative regulatory BCR signaling molecules. In peripheral blood memory B cells, however, complete deprivation from IL-4 in cell culture medium for 48 h resulted in a concomitant decrease of mRNA levels of BLK, BTK, BLNK, and SYK with markedly increased expression levels of inhibitory IgSF receptors (Fig. 5) as compared with expression levels in ex vivo analyzed peripheral blood memory B cells (for comparison, see amplification products in Fig. 3, at 32 cycles for LAIR1 and 28 cycles for the other genes studied here). Loss of positive and gain of negative regulatory BCR signaling molecules upon withdrawal of IL-4 in peripheral blood memory B cells indicates that gene regulation through IL-4R requires continuous presence of its ligand. However, serum levels of IL-4 are low in healthy individuals (i.e., <0.1 pg/ml; reference 38), which suggests that peripheral blood memory B cells are able to respond also in the presence of low concentrations of IL-4. It is indeed conceivable, that memory B cells have acquired higher responsiveness to IL-4 (e.g., by up-regulation of IL-4R) during maturation within GCs, in which IL-4–producing TH2-cells are highly concentrated (62). Treatment of memory B cells with IL-4 at high concentrations induced a slight increase of mRNA levels of BLK, BTK, BLNK, and SYK but a marked reduction of mRNA levels of the inhibitory IgSF receptors LIRB1, LIRB2, LIRB5, SigLeC5, SigLeC8, LAIR1, and CD66. IL-4 treatment did not affect expression levels of other inhibitory BCR signaling molecules in memory B cells including CSK, SHIP, and SHP1, which are expressed at constitutively lower levels in memory than in naive B cells (Fig. 5). Consistent with low-level expression of IL-4R in naive B cells (Figs. 2 A, 3, and 4), IL-4 can induce transcriptional repression of inhibitory IgSF receptors in memory but not naive B cells. Inhibition of IL-4R–dependent signals by a neutralizing antibody, which was added after preincubation with high concentrations of IL-4, only slightly reduced mRNA levels of the positive mediators of BCR signaling BLK, BTK, BLNK, and had no effect on expression levels of the negative regulatory receptors CSK, SHIP, and SHP1. However, inhibitory IgSF receptors including LIRB1, LIRB2, LIRB5, SigLeC5, SigLeC8, LAIR1, and CD66 were markedly down-regulated in memory B cells in the presence of neutralizing anti–IL-4R antibody, and reached similar expression levels as in naive B cells. Thus, down-regulation of inhibitory IgSF receptors in memory B cells largely depends on IL-4R engagement.

**Figure 5.** Regulation of inhibitory IgSF receptors in memory B cells by IL-4. Naive and memory B cells were purified from peripheral blood and cultured either in medium alone, or with IL-4. In another set of experiments, memory B cells were cultured in the presence of a neutralizing anti–IL-4R antibody, which was added after 8 h of preincubation with IL-4. The left and center panels show amplification products of semi-quantitative RT-PCR for positive regulatory PTKs (BLK, BTK, SYK), the linker molecule BLNK, the negative regulatory PTK CSK, the inhibitory PTPs SHIP and SHP1, and the inhibitory IgSF receptors LIRB1, LIRB2, LIRB5, SigLeC5, SigLeC8, and CD66. In the right panel, the genomic loci of these genes are indicated.

**Abbreviations:** PTK, protein tyrosine kinase; IgSF, immunoglobulin superfamily; PTP, protein tyrosine phosphatase

**Clustering of IgSF Genes Overexpressed in Naive B Cells to a Region on Chromosome 19.** As expression levels of other inhibitory BCR signaling molecules such as CSK, SHIP, and SHP1 remain stable in the presence or absence of IL-4R signaling, transcriptional repression induced by IL-4 in memory B cells seems to be specific for inhibitory IgSF receptors. In this regard, it is notable that the genes coding for the ITIM-bearing IgSF receptors studied here and also many inhibitory killer cell Ig-like receptors are arranged in a cluster within a 2.9 Mbp region on chromosome 19 (19q13.2–q13.4; see Fig. 5). This cluster of genes also includes the inhibitory IgSF receptors CD22, GP6 and CD33, which are highly expressed in naive but not GC and memory B cells (Figs. 2 C, 3, and 4). Therefore, it is tempting to speculate that this cluster on chromosome 19 harbors a large number of IgSF genes sharing a common mechanism of transcriptional regulation in that they can be silenced by IL-4R–dependent signals.

Differential responsiveness to IL-4 in naive and memory B cells suggests that human B cells may acquire sensitivity to IL-4 during the GC reaction, while IL-4R–dependent signaling itself facilitates transduction of signals initiated from the BCR. Further studies are needed to identify other mediators of the relief of the “inhibition-phenotype” in human B cells after antigen-encounter in the GC.

**Regulation of BCR Responsiveness by IL-4R Signals in Memory B Cells.** Having shown that IL-4 can down-regulate inhibitory IgSF receptors in memory but not naive B cells (Fig. 5), we studied the effect of IL-4R signals on BCR–dependent Ca2+ mobilization in memory B cells. Af-
ter preincubation of memory B cells in PBMC-conditioned medium in the presence or absence of human recombinant IL-4 or an inhibitory anti-IL-4Rα antibody, memory B cells were challenged with anti–human IgG + IgM F(ab')2 fragments and Ca

mobilization was measured. Addition of IL-4 beyond physiological concentrations had no effect on BCR responsiveness of memory B cells (Fig. 6 C). However, inhibition of IL-4Rα resulted in a decrease of the peak size and a rapid decline of the calcium signal, which suggests that integrity of BCR responsiveness in memory B cells requires signals through the IL-4R (Fig. 6 C). This was expected, as inhibition of IL-4R signaling resulted in reexpression of inhibitory IgSF molecules in memory B cells (Fig. 5). The failure of supraphysiological IL-4 concentrations to further augment BCR-dependent Ca

mobilization in memory B cells suggests that already low IL-4 concentrations (e.g., as in human serum) may be sufficient to maintain full BCR responsiveness to antigen.

Regulation of BCR Responsiveness by the IgSF Receptor LIRB1 in Naive and Memory B Cells. As IL-4 can repress inhibitory IgSF receptors in memory but not naive B cells, we next investigated the direct consequences of IgSF receptor signaling on the responsiveness of the BCR in both B cell subsets. As an example for inhibitory IgSF receptors, we chose LIRB1, which is most prominently expressed in naive B cells (45 tags; Figs. 2 C, 3, and 4) but missing in the SAGE profiles for GC and memory B cells (Figs. 2 C, 3, and 4). To determine how LIRB1 can modify BCR responsiveness, changes of cytoplasmic Ca

concentration in response to BCR engagement were measured. To this end, naive and memory B cells were preincubated in supernatants conditioned by LPS-stimulated PBMCs in the presence or absence of agonistic (clone GVI/75 cross-linked by goat anti–human IgG) or inhibitory (clone HP-F1 non-cross-linked) antibodies against LIRB1. After LPS treatment, PBMCs release both levels of soluble MHC class I molecules, which can serve as natural ligand for LIRB1 (13, 14). Naive and memory B cells were cultured in supernatants conditioned by LPS-stimulated PBMCs in the presence of either an agonistic (clone GVI/75 cross-linked by goat anti–human IgG) or no further reagents (black curve, none). For quantitation, area under curve (AUC) values were calculated. Statistically significant differences from controls (black curves; none) with P < 0.05 were determined using Fisher’s exact test and indicated by asterisks.

Figure 6. Regulation of BCR-dependent Ca

mobilization by LIRB1 and IL-4 in naive and memory B cells. Naive (A) and memory (B) B cells were preincubated for 24 h in medium, which had been conditioned by LPS-stimulated PBMCs, in the presence of either an antagonistic (light gray curve, αLIRB1 HP-F1) or an agonistic antibody to LIRB1 cross-linked by goat anti–mouse IgG serum (dark gray curve, αLIRB1 GVI/75-GAM) or no antibody (black curve, none). Naive and memory B cells were stimulated with anti–human IgM F(ab')2 and anti–human IgG + IgM F(ab')2 fragments, respectively, at the indicated times (arrows) and changes of intracellular Ca

concentrations in response to BCR engagement were measured by confocal microscopy. Whereas treatment of naive and memory B cells with an anti-CD3 antibody had no effect on intracellular Ca

levels (not shown), stimulation with anti–human IgM F(ab')2 and anti–human IgG + IgM F(ab')2 fragments increased the concentration of cytoplasmic Ca

in naive and memory B cells (Fig. 6). While stimulation (GAM-cross-linked GVI/75 antibody) and inhibition (noncross-linked HP-F1 antibody) of LIRB1 significantly affected the peak amplitude and duration of the calcium signal in naive B cells (Fig. 6 A), agonistic and antagonistic antibodies had no significant effect on BCR-dependent Ca

mobilization in memory B cells (Fig. 6 B). As expected, the overall signal intensity of BCR engagement was significantly higher in memory (peak [Ca

concentration 220 nmol/L) than in naive B cells (peak [Ca

concentration 140 nmol/L). Also, steady-state levels of cytoplasmic Ca

were higher in memory (~100 nmol/L) as compared with naive B cells (~50 nmol/L). Interestingly, occupancy of LIRB1 by the inhibitory antibody raised Ca

mobilization in response to BCR engagement to similar levels as in memory B cells (Fig. 6 A and B). That inhibition of LIRB1 alone could nearly restore BCR responsiveness in naive B cells in vitro does not rule out an important contribution of other inhibitory IgSF receptors identified in this study to silencing of BCR signals in vivo. This is supported by a number of animal models, in which inactivation of a single gene similarly resulted in profound changes of BCR responsiveness (63–66). That other inhibitory
IgSF receptors, which were found specifically expressed in naive B cells by SAGE, RT-PCR and flow cytometry (Figs. 2 C, 3, and 4), have a similar effect on BCR signals in naive B cells appears likely but remains to be established. Conversely, engagement of LIRB1 by the agonistic cross-linked antibody further suppressed and shortened the calcium signal in naive B cells initiated by BCR ligation (Fig. 6 A). None of these effects could be seen in memory B cells (Fig. 6 B), which is consistent with absence of LIRB1 expression in memory B cells (Fig. 2 C, 3, and 4).

We conclude that IL-4 is essential to down-regulate inhibitory IgSF receptors in antigen-experienced B cells. As inhibitory IgSF receptors can indeed silence BCR signals, their repression by IL-4 is required to maintain an intense signaling capacity and responsiveness of the BCR to antigen.

Concluding Remarks. Unlike naive or memory B cells, pre- and GC B cells exhibit a particular propensity to apoptosis in that they enter a readily initiated apoptosis program unless they are rescued by survival signals from the (pre)-BCR. Intensified activation signaling in the surviving pre and GC B cells can, therefore, be an effect of selection. Neither naive nor memory B cells are directly involved in selection processes. In the case of naive and memory B cells, the distinct gene expression pattern of activating and inhibitory BCR signaling molecules is probably largely cell autonomous and marks a constitutive difference between the two subsets.

In naive B cells, attenuation of BCR signals (e.g., by inhibitory IgSF receptors) may prevent the mature activation upon antigen encounter. Conversely, amplification of BCR activity may contribute to facilitated responsiveness of memory B cells (Fig. 6 A). Resulting from the PCRs, for example, activating expression levels of activating BCR signaling molecules in memory B cells also tend to be higher than those in pre-B and GC B cells (Fig. 2, A and B). High expression levels of costimulatory molecules may reflect activation in pre-B and GC B cells and sensitization and increased responsiveness to antigen in memory B cells.

A constitutively active signaling machinery in memory B cells may not only increase responsiveness to but also confer independence from BCR cross-linking by antigen. Indeed, the capacity to persist independently from the immunizing antigen was thought to define “true immunological memory” (68) as opposed to survival within a chronic immune response, in which the immunizing antigen can be retained and presented for extended periods of time by follicular dendritic cells within GCs. To ensure persistence of B cell memory, perpetuation of a B cell–autonomous “maintenance signal” would be required in the former but not in the latter case. Recent data suggest that the continuous presence of the immunizing antigen is indeed dispensable for the survival of memory B cells provided that a functional BCR is expressed on the cell surface to initiate the “maintenance signal” (68). The concept that the BCR in memory B cells has distinct intrinsic signaling properties is further supported by recent data on a burst-enhancing role of the membrane spanning region of IgG in memory B cells (69). Earlier findings suggested that, unlike IgM (three cytoplasmic residues), the cytoplasmic tail of IgG1 (28 residues) as such can contribute to signal transduction through the BCR, presumably involving a tyrosine-based motif within Cγ1 (70).

Although both naive and memory B cells critically depend on continuous survival signals from a functional BCR (2), we show in a genome-wide gene expression analysis that BCR–dependent signals in naive and memory B cells differ fundamentally from each other. Conceivably, these differences may owe to some extent to IL-4–dependent ablation of negative regulatory signals from inhibitory receptor molecules. However, IL-4R signals, although required for transcriptional suppression of inhibitory IgSF receptors and for integrity of BCR–dependent Ca\(^{2+}\) mobilization in memory B cells, had no effect on their naive precursors. Therefore, the mechanism establishing restraint of BCR signals in naive B cells was derived upon antigen-encounter within the follicles and awaits further investigation.

Immunization was supported by the Deutsche Forschungsgemeinschaft through SFB 502 and a Heisenberg award to R. Muschen is supported by the Cancer Research Institute, New York, NY, through the Tumor Immunology Program. This work was supported in part by the Deutsche Forschungsgemeinschaft through the Emmy-Noether-Programm (to M. Mischten; MU1616/2-1 and MU1616/2-2), Harold and Lelia Y. Mathers Foundation (S.M. Wang and J.D. Rowley), SFB 502 and a Heisenberg award to R. Küppers, by the Köln Fortune Program/Faculty of Medicine, University of Cologne, and the German José Carreras Leukemia Foundation (grant to M. Mischten).
B Cell Receptor Signaling in Naive B Cells

13966–13971.
8. Müschen, M., S. Lee, G. Zhou, N. Feldhahn, V.S. Barath, J. Chen, C. Moers, M. Krönke, J.D. Rowley, and S.M. Wang. 2002. Molecular portraits of B cell lineage commitment. *Proc. Natl. Acad. Sci. USA.* 99:10014–10019.
9. Müschen, M., D. Re, B. Jungnickel, V. Diehl, K. Rajewsky, and R. Küppers. 2000. Somatic mutation of the CD95 gene in human B cells as a side-effect of the germinal center reaction. *J. Exp. Med.* 192:1833–1840.

10. Müschen, M., K. Rajewsky, A. Bräuninger, A.S. Baur, J.J. Oudejans, A. Roers, M.-L. Hansmann, and K. Rajewsky. 2001. The follicular versus marginal zone B lymphocyte cell fate decision is regulated by the CD27 cell surface antigen. *Proc. Natl. Acad. Sci. USA.* 98:13866–13871.
11. Nagumo, H., K. Agematsu, N. Kobayashi, K. Shinozaki, S. AIOLOS, A. Brieva, J.A., L.M. Villar, G. Leoro, J.C. Alvarez-Cermeno, and J. Georgopoulos, and S. Pillai. 2001. The follicular versus marginal zone B lymphocyte cell fate decision is regulated by the CD27 cell surface antigen. *Proc. Natl. Acad. Sci. USA.* 98:13866–13871.
12. Satoh, H., L.A. Blatter, and D.M. Bers. 1997. Effects of Ca^2+ on the germinal center reaction. *J. Exp. Med.* 191:387–394.
13. Banham, A.H., M. Colonna, M. Cella, K.J. Micklem, K. Pulmon. 2001. Different process of class switching and somatic hypermutation; a novel analysis by CD27+ naive B cells. *Blood.* 99:567–575.
14. Colonna, M., F. Navarro, T. Bellon, M. Llano, P. Garcia, J. Samaridis, L. Angman, M. Cella, and J.V. Ravetch. 1997. Deletion of B7-1 and B7-2 from B cells by rapid up-regulation of B7-1 and B7-2. *Immunity.* 7:90–293–301.
15. Munro, N.G., and J.C. Cambier. 1983. Sorting of B lymphoblasts based upon cell diameter provides cell populations enriched in different stages of cell cycle. *J. Immunol. Methods.* 63:45–56.
16. Brieva, J.A., L.M. Villar, G. Leoro, J.C. Alvarez-Cermeno, E. Roldan, and P. Gonzalez-Porque. 1990. Soluble HLA class I antigen secretion by normal lymphocytes: relationship with cell activation and effect of interferon-gamma. *Clin. Exp. Immunol.* 82:390–395.
17. Colonna, M., F. Navarro, T. Bellon, M. Llano, P. Garcia, J. Samaridis, L. Angman, M. Cella, and M. Lopez-Botet. 1997. A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphocytes and clonogenic cells. *J. Exp. Med.* 186:1809–1818.
18. Banham, A.H., M. Colonna, M. Cell, A.C. Willis, and D.Y. Ma. 2001. CD85a as an inhibitor of the CD85k antigen as ILT2, an inhibitory receptor of the immunoglobulin superfamily. *J. Biol. Chem.* 276:42070–42076.
19. Williams, D.A. 1997. Imaging with laser confocal scanning cytometry. *Cytometry.* 28:151–157.
20. Petrov, A., L.A. Blatter, and D.M. Bers. 1997. Effects of Ca^2+ on the germinal center reaction. *J. Exp. Med.* 191:387–394.
21. Kirstetter, P., M. Thomas, A. Dietrich, P. Kastner, and S. Chan. 2002. IKAROS is critical for B cell differentiation and function. *Eur. J. Immunol.* 32:720–730.
22. Ono, M., H. Okada, S. Bolland, S. Yanagi, T. Kurosaki, and J.V. Ravetch. 1997. Deletion of SHIP or SHP1 reveals two distinct pathways for inhibitory signaling. *Cell.* 90:293–301.
23. Cariappa, A., M. Tang, C. Parmg, E. Nebelitsky, M. Carroll, K. Georgopoulos, and S. Pillai. 2001. The follicular versus marginal zone B lymphocyte cell fate decision is regulated by AIOLOS, BTK, and CD21. *Immunity.* 14:603–615.
24. Klein, U., K. Rajewsky, and R. Küppers. 1998. Human immunoglobulin (IgM)+IgD+ peripheral B blood cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. *J. Exp. Med.* 188:1679–1689.
25. Liu, Y.J., C. Barthely, O. de Bouteiller, C. Arpin, I. Durand, and J. Banchereau. 1995. Memory B cells from human tonsils colonize mucosal epithelium and directly present antigen to T cells by rapid up-regulation of B7-1 and B7-2. *Immunity.* 2:239–248.
26. Arpin, C., J. Banchereau, and Y.J. Liu. 1997. Memory B cells are biased towards terminal differentiation: a strategy to prevent repertoire freezing. *J. Exp. Med.* 186:931–940.
27. Klein, U., R. Küppers, and K. Rajewsky. 1997. Evidence for a large compartment of IgM-expressing memory B cells in humans. *Blood.* 89:1288–1298.
28. Galibert, L., N. Burdin, B. de Saint-Vis, P. Garonne, C. Van Kooten, J. Banchereau, and F. Rouset. 1996. CD40 and B cell antigen receptor dual triggering of resting B lymphocytes turns on a partial germinal center phenotype. *J. Exp. Med.* 183:77–85.
29. Xu, S., and K.P. Lam. 2001. B-cell maturation protein, which binds the tumor necrosis factor family members BAFF and APRIL, is dispensable for humoral immune responses. *Mol. Cell. Biol.* 21:4067–4074.
30. Schiement, B., J.L. Gommerman, K. Vora, T. Cachero, H. Chiu, T. Shulsm-Morskaya, M. Dobles, E. Frew, and M.L. Scott. 2001. An essential role for BAFF in the normal development of B cells through a BCA-4 independent pathway. *Science.* 293:2111–2114.
31. Hatzoglou, A., J. Roussel, M.F. Bourgeade, E. Rogier, C. Madry, J. Inoue, O. Devergne, and A. Tsapis. 2000. BCMA associates with TRAF1, TRAF2, and TRAF3 and activates NF-κB and p38 mitogen-activated protein kinase. *J. Immunol.* 165:1322–1330.
32. Ceci, M., S. Yang, M. Roose-Girma, S. Erickson, T. Baker, D. Tuma, Y. Shi, V.M. Dixit. 2001. Activation of an immunoglobulin superfamily member encoded in the human chromosome 17. *Nat. Immunol.* 2:119–125.
33. Ono, M., H. Okada, S. Bolland, S. Yanagi, T. Kurosaki, and J.V. Ravetch. 1997. Deletion of SHIP or SHP1 reveals two distinct pathways for inhibitory signaling. *Cell.* 90:293–301.
34. Cariappa, A., M. Tang, C. Parmg, E. Nebelitsky, M. Carroll, K. Georgopoulos, and S. Pillai. 2001. The follicular versus marginal zone B lymphocyte cell fate decision is regulated by AIOLOS, BTK, and CD21. *Immunity.* 14:603–615.
35. Klein, U., K. Rajewsky, and R. Küppers. 1998. Human immunoglobulin (IgM)+IgD+ peripheral B blood cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. *J. Exp. Med.* 188:1679–1689.
36. Liu, Y.J., C. Barthely, O. de Bouteiller, C. Arpin, I. Durand, and J. Banchereau. 1995. Memory B cells from human tonsils colonize mucosal epithelium and directly present antigen to T cells by rapid up-regulation of B7-1 and B7-2. *Immunity.* 2:239–248.
37. Arpin, C., J. Banchereau, and Y.J. Liu. 1997. Memory B cells are biased towards terminal differentiation: a strategy to prevent repertoire freezing. *J. Exp. Med.* 186:931–940.
38. Klein, U., R. Küppers, and K. Rajewsky. 1997. Evidence for a large compartment of IgM-expressing memory B cells in humans. *Blood.* 89:1288–1298.
39. Galibert, L., N. Burdin, B. de Saint-Vis, P. Garonne, C. Van Kooten, J. Banchereau, and F. Rouset. 1996. CD40 and B cell antigen receptor dual triggering of resting B lymphocytes turns on a partial germinal center phenotype. *J. Exp. Med.* 183:77–85.
40. Xu, S., and K.P. Lam. 2001. B-cell maturation protein, which binds the tumor necrosis factor family members BAFF and APRIL, is dispensable for humoral immune responses. *Mol. Cell. Biol.* 21:4067–4074.
