Discriminating gene expression profiles of memory B cell subpopulations

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Morphologically and functionally distinct subpopulations of human memory B (B_{Mem}) cells are identifiable by either their expression of CD27 or Fc receptor–like 4 (FCRL4), an immunoglobulin domain containing a receptor with strong inhibitory potential. We have conducted comparative transcriptome and proteome analyses of FCRL4$^+$ and FCRL4$^-$ B_{Mem} cells and found that these two subsets have very distinctive expression profiles for genes encoding transcription factors, cell-surface proteins, intracellular signaling molecules, and modifiers of the cell-cycle status. Among the differentially expressed transcription factors, runt-related transcription factor 1 (RUNX1) transcript levels were up-regulated in FCRL4$^-$ cells, whereas RUNX2 transcripts were preferentially detected in FCRL4$^+$ cells. In vitro evidence for FCRL4 promoter responsiveness and in vivo promoter occupancy suggested that RUNX transcription factors are involved in the generation of these B_{Mem} cell subpopulations. A distinctive signature profile was defined for the FCRL4$^+$ B_{Mem} cells by their expression of CD11c, receptor activator for nuclear factor κB ligand, and FAS cell-surface proteins, in combination with increased levels of *SOX5*, *RUNX2*, *DLL1*, and *AICDA* expression. We conclude that this recently identified subpopulation of B_{Mem} cells, which normally resides in epithelial tissue–based niches, may serve a unique role in mucosal defense and, conversely, as a target for neoplastic transformation events.

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Abbreviations used: AICDA, activation-induced cytidine deaminase; B_{Mem}, memory B; CBFβ, core-binding factor β; ChIP, chromatin immunoprecipitation; DLL1, delta-like 1; FCRL, Fc receptor like; GC, germinal center; HCK, hemopoietic cell kinase; LCK, lymphocyte-specific protein tyrosine kinase; RANKL, receptor activator for NF-κB ligand; RUNX, runt-related transcription factor; SHP, Src homology 2 domain–containing phosphatase; SOX5, SRY-box 5; XBP1, X-box binding protein 1.

Protective immunity elicited by T cell–dependent antigens may last for decades without further exposure to the immunizing antigen (1, 2), and the memory B (B_{Mem}) cells serve an important role in sustaining humoral immunity. A defining characteristic of B_{Mem} cells is their ability to respond rapidly upon reencounter with their cognate antigens. Most B_{Mem} cells in humans are characterized by their expression of CD27 and switched Ig isotype (3–5). However, recent reports describe an additional population of tissue-based B_{Mem} cells that do not express CD27 (6–9) but instead express Fc receptor–like 4 (FCRL4) (7). FCRL4 is a member of a recently identified family of transmembrane proteins with immunoregulatory potential, five of which display preferential B cell expression (for review see reference 10). Originally identified as a translocation fusion partner in a myeloma cell line, FCRL4 was named immunoreceptor superfamily translocation-associated 1 (11), and an independent identification through its sequence similarity to known Fc receptors led to the name Fc receptor homologue 4 (12). A unified nomenclature was recently adopted in which this gene was renamed *FCRL4* (13).

FCRL4 expression is restricted to a subpopulation of B_{Mem} cells with a distinctive phenotype and tissue localization pattern (7, 14). Its intracellular domain contains three consensus immunoreceptor tyrosine-based inhibition motifs, and coligation of FCRL4 with the BCR aborts BCR-mediated signaling via recruitment of the Src homology 2 domain–containing phosphatase 1 (SHP-1) and/or SHP-2 tyrosine phosphatases (15).

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The FCRL4$^+$ cells are rarely seen in the bone marrow, blood, and spleen, but are found instead in the crypt epithelium and perifollicular regions of the palatine tonsils, intestinal lymphoid tissues, and mesenteric lymph nodes, wherein their numbers are increased in patients with lymphadenitis caused by *Toxoplasmosis gondii*, HIV-1, and EBV infections (7, 14, 16). Most of the FCRL4$^+$ tissue-resident cells do not express CD27, the currently favored marker for human B_{Mem} cells. Although the FCRL4$^-$/CD27$^+$ B_{Mem} cells respond vigorously to in vitro stimulation by T cell–derived cytokines (IL-2 and IL-10) and CD40L or BCR ligation, the FCRL4$^+$ cells preferentially respond to T cell cytokines and CD40L alone (7). The relatively large FCRL4$^+$ B_{Mem} cells and the relatively small FCRL4$^-$ B_{Mem} cells thus represent morphologically, topographically, and functionally distinguishable subpopulations.

To gain additional insight into their differentiation status and function, we examined the transcript expression profiles of these two B_{Mem} cell subpopulations. This transcriptome analysis revealed a surprisingly large number of differentially expressed genes. Using this gene expression data as a starting point, we obtained evidence indicating that differences in transcription factors, cell-surface receptors, and cell-cycle status characterize the differentiation profiles that distinguish these two B_{Mem} cell subpopulations.

RESULTS

Transcriptome profiles differ for FCRL4$^+$ and FCRL4$^-$ B_{Mem} cells

A comparison of transcripts expressed by FCRL4$^+$ versus FCRL4$^-$ B_{Mem} cells was conducted by a gene array analysis of tonsillar tissues from three young donors, none of whom showed signs of illness at the time of tonsillectomy. This comparison led to the identification of 164 transcripts that were expressed with a greater than fourfold difference by the two

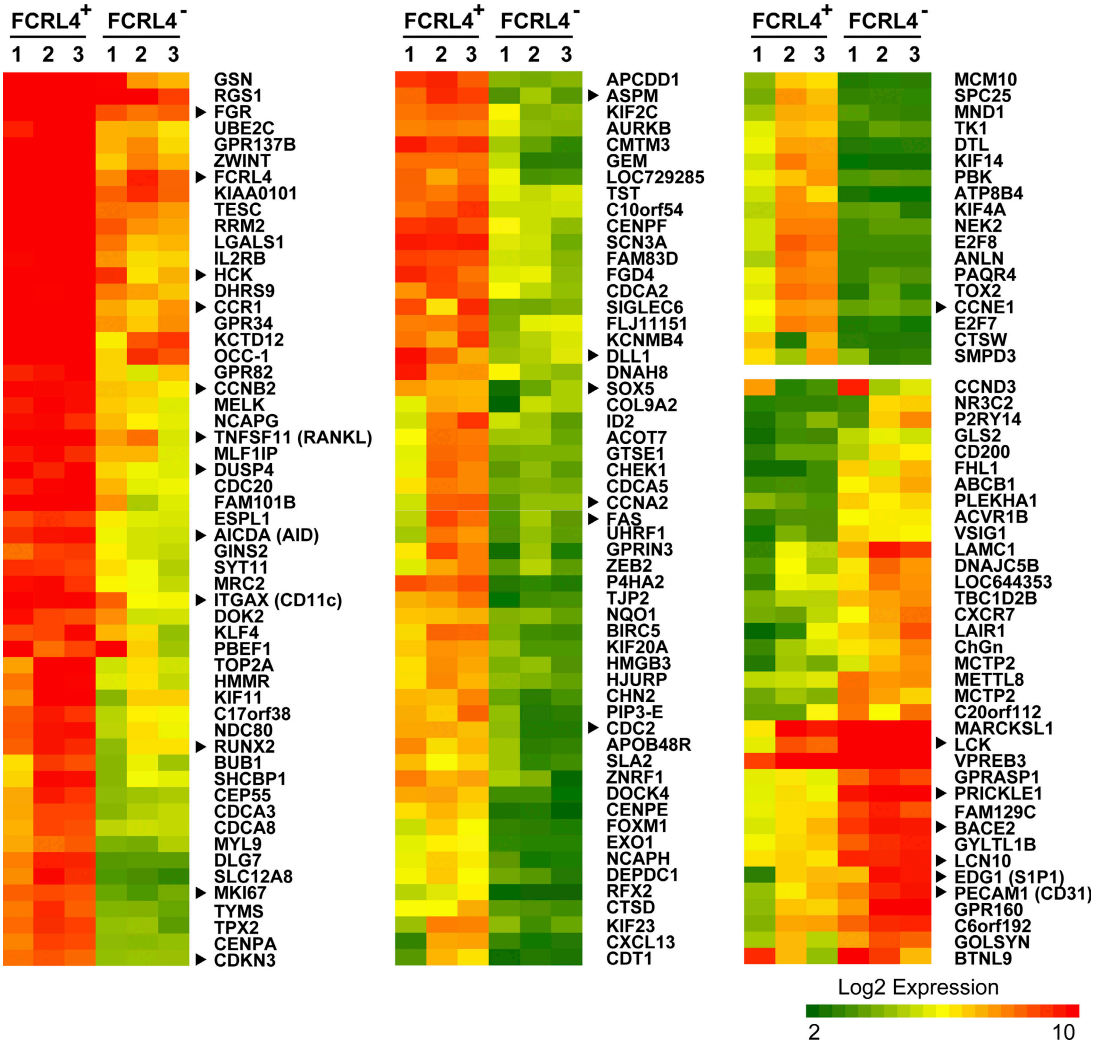

Figure 1. Transcriptome analysis of FCRL4$^+$ and FCRL4$^-$ B_{Mem} cells. The transcripts differentially expressed by FCRL4$^+$ and FCRL4$^-$ B_{Mem} from three independent tonsillar cell preparations are displayed. Up- and down-regulated transcripts are indicated in red and green, respectively. The magnitude of expression is depicted by the color bar. Arrowheads indicate transcripts whose expression was validated by quantitative RT-PCR and/or by FACS analysis of other tonsillar samples.

B_{Mem} cell populations. The large array of differentially expressed genes revealed in this analysis included ones encoding transcription factors, cell-cycle modulators, cell-surface molecules, and components of signal transduction cascades. Fig. 1 shows a heat map of the differentially regulated transcripts. The majority of these up-regulated genes were identified in the FCRL4$^+$ cells. Not surprisingly, FCRL4 was among the strongly up-regulated transcripts in the FCRL4$^+$ population, as were CCR1 transcripts, in keeping with a previous analysis (7). Surprisingly, one of the strongly up-regulated transcripts encoded receptor activator for NF-κB ligand (RANKL), and this unanticipated finding was confirmed by quantitative RT-PCR analysis and measurement of protein expression in other tonsil samples (Fig. 2 A). Quantitative RT-PCR analyses likewise confirmed the higher expression levels of runt-related transcription factor 2 (RUNX2) and SRY-box 5 (SOX5) for the FCRL4$^+$ B_{Mem} cells and relatively higher RUNX1 expression by FCRL4$^-$ B_{Mem} cells (Fig. 2 B). The differential expression patterns observed for several src kinase family members were similarly validated: FCRL4$^+$ cells expressed higher levels of hemopoietic cell kinase (HCK) and FGR, but lower levels of lymphocyte-specific protein tyrosine kinase (LCK), than FCRL4$^-$ cells. Although LCK is typically considered a T cell–specific src family kinase, LCK expression in B cell lines and primary B cells has also been observed (17–20). As would be anticipated, significant differences in transcription levels were not detected for the prototypical B cell src kinase LYN (Fig. 2 B, top). Overall, this transcriptome analysis revealed a relatively large number of transcripts (128) with increased levels of expression in FCRL4$^+$ cells, and a more limited set of transcripts (36) with increased expression levels in FCRL4$^-$ cells.

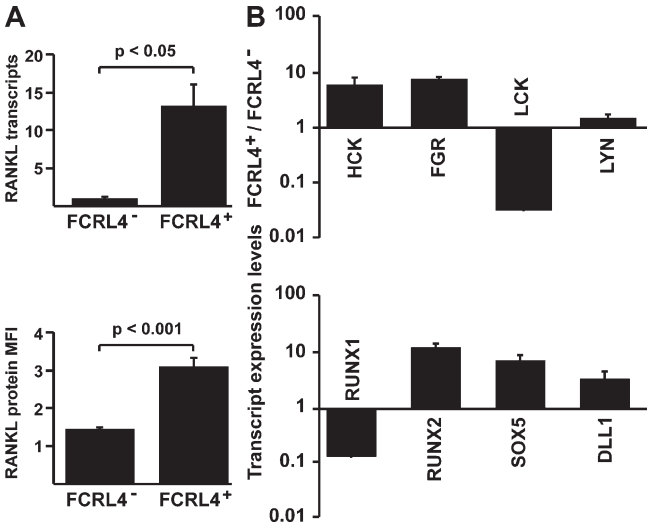

Figure 2. Genes differentially expressed by FCRL4$^+$ and FCRL4$^-$ B_{Mem} cells. (A) RANKL transcript (top) and protein (bottom) levels in FCRL4$^+$ and FCRL4$^-$ B_{Mem} cells. RANKL mRNA levels were determined by quantitative RT-PCR normalized to *RP-2*. Values represent mean ± SEM ($n = 3$), and statistical significance was determined using the paired Student's *t* test. RANKL protein was quantitated by examining the mean fluorescence intensity levels for RANKL on FCRL4$^+$ and FCRL4$^-$ B_{Mem} cells, normalized to the mean fluorescence intensity levels of isotype control antibodies in the corresponding cell populations. Values represent mean ± SEM ($n = 16$), and statistical significance was determined using the paired Student's *t* test. (B) Quantitative RT-PCR analysis of the indicated src family kinases (top), or DLL1 and transcription factor transcripts (bottom) in FACS-sorted FCRL4$^+$ and FCRL4$^-$ B_{Mem} cells. Values represent mean ± SEM ($n = 4$).

Distinctive surface marker profile of FCRL4$^+$ cells

We performed quantitative RT-PCR and FACS profile analyses to verify the differential expression of transcripts for cell-surface receptors that might be useful in distinguishing the two B_{Mem} cell populations (Fig. 3). Flow cytometric immunofluorescence analysis confirmed that the FCRL4$^+$ cells express relatively high levels of CD95 (FAS) in comparison with FCRL4$^-$ cells. Interestingly, the FCRL4$^+$ B_{Mem} cells expressed CD11c, and most were negative for cell-surface CD11b, whereas the opposite was true for the FCRL4$^-$ B_{Mem} cells. The FCRL4$^-$ cells also preferentially expressed CD31, and a substantial subpopulation of these cells expressed CD23. In accord with previous results (7), FCRL4$^+$ B_{Mem} cells expressed higher surface levels of CD20 and lower levels of CD21 than their FCRL4$^-$ counterparts. Finally, in keeping with the relatively high levels of RANKL mRNA expression, we observed higher cell-surface RANKL expression levels for the FCRL4$^+$ B_{Mem} cells by immunofluorescence analysis (Fig. 2 A and Fig. 3). Thus, in addition to their expression of FCRL4, this subpopulation of B_{Mem} cells was found to have the following characteristic cell-surface profile: CD11c$^+$, CD20^{hi}, CD31$^-$, CD95$^+$, and RANKL$^+$.

Cell-cycle status of FCRL4$^+$ versus FCRL4$^-$ B_{Mem} cells

The gene array data indicated relatively high expression levels in the FCRL4$^+$ cells for several genes regulating entry and progression through the cell cycle. Quantitative RT-PCR confirmed that these transcripts, including several cyclins as well as the cell division cycle 2 kinase subunit of the M-phase promoting factor, were increased in the FCRL4$^+$ subpopulation of B_{Mem} cells (Fig. 4). Ki-67, a nuclear protein preferentially expressed by cycling cells, was among the transcripts with increased expression levels in FCRL4$^+$ cells (Fig. 1). Using Ki-67 and propidium iodide costaining, which allows the discrimination of cells in the G_0 versus the G_1 phase of the cell cycle, we found a substantial fraction of the FCRL4$^+$ population (42.8% ± 18.3 SEM; $n = 4$) to be in the G_1 phase; smaller fractions of S-phase and G_2/M-phase cells were also observed (Fig. 4 B). In contrast, the cells in the FCRL4$^-$ population were almost all in the G_0 phase (95.8% ± 1.1 SEM; $n = 4$). In the representative experiment shown in Fig. 4 B, note that cells in the G_0 phase are located in the bottom left quadrant of the FACS profile and cells in the G_1 phase are in the top left quadrant; cells in the S, G_2, and M phases of cell cycle are located in the top right quadrant. In conclusion, the findings of heightened expression of genes regulating entry and progression through the cell cycle provide evidence for prior activation of the relatively large FCRL4$^+$ B_{Mem} cells.

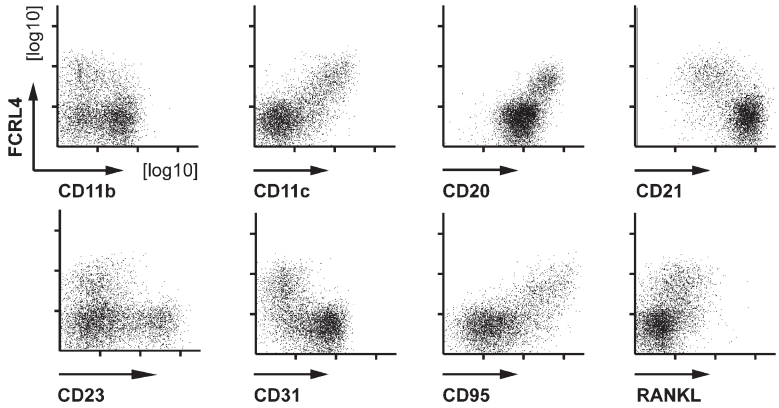

Figure 3. Expression of cell-surface antigens on human B_{Mem} cell populations. FACS analysis of tonsillar lymphocytes was performed for the indicated markers by gating on the B_{Mem} cell population ($CD19^+$, IgD^-, $CD38^-$). Representative FACS profiles from at least 12 analyzed tonsillar preparations are depicted.

Differential transcription factor expression by B_{Mem} cell subpopulations

One of the goals in this comparison of transcripts expressed by $FCRL4^+$ versus $FCRL4^-$ cells was to gain a better view of the differentiation status of these B_{Mem} cell subpopulations. Differentially expressed transcription factors were therefore of particular interest. The SOX5 transcription factor was among the strongly up-regulated transcripts observed for $FCRL4^+$ cells in the gene array analysis (Fig. 1). This member of the SRY-box–containing family of transcription factors was expressed in $FCRL4^+$ cells at ~12-fold higher levels than in $FCRL4^-$ cells, a finding that was confirmed by quantitative RT-PCR (Fig. 2 B). We found that, in addition to SOX5, the RUNX2 transcription factor was preferentially up-regulated in $FCRL4^+$ cells. In contrast, quantitative RT-PCR indicated that RUNX1 was expressed at higher levels in $FCRL4^-$ cells, although just outside the parameters used for inclusion of differentially expressed transcripts in the gene array assessment. Expression of the *RUNX* genes is regulated by two separate promoters. In the B_{Mem} cell populations analyzed, the RUNX1 transcripts were initiated from the distal promoter (P1) in $FCRL4^-$ cells, whereas transcription of three different RUNX2 isoforms was initiated from the proximal promoter (P2) by cells of the $FCRL4^+$ population (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20072682/DC1).

FCRL4 promoter responsiveness to Runx transcription factors

To address the possibility that the *FCRL4* gene is regulated by these differentially expressed transcription factors, we cloned the putative promoter region, a genomic fragment extending 2,600 bp upstream of the initiating ATG, into a luciferase reporter construct. Sequence analysis of this putative promoter region identified eight potential RUNX binding sites and one SOX5 binding site (Fig. 5 A). Cotransfection of the FCRL4 reporter plasmids with RUNX1 or RUNX2 expression vectors resulted in little or no detectable activation. However, the putative *FCRL4* promoter activated the reporter gene robustly after cotransfection of RUNX1 with core-binding factor β (CBFβ), its normal heterodimeric partner. Conversely, limited reporter gene activity was observed

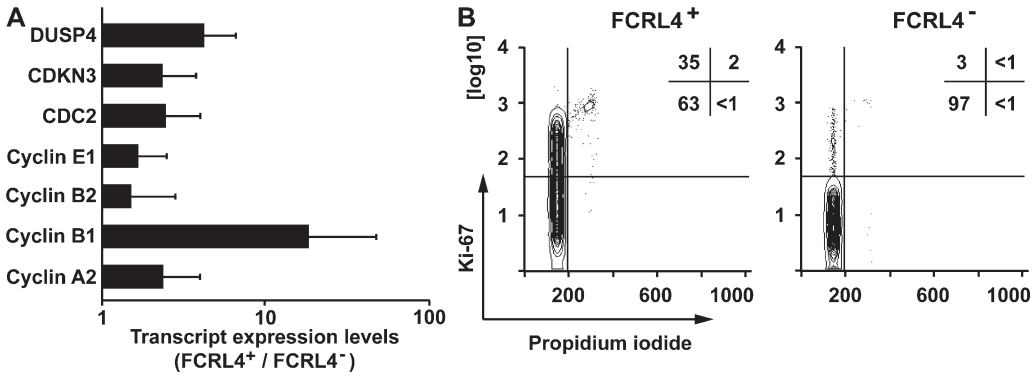

Figure 4. Cell-cycle phenotype of $FCRL4^+$ and $FCRL4^-$ B_{Mem} cells. (A) Quantitative RT-PCR analysis of the indicated transcripts. Values were normalized to RP2 expression and are displayed as the mean ratio of $FCRL4^+/FCRL4^-$ ± SEM (n = 5 independent PCRs on independent tonsil preparations). (B) Staining of FACS-sorted $FCRL4^+$ and $FCRL4^-$ B_{Mem} cells for DNA content and Ki67 expression. Shown is a representative example of four independent tonsillar B_{Mem} cell preparations. Numbers in the top right of each FACS blot indicate the percentage of cells in each quadrant.

after cotransfection of the RUNX2-isoforms with CBFβ. Cotransfection of SOX5 with CBFβ and either RUNX1 or RUNX2 did not lead to any difference in reporter gene activity (Fig. 5 B). As a control we transfected the paired box 5 B cell transcription factor, which has no binding sites in the *FCRL4* promoter, and observed no promoter activation (Fig. 5 B).

Because the reporter gene activation assays indicated the potential of RUNX1 and, to a lesser degree, RUNX2 to regulate the *FCRL4* promoter, we performed chromatin immunoprecipitation (ChIP) followed by quantitative PCR to determine whether RUNX transcription factors occupy the *FCRL4* promoter in primary tonsillar B_{Mem} cells. Significant RUNX2 binding to *FCRL4* promoter sequences was not detected in these experiments (unpublished data). However, we found that endogenous RUNX1 bound to promoter region sequences of *FCRL4* in FCRL4$^-$ cells but not in FCRL4$^+$ cells (Fig. 5 C). These findings are consistent with a functional role for RUNX1 in the differential transcriptional regulation of the B_{Mem} cell subpopulations.

Signature transcript expression profiles for B_{Mem} cell populations

In a search for genes preferentially expressed in one or the other of these two subpopulations of B_{Mem} cells and not in other stages of B cell differentiation, we selected a panel of the differentially expressed genes for comparative analysis at all stages in the differentiation of peripheral B cells (Fig. 6). Notch ligand delta-like 1 (DLL1) was found to be expressed at relatively high levels by naive B cells as well as FCRL4$^+$ B_{Mem} cells. Interestingly, the expression levels of activation-induced cytidine deaminase (AICDA), more commonly known as AID, in FCRL4$^+$ cells approached those observed for the pre–germinal center (GC) and GC B cells, even though the GC-specific transcription factor B cell lymphoma 6, which serves to maintain the proliferative status of GC B cells, was not expressed above background levels by either B_{Mem} cell subpopulation. We also confirmed that neither of the B_{Mem} cell subpopulations expressed B lymphocyte–induced maturation protein 1 (Fig. 6) or X-box binding protein 1s (XBP1s [the spliced isoform of XBP1]; not depicted) transcripts, which are expressed exclusively by cells undergoing plasma cell differentiation. In addition to their preferential expression of FCRL4, the FCRL4$^+$ B_{Mem} cells also proved to be distinguishable from B cells at other stages of differentiation by their expression of RANKL, RUNX2, and SOX5. In contrast, although the higher expression patterns for lipocalin 10, prickle-like 1, β-site amyloid precursor protein–cleaving enzyme 2, and endothelial differentiation gene 1 transcripts in FCRL4$^-$ B_{Mem} cells could be confirmed by quantitative RT-PCR, none of these transcripts were found to be exclusively expressed by this subpopulation of B lineage cells. In conclusion, this analysis provides a distinctive gene expression signature for the FCRL4$^+$ B_{Mem} cells, featuring the preferential expression of *RANKL*, *SOX5*, and *RUNX2* in combination with shared expression of *AICDA*.

DISCUSSION

This comparative analysis of the transcripts preferentially expressed by FCRL4$^+$ B_{Mem} cells versus those expressed by the FCRL4$^-$ B_{Mem} cells reveals intriguing differences in their gene expression profiles. The differentially expressed transcripts fall into several categories: transcription factors, DNA-modifying enzymes, cell-cycle regulators, adhesion molecules, homing

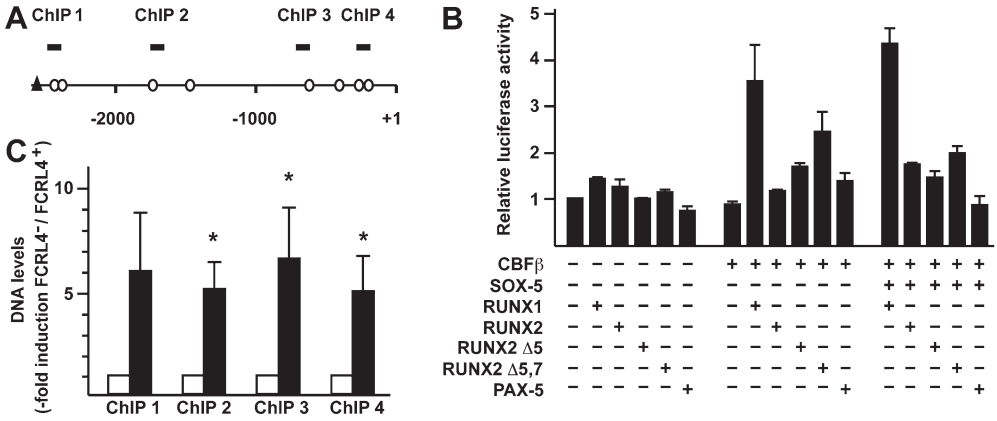

Figure 5. RUNX1 responsiveness of the *FCRL4* gene promoter. (A) Analysis of the putative promoter sequence of the *FCRL4* gene. Locations are indicated for potential RUNX (open circles) and SOX5 (closed triangle) binding sites. (B) Luciferase reporter gene studies on transiently transfected 293T cells. Cells were transfected with the putative promoter of the *FCRL4* gene controlling expression of the luciferase reporter gene and expression vectors containing the indicated transcription factors. RUNX2 Δ5 and RUNX2 Δ5,7 are splice isoforms that lack exon 5 and exons 5 and 7, respectively (see also Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20072682/DC1). Values represent mean ± SD ($n = 5$). (C) ChIP assay of RUNX1 binding in FACS-sorted FCRL4$^+$ and FCRL4$^-$ B_{Mem} cells. Positions of the amplified PCR fragments are indicated by closed rectangles in A. Open bars indicate signals obtained from FCRL4$^+$ cells, and shaded bars indicate signals obtained from FCRL4$^-$ cells. All values were normalized to the PCR signal obtained from control immunoprecipitates (anti–SHP-1), as well as to unrelated, nonspecifically copurified DNA (CCR4). Values represent mean ± SEM ($n = 5$). Statistical significance of $P < 0.05$ is indicated by an asterisk.

receptors, signal transduction intermediates, apoptosis-regulating molecules, and receptors that may induce activation and differentiation by cells of other lineages.

One defining characteristic of the FCRL4$^+$ and FCRL4$^-$ populations of B_{Mem} cells is their reciprocal expression of the RUNX1 and RUNX2 transcription factors that have important developmental roles. Our data suggest that these transcription factors may also influence B_{Mem} cell differentiation decisions. The three mammalian *Runx* genes are homologues to the *Drosophila runt* gene that has an essential role in establishing the embryonic segmental body pattern (21). All three of these highly conserved genes encode transcription factors that recognize the same DNA binding sequence, and each heterodimerizes with the CBFβ transactivation unit (for review see references 22, 23). RUNX1 appears to be important for hemopoietic development because mice with targeted disruption of the *Runx1* locus die early in embryogenesis without definitive hemopoiesis (24). However, inducible *Runx1* knockout studies suggest that this gene does not affect general hemopoiesis in adult life, where its absence instead leads to reduced lymphocyte numbers and a myeloproliferative phenotype (25). Runx1 cooperates with early B cell factor to activate the *mb-1* gene during B lineage differentiation (26). Runx2, a master regulator for bone development (27, 28), may similarly contribute to the regulation of many genes in addition to those controlling osteogenesis (29).

Our analysis of B_{Mem} cell subpopulations suggests that differential expression of the RUNX1 and RUNX2 transcription factors could modulate expression of *FCRL4* itself. Alternatively, they may influence other target genes that determine the distinctive B_{Mem} cell differentiation patterns. We identified multiple potential RUNX binding sites in the predicted promoter region 2.6 kb upstream of the *FCRL4* gene, and reporter gene analysis indicated RUNX1/CBFβ responsiveness for this putative FCRL4 promoter region. Preferential RUNX2 mRNA expression was observed for the FCRL4$^+$ B_{Mem} cells and, when overexpressed in reporter gene expression studies, RUNX2 activated the *FCRL4* promoter, albeit to a modest extent. However, we were unable to demonstrate the involvement of this transcription factor in the regulation of FCRL4 expression. The same was true for SOX5, the other transcription factor for which we found elevated expression in FCRL4$^+$ B_{Mem} cells. Our findings to date are thus more consistent with the possibility that other genes whose transcripts make up the distinctive transcriptome of FCRL4-bearing B_{Mem} cells are influenced by these two transcription factors. Somewhat paradoxically, ChIP assays indicated RUNX1 occupancy of the *FCRL4* promoter region in primary FCRL4$^-$ B_{Mem} cells, a finding suggesting that RUNX1 may negatively regulate this promoter in vivo. A negative regulatory role for RUNX1 has indeed been described in lymphocytes, most recently with the finding of Th-inducing POZ/Krüppel-like factor repression in CD8$^+$ T cells (30). Although RUNX1 can associate with the corepressor mSin3A in the absence of extracellular signal-regulated kinase–mediated RUNX1 phosphorylation, the corepressor

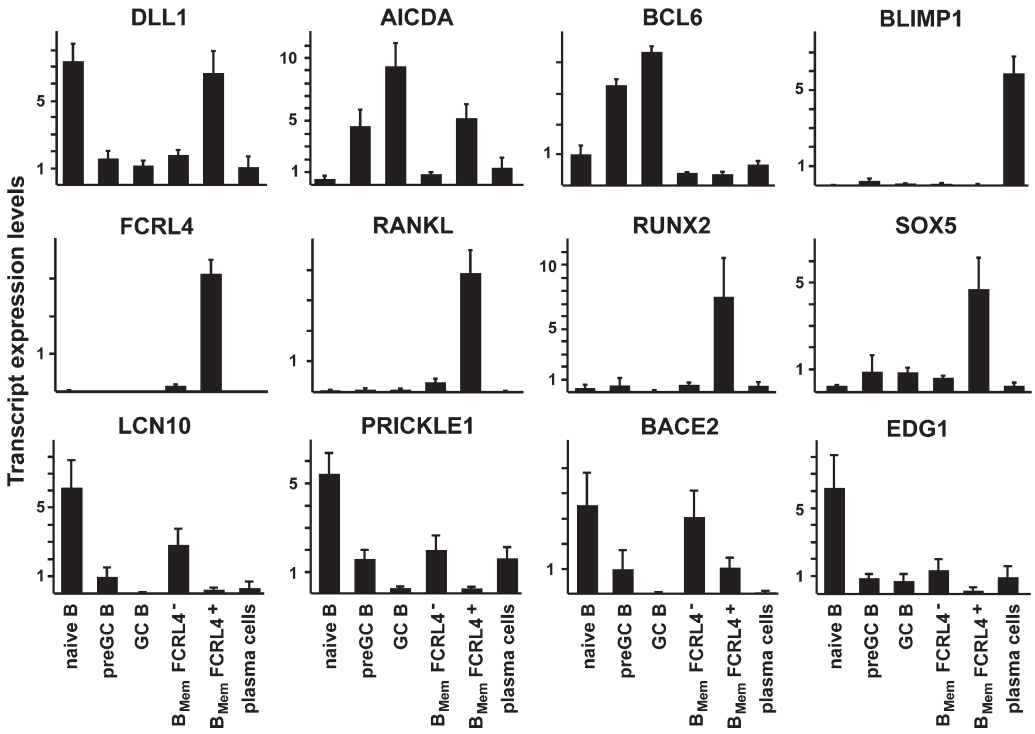

Figure 6. Differentially regulated transcripts in FCRL4$^+$ and FCRL4$^-$ B_{Mem} cells in comparison with other stages in B cell differentiation. Analysis of expression levels of the indicated transcripts in naive B, pre–GC B (preGC B), GC B (GC B), FCRL4$^+$ and FCRL4$^-$ B_{Mem}, and plasma cells. All values are normalized to RP2 expression. Error bars indicate one SEM (n = 3).

interaction is lost and protein stability is diminished after mitogen-activated protein kinase–mediated phosphorylation of serine residues 249 and 266 (31). A negative RUNX1 regulatory function would thus be favored by the G_0 cell-cycle status of most FCRL4$^-$ cells, during which limited extracellular signal-regulated kinase activity would be anticipated.

A significant fraction of the differentially expressed transcripts that we identified encode genes involved in regulation of the cell cycle. The pronounced up-regulation of these genes in the FCRL4$^+$ B_{Mem} cells accords with our demonstration that ~35% of the cells in this population are in the G1 phase of the cell cycle. The observation that tissue-resident FCRL4$^+$ cells often express nuclear Ki-67 further supports the notion that these cells have recently undergone activation. These indications of a primed cell status could reflect the physical localization of the FCRL4$^+$ B_{Mem} cells in the mucosal tissues in close proximity to the natural microflora and potential pathogen invaders.

The differential expression patterns for CD11b and CD11c provide additional discriminating cell-surface markers for distinguishing these B_{Mem} cell subpopulations. The FCRL4$^+$ cells preferentially express CD11c and have lower levels of CD11b. Indeed, FCRL4 and CD11c expression are entirely concomitant on primary tonsillar B cells. Although commonly used as a dendritic cell marker, CD11c is also expressed by activated B cells and B cell leukemias (32, 33). Through their heterodimerization with CD18, the CD11b and CD11c transmembrane proteins function both as complement receptors and adhesion molecules. The ligands identified for CD11b and CD11c, including iC3b, fibrinogen, and intracellular adhesion molecules 1 and 2, are largely shared. One interesting difference is that CD11c recognizes acidic residues on fibrinogen with much higher affinity than does CD11b (34). The accessibility of negatively charged residues is notably increased in structurally compromised proteins, such as the proteolytically cleaved fibrinogen that accompanies infection. Although fibrinogen is synthesized primarily by hepatocytes, epithelial cells also have the capacity to produce fibrinogen (35, 36). The expression of CD11c on FCRL4$^+$ B_{Mem} cells could therefore contribute to their preferential localization in lymphoid tissues along the mucosal borders. Similarly, their heightened expression of chemokine receptors, CCR1 and CCR5, may support homing of the FCRL4$^+$ B_{Mem} cells to tissues with ongoing inflammation. The increased expression levels of FGR and HCK by the FCRL4$^+$ B_{Mem} cells is also noteworthy in this context, in that these src family kinases have been implicated in the regulation of signaling induced by macrophage inflammatory protein 1α, one of the ligands for CCR1 and CCR5 (37). Another distinctive cell-surface feature of FCRL4$^+$ cells is the absence of CD31 (platelet/endothelial cell adhesion molecule 1), whereas most FCRL4$^-$ B_{Mem} cells express this receptor. CD31 on circulating leukocytes functions as a facilitator of extravasation, in addition to its immunoregulatory activity on BCR signaling (38, 39). Its absence on the FCRL4$^+$ B_{Mem} cells thus could contribute to their preferential tissue-based localization. Similarly, the lack of EDG1 expression, a gene demonstrated to be crucial for lymphocyte egress from lymphoid tissues (40), would also favor the tissue-resident status of this B_{Mem} cell population.

It is intriguing that the FCRL4$^+$ cells express higher levels of CD95 (FAS) than the FCRL4$^-$ B_{Mem} cells in that elimination of lymphocytes is a key requirement both for the development of an appropriate lymphocyte repertoire and for the termination of an immune response. The elevated FAS level may not necessarily translate into increased potential to undergo apoptosis, however, because IL-4 and BCR ligation can counteract FAS-induced B cell apoptosis (41). The comparatively high level of AICDA expression found to characterize the FCLR4$^+$ B_{Mem} cells was also surprising, because increased levels of FAS and AICDA expression typify GC B cells. The shared absence of CD38 on both the FCRL4$^+$ and FCRL4$^-$ subpopulations of B_{Mem} cells and their limited expression of B cell lymphoma 6, B lymphocyte–induced maturation protein 1, and XBP1s transcripts nevertheless suggests that neither B_{Mem} cell subpopulation has recently left the GC nor is undergoing terminal plasma cell differentiation. Although AICDA expression by B cells is indispensable for somatic hypermutation and class switch recombination within the GC, this enzyme may also be expressed by B cells outside of the GC, notably by large interfollicular B cells with many similarities to FCRL4-bearing B_{Mem} cells (42–45). This raises the issue of whether FCRL4$^+$ B_{Mem} cells may merely maintain AICDA expression after their exit from the GC or have been induced elsewhere to express AICDA to undergo somatic hypermutation and class switch recombination. The latter possibility gains support from recent studies suggesting that B_{Mem} cells may also be generated outside the GC (42, 46). Irrespective of the derivation of FCRL4$^+$ cells, AICDA-induced genomic instability could potentially put them at increased risk for chromosomal translocation events and malignant transformation. In that regard, it is interesting to note that *FCRL4* was originally identified in a myeloma cell line as a gene involved in a translocation event on chromosome 1q21, a genetic region known as a "hot spot" for the somatic mutations and chromosomal translocations often found in B cell malignancies (11).

Our studies emphasize the differences between FCRL4$^+$ and FCRL4$^-$ B_{Mem} cells, but the possibility of a precursor–progeny relationship is raised by certain shared features. Although most FCRL4$^+$ B_{Mem} cells are CD27$^-$ and virtually all of the FCRL4$^-$ B_{Mem} cells are CD27$^+$, some of the FCRL4$^+$ cells express CD27 (7). This finding could suggest a CD27$^+$ B_{Mem} cell transition to become a FCRL4$^+$ B_{Mem} cell, or vice versa (7). As one means of testing the first possibility, we examined the ability of FCRL4$^-$ cells to express FCRL4 after in vitro treatment with a variety of B cell stimuli, but without success (unpublished data). Nevertheless, in lieu of knowledge about the essential physiological conditions that influence the differentiation of these B_{Mem} cell subpopulations, a precursor–progeny relationship remains a possibility.

The FCRL4$^+$ B_{Mem} cells were notably characterized by their increased levels of RANKL and DLL1. Animal models

with targeted deletions in the *Dll1* and *Rankl* genes have severe developmental abnormalities of the immune system, thereby demonstrating the importance of these ligands for a functional immune system (47–49). Unlike T cells, which are well recognized for their ability to directly and indirectly influence the activation and differentiation status of many cell types, B cells are known primarily for their ability to produce antibodies. Nevertheless, B cells may indirectly influence other cells as well, for example, by secreting the cytokines IL-4, IL6, IL-12, and IFN-γ (50). The prominent expression of DLL1 and RANKL by FCRL4$^+$ B_{Mem} cells implies that they also exert an instructive function through interaction with other cells expressing the cognate receptors for these ligands. We conclude that the distinctive signature expression patterns for receptors (CD11c, FCRL4, CCR1, CCR5, RANKL, and DLL1), signal transduction molecules (FGR and HCK), transcription factors (RUNX2 and SOX5), and AICDA, in combination with the differential responsiveness to cytokines and BCR ligation that characterize these B_{Mem} cell subpopulations (7), indicate a unique differentiation status for the FCRL4$^+$ B_{Mem} cells.

The potent inhibitory potential of FCRL4 for BCR-mediated signaling (15) implies a physiological role in immune responses of this distinctive subpopulation of B_{Mem} cells. Conversely, FCRL4 dysfunction may contribute to B cell–mediated immunopathology. Although the B cells that express FCRL4 are normally retained in lymphoid tissues belonging to the mucosal immune system (7, 14, 16), they may migrate elsewhere under disease conditions. In HIV-1–infected patients, for example, wherein B cell compartmental perturbations characterized by polyclonal B cell activation and hypergammaglobulinemia may accompany the progressive depletion of CD4$^+$ T cells (51), a prominent subpopulation of CD21^{low}, CD95^{high} peripheral blood B cells that is partially positive for Ki-67 has been previously described (52). These cells have been recently shown to be functionally impaired FCRL4$^+$ B cells (Fauci, A.S., personal communication). Given the potent immunoregulatory potential of FCRL4, it will be interesting to determine if its dysfunction or disengagement contributes to the hyperactivation and displacement of B cells that are observed in chronic HIV-1 infection. In conclusion, the results of this study further define the unique features that characterize the FCRL4$^+$ subpopulation of B_{Mem} cells and emphasize their likely engagement in humoral immune defense of the epithelial boundaries of the body.

MATERIALS AND METHODS

Materials and reagents. Antibodies used for FACS analysis were purchased from BD Biosciences unless otherwise indicated. Anti–Ki-67 was obtained from Vector Laboratories. $F(ab')_2$ fragments of FCRL4 antibodies were described previously (7) Antibodies to RUNX1, RUNX2, and SHIP were obtained from Santa Cruz Biotechnology, Inc. Anti-RANKL antibodies were purchased from eBioscience.

Isolation of tonsillar B_{Mem} cells. Tonsil tissue samples were obtained from the human tissue procurement service of the University of Alabama at Birmingham with Institutional Review Board approval. Single-cell suspensions were generated by tissue mincing, filtration through a 70-μm wire mesh, and centrifugation on a Ficoll-Hypaque gradient. Tonsillar B cells were negatively selected using the B cell isolation kit II (Miltenyi Biotec) according to the manufacturer's instructions. The isolated cells were typically >99.5% CD19$^+$ B cells. The resulting tonsillar B cells were stained for CD38 and IgD expression, and, to further exclude potential contamination with non–B cells, also with anti-CD19 antibodies. B_{Mem} cells were identified as CD19$^+$, CD38$^-$, IgD$^-$ cells. Costaining with biotinylated $F(ab')_2$ fragments of FCRL4 antibodies and streptavidin-allophycocyanin allowed the purification of FCRL4$^+$ and FCRL4$^-$ B_{Mem} cells using a FACS sorter (MoFlow; Dako).

Transcript analysis of FCRL4$^+$ and FCRL4$^-$ B_{Mem} cells. Total RNA from ∼250,000 cells from each purified population was extracted using an RNeasy kit (QIAGEN), and RNA integrity was assessed using a bioanalyzer (Agilent Technologies). cDNA synthesis, aRNA amplification, biotinylation, and fragmentation were performed with a Two-Cycle Target Labeling kit (Affymetrix). 15 μg of labeled samples were added to the hybridization cocktail and hybridized with Human Genome U133 Plus 2.0 GeneChips (Affymetrix) at 45°C for 16 h, as described in the manufacturer's instructions. Washing and streptavidin-phycoerythrin staining were conducted using a GeneChip Fluidics Station (Affymetrix); the chips were scanned using a GeneChip Scanner 3000 (Affymetrix). The normalized hybridization intensity for each probe set was calculated using the GC-RMA method (53) implemented in the GeneSpring software package (Agilent Technologies) as the default setting. Statistical analysis of the gene expression between FCRL4$^+$ and FCRL4$^-$ cells was performed with the Rank products method (54) implemented in the Bioconductor package (http://www.bioconductor.org). Genes for which the percentage of false positives was <0.05 were considered as significantly differentially expressed. The microarray data were deposited in the Center for Information Biology Gene Expression Database (available at http://cibex.nig.ac.jp/) under accession no. CBX40.

Quantitative RT-PCR. Transcripts found to be differentially expressed by gene array were confirmed by quantitative RT-PCR using the SYBR-Green PCR Master Mix (Applied Biosystems) on a sequence detection system (7900HT; Applied Biosystems). To this end, FCRL4$^+$ and FCRL4$^-$ B_{Mem} cells (CD19$^+$, CD38$^-$, IgD$^-$) were purified using a MoFlow FACS sorter. RNA was prepared using the QIAGEN RNeasy kit, and random-primed cDNA was generated using SuperScript II (Invitrogen), according to the manufacturer's instructions. To prevent the potential contamination of the RNA samples with genomic DNA, all RNA preparations were DNase treated. Gene-specific primers (primer sequences are available in Table S1, available at http://www.jem.org/cgi/content/full/jem.20072682/DC1) for the quantitative PCR were designed to overlap exon–intron borders to further reduce the possibility of amplification of potentially contaminating genomic DNA. Because the *GAPDH* and *β-actin* genes, which are commonly used for normalization purposes, can be subject to transcriptional regulation, we normalized all samples to *RNA polymerase II* (*RP-2*) expression, because *RP-2* expression is relatively independent of transcriptional regulation in different cell types and tissues (55).

FACS analysis. FACS analysis was performed with a FACS Cyan instrument (Dako). Purified tonsillar cells were blocked in the presence of 5% normal mouse serum and incubated with the antibodies indicated in the figures. For each of the cell-surface markers, at least eight independent tonsillar cell preparations were analyzed. For cell-cycle analysis, purified FCRL4$^+$ and FCRL4$^-$ memory cells were fixed in 80% ice-cold ethanol. After fixation, the cells were incubated for 15 min with FITC-labeled Ki-67 antibodies or isotype-matched control antibodies. The cells were then washed and resuspended in PBS containing 10 mg/ml RNaseA, followed by an additional incubation period of 10 min at 37°C. Propidium iodide was added to a final concentration of 20 μg/ml before analysis of the cells by flow cytometry.

Cloning of the FCRL4 promoter region and of the RUNX and SOX transcription factor cDNAs. To clone the putative promoter region of the *FCRL4* gene, we PCR amplified 2,600 bp of DNA upstream of the initiating ATG. As a template, we used clone RP11-369I17, which contains the relevant genomic sequences. The PCR product was cloned into pBlueScript and verified by DNA sequencing before cloning into the luciferase reporter gene vector pGL2 (Promega). The SOX5 transcription factor was PCR amplified using clone 10435684 (American Type Culture Collection) as a template. The PCR product was cloned into pBlueScript, sequenced to verify sequence fidelity, and finally cloned into the bicistronic expression vector pIRES-hrGFP II (Stratagene), which expresses the gene of interest upstream of an IRES element and GFP. Cloning of the RUNX1 and RUNX2 isoforms was performed by nested RT-PCR using random-primed cDNA from FACS-sorted FCRL4$^+$ and FCRL4$^-$ B_{Mem} cell populations. Because *RUNX* genes can be transcribed from two promoters, one of which is located in the first intron, we used two different primer sets to amplify RUNX transcripts. All PCR products were cloned into pBlueScript for sequence verification, followed by cloning into the eukaryotic expression vector pIRES-hrGFPII (Stratagene). Primer sequences for the described PCR reactions are listed in Table S1.

Reporter gene expression studies. Transient transfections of 293T cells were performed using the polyethyleneimine (PEI; Sigma-Aldrich) method. In brief, 3×10^5 293T cells per well were plated 24 h before transfection in 12-well plates. For transfection, 0.5 μg of luciferase reporter construct and 1.5 μg of plasmid DNA encoding the transcription factors indicated in the figures were mixed with 4 μg PEI in a volume of 330 μl of serum-free medium and incubated at room temperature for 10 min. After this incubation period, 660 μl of culture medium containing 2% FCS was added, and the DNA-PEI suspension was added directly onto the cells. After incubation for 3 h at 37°C, 2 ml of culture medium containing 10% FCS was added and the cells were cultured for an additional 40 h before cell lysis. To assay luciferase activity in the transfected cells, the cells were lysed in 70 μl of reporter lysis buffer, according to the manufacturers protocol (Promega), and luciferase activity was determined using a luminometer (TD-20/20; Turner Designs). Luciferase values were normalized to protein concentration using the BCA protein assay reagent (Thermo Fisher Scientific). Data represent the mean ± SD of five independent experiments.

ChIP assays. ChIP assays were performed according to a modified protocol described in reference 56. In brief, purified FCRL4$^+$ and FCRL4$^-$ tonsillar B_{Mem} cells were fixed in 1% paraformaldehyde for 15 min at room temperature, followed by inactivation of residual paraformaldehyde in 125 mM glycine. The cells were washed twice with PBS and lysed in 500 μl of lysis buffer (5 mM Pipes [pH 8], 85 mM KCl, 0.5% NP-40) in the presence of protease inhibitors. After centrifugation, the pellet was resuspended in 200 μl of nuclear lysis buffer (50 mM Tris [pH 8], 10 mM EDTA, 1% SDS) in the presence of protease inhibitors, followed by the addition of 800 μl of dilution buffer (20 mM Tris [pH 8], 0.01% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl). The genomic DNA was sheared by five sonication pulses of 30 s each, resulting in a mean length of DNA fragments of 600–1,000 bp. Immunoprecipitation with anti-RUNX1, anti-RUNX2, or control anti-SHIP1 antisera was preceded by a preclearing step using salmon sperm–absorbed protein A beads. The beads were washed five times with HEGN buffer (10 mM Hepes [pH 7.9], 1 mM EDTA, 150 mM NaCl, 10% glycerol) in the presence of protease inhibitors, followed by elution and reversal of cross-linking by overnight incubation at 68°C in elution buffer (50 mM Tris [pH 7.5], 10 mM EDTA, 1% SDS). After proteinase K digest (0.5 mg/ml) and phenol/chloroform extraction, the DNA was ethanol precipitated, washed with 70% ethanol, and resuspended in 30 μl of 10 mM Tris (pH 8). All samples were analyzed by two independent PCR reactions, each performed in duplicate and normalized to the signal obtained in the control immunoprecipitation using anti-SHIP1 antisera, as well as by the signal obtained from amplification of copurification-unrelated DNA (input control). For this purpose, we performed quantitative PCR for CCR4, a gene that is not expressed in either memory subpopulation (7). Statistical significance was determined using the two-tailed Student's *t* test.

Online supplemental material. Fig. S1 shows a diagram of the amplified RUNX1 and RUNX2 splice isoforms. The exon–intron structures of the genes, and the positions of the promoters and the primers used for PCR amplification are indicated. Table S1 lists the oligonucleotide sequences for PCR reactions performed in this study. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20072682/DC1.

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