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Anergic Responses Characterize a Large Fraction of Human Autoreactive Naive B Cells Expressing Low Levels of Surface IgM

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B cell anergy represents an important mechanism of peripheral immunological tolerance for mature autoreactive B cells that escape central tolerance enforced by receptor editing and clonal deletion. Although well documented in mice, the extent of its participation in human B cell tolerance remains to be fully established. In this study, we characterize the functional behavior of strictly defined human naive B cells separated on the basis of their surface IgM (sIgM) expression levels. We demonstrate that cells with lower sIgM levels (IgMlo) are impaired in their ability to flux calcium in response to either anti-IgM or anti-IgD cross-linking and contain a significantly increased frequency of autoreactive cells compared with naive B cells with higher levels of sIgM. Phenotypically, in healthy subjects, IgMlo cells are characterized by the absence of activation markers, reduction of costimulatory molecules (CD19 and CD21), and increased levels of inhibitory CD22. Functionally, IgMlo cells display significantly weaker proliferation, impaired differentiation, and poor Ab production. In aggregate, the data indicate that hyporesponsiveness to BCR cross-linking associated with sIgM downregulation is present in a much larger fraction of all human naive B cells than previously reported and is likely to reflect a state of anergy induced by chronic autoantigen stimulation. Finally, our results indicate that in systemic lupus erythematosus patients, naive IgMlo cells display increased levels of CD95 and decreased levels of CD22, a phenotype consistent with enhanced activation of autoreactive naive B cells in this autoimmune disease. The Journal of Immunology, 2011, 186: 000–000.
slgM, representing up to 30% of all naive cells, display an anergic phenotype and are substantially enriched in autoreactivity against HEp-2 cell Ags. The evidence presented indicates that anergic responses correlate with the level of downregulation of slgM and may therefore account for the high variability in slgM expression observed in human naive B cells. Finally, we present evidence indicating that this anergic phenotype is attenuated in patients with systemic lupus erythematosus (SLE/lupus), in whom down-regulation of slgM appears to be secondary to naive B cell activation.

**Materials and Methods**

**Human samples**

PBMCs were isolated by gradient centrifugation at 20°C using Ficol-Paque (Amersham Biosciences).

**Flow cytometry**

Single-cell suspensions (2 × 10^6/sample) were labeled at 4°C with predetermined optimal concentrations of fluorochrome-conjugated mAbs and pair-matched fluorescence minus one controls. The following Abs were used: FcR blocking reagent (Miltenyi Biotech), anti-CD19–allophycocyanin-Cy7 or PE-Cy5 (clone S525C1), anti-CD27–allophycocyanin or biotinylated (O323) protein, anti-IgD–PE (IA6-2), anti-IgM–PE-Cy5 (G20-127), anti-IgG–PE-Cy5 or PE (G18-145), anti-CD22–allophycocyanin (S-HCL-1), anti-CD24–FITC (ML5), anti-CD32–Alexa Fluor 647 (a generous gift from Dr. Robert Carter), anti-CD38–PE-Cy7 or allophycocyanin-Cy5.5 (H1T2), anti-CD69–PE-Cy7 (FN50), anti-CD80–biotinylated (BD10.4), anti-CD86–biotinylated (IT2.2) or FITC (2331-FUN-1), anti-CD95–biotinylated or FITC (DX2), and streptavidin (SA)–PE-Cy7 (BD Biosciences-Pharmingen; ebioscience). In total, 5 × 10^5–10^6 events were collected for each sample on an LSRII (10) flow cytometer. Data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

**Cell sorting**

PBMCs were stained with MitoTracker Green (MTG; Invitrogen), FcR blocking reagent (Miltenyi Biotech), anti-CD19–allophycocyanin-Cy7 or PE-Cy5 (clone S525C1), anti-CD27–allophycocyanin or biotinylated (O323) protein, anti-IgD–PE (IA6-2), anti-IgM–PE-Cy5 (G20-127), anti-IgG–PE-Cy5 or PE (G18-145), anti-CD22–allophycocyanin (S-HCL-1), anti-CD24–FITC (ML5), anti-CD32–Alexa Fluor 647 (a generous gift from Dr. Robert Carter), anti-CD38–PE-Cy7 or allophycocyanin-Cy5.5 (H1T2), anti-CD69–PE-Cy7 (FN50), anti-CD80–biotinylated (BD10.4), anti-CD86–biotinylated (IT2.2) or FITC (2331-FUN-1), anti-CD95–biotinylated or FITC (DX2), and streptavidin (SA)–PE-Cy7 (BD Biosciences-Pharmingen; ebioscience). In total, 5 × 10^5–10^6 events were collected for each sample on an LSRII (10) flow cytometer. Data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

**Intracellular calcium measurements**

B cells were purified by negative selection from heparinized PBL according to the manufacturer’s protocol (Human B Cell Enrichment Cocktail, RosetteSep; StennCell Technologies). To identify naive B cell populations of interest, IgM^−^, IgM^+^, and IgM^+^, the cells were stained with an exclusion mixture of biotinylated non-B cell markers (CD14, CD16, CD36, CD43 and CD235a Abs; Miltenyi Biotech) as well as anti-CD27 and anti-IgG to gate out remaining non-B cells and memory B cells at the time of the calcium measurements. Calcium responses were measured on a BD FACS Vantage SE with UV excitation (BD Biosciences). Data were collected and displayed as the relative ratio of intensities of Indo fluorescence (Ca-bound Indo violet emission 405 nm/free Indo blue emission 485 nm) for each cell over time and analyzed with FlowJo software (Tree Star). Samples were analyzed for a 50–60 s baseline in the respective gated naive B cell populations (IgM^+^, IgM^+^) at 37°C followed by the addition of 20 μg/ml F(ab')2 goat anti-human IgM or anti-IgD (Jackson Immunoresearch Laboratories).

**Recombinant mAbs**

Single-cell sorting. PBMCs were stained with MTG (Invitrogen), FcR blocking reagent (Miltenyi Biotech), anti-CD19–allophycocyanin-Cy7, anti-IgD–PE, anti-IgM–PE-Cy5 (BD Biosciences) and anti-IgM–allophycocyanin (Southern Biotechnology Associates), anti-CD27–biotin, and SA–PE-Cy7 in cell sorting buffer (0.5% BSA in PBS) and then sorted using the gating scheme shown in Fig. 2. Following gating on single lymphocytes and CD19-pos, cells were gated on MitoTracker-neg, CD27-neg, and IgD-pos, and these cells were subdivided into three fractions (cells with the highest slgM levels [IgM^+^], IgM intermediate [IgM^+^], and cells with lower slgM levels [IgM^−^]) based on slgM expression levels.

**In vitro cultures**

CFSE proliferation assay. After sort purification, 3 × 10^5 cells of each fraction from the naive compartment were loaded with 0.4 μM CFSE (Invitrogen) for 5 min at 37°C and cultured untreated (media alone) or treated with CpG oligodeoxynucleotide 2006 (Oligos Etc, Wilsonville, OR; 2.5 μg/ml), anti-IgM F(ab')2 (2.5 μg/ml), and IL-2 (10 ng/ml). Cultured cells were collected at various time points (from days 3–5) and analyzed for cell division using flow cytometry. The total cell numbers in each division (n) were determined using a proliferation-fitting model from FlowJo (Tree Star). The precursor cohort in each peak was calculated by dividing the number of cells in each division, n, by 2^i, where i represents the division number of the CFSE peak (11, 12). To estimate the time required for a cell that has divided once to go through subsequent divisions, we calculated the average division index at each time point [i.e., Σ (i × n)/Σ (n/2^i)] and plotted them against time. The inverse of the slope of the linear regression line fit through each data set gives an indication of time to subsequent division (12).

**Flow analysis of activation markers.** After sort purification, 3 × 10^5 cells of each fraction were placed in culture with anti-IgM alone, anti-IgM plus IL-2, or anti-IgM plus CpG and IL-2 or left untreated for 18 h and then stained with SytoxBlue, anti-CD69, and anti-CD86. Ab and autoantibody analysis. After sort purification, 3 × 10^5 cells were cultured in medium alone (untreated) or treated with CpG (2.5 μg/ml) plus anti-IgM F(ab')2 (2.5 μg/ml) and IL-2 (10 ng/ml) or with a CD40L-expressing murine cell line and IL-21 (50 ng/ml). Culture supernatants were collected on day 7 and tested for total IgM Ab production by ELISA.

Sorted cells were cultured with ODN2006 CpG (2.5 μg/ml) plus anti-IgM F(ab')2 (2.5 μg/ml) and IL-2 (10 ng/ml) as described above. Cells were harvested on day 4 and placed in multicwell ELISPOT plates for the detection of IgM production using goat anti-human IgM F(ab')2, at 10 μg/ml (Biosource International) or for the detection of autoantibodies binding to nuclear and cytoplasmic autoantigens. The latter was accomplished by coating ELISPOT plates with HEp-2 extracts conventionally used for the detection of autoreactivity in immunofluorescence and ELISA assays (7) using the equivalent of 10^5 cells/extract/plate. Similar methods have been used by others to measure Ab reactivity against malaria Ags and human brain extracts (13). After extensive washing, spot-forming cells producing either total IgM or autoreactive IgM were detected by incubation with alkaline phosphatase-conjugated goat anti-human IgM (μ-chain specific; Jackson Immunoresearch Laboratories) for 2 h and developed with VECTOR Blue Alkaline Phosphatase Substrate Kit III (Vector Laboratories, Burlingame, CA). Spots in each well were counted using a CTL ImmunoSpot plate reader and counting software (Cellular Technology Limited, Cleveland, OH).

**Human naive B cell anergy**

Human naive B cells express a relatively homogenous level of expression of slgD but heterogeneous expression levels of slgM.
with mean fluorescence intensity (MFI) values ranging from undetectable (background staining) to the 4th or -5th log decade. In some transgenic mice, low slgM is a hallmark of anergic B cells, and as many as 50% of all transitional B cells in wild-type mice have low slgM and represent anergic autoreactive B cells (6). To test the functional consequences of slgM levels in human B cells, we determined the ability of naive cells to respond to BCR crosslinking. We compared the response of naive cells at the lower end of slgM expression (IgMlo) with naive cells expressing higher levels of slgM. As shown in Fig. 1, IgMlo cells displayed both significantly lower global Ca2+ oscillations and a significantly decreased frequency of responding cells. Comparable differences were consistently observed irrespective of whether cells were stimulated through slgM (Fig. 1B) or slgD (Fig. 1C) despite relatively similar levels of slgD expression between the compared populations (Fig. 2B). Importantly, the data indicated a strong correlation between slgM expression level and the degree of response to either type of stimulation (Fig. 1E). Overall, these data suggest that the level of slgM expression plays a significant role in determining the response of human naive B cells and that hyporesponsiveness to Ag ligation is not rescued in these cells by IgD signaling.

A previous report by Duty et al. (9) showed that a subset of BND represent on average 2.5% of all peripheral blood B cells. Accordingly, to assess the contribution of this subset to the overall behavior of IgMlo cells, experiments were repeated excluding BND from the analysis. Fig. 1F and 1G show that after exclusion of BND, IgMlo cells were consistently hyporesponsive in response to anti-IgM (Fig. 1F) and anti-IgD (Fig. 1G) as compared with control IgMhi cells.

To determine the fraction of naive cells that were hyporesponsive, we calculated the ED90 Ca2+ flux values (the slgM MFI at which the frequency of responding cells achieved 90% of the maximum responses). Based on this ED90 cutoff value, we determined that BCR hyporesponsive cells were contained in the 15–30% fraction of naive cells at the lower end of slgM expression (n = 12–14) (Fig. 1E). Accordingly, for further phenotypic and functional analyses, we consistently defined, and purified when needed, IgMlo cells as the dimmest 20% of naive B cells and compared them to naive cells expressing higher slgM (IgMhi).

**Phenotypic characterization of human IgMlo naive B cells**

We have recently reported that late transitional cells express slgM levels that are similar to naive cells and that the two populations cannot be clearly separated on the basis of conventional markers including CD10 (15). However, early transitional cells (T1 and T2), which are hyporesponsive to BCR stimulation (15), are unlikely to contribute significantly to the signaling results observed for IgMlo cells because, in healthy peripheral blood, transitional cells express significantly higher levels of slgM. To ensure a rigorous phenotypic and functional analysis of naive IgMlo cells, we took advantage of the observation that, in contrast to transitional and memory B cells, naive B cells express a functional ATP-binding cassette transporter (ATP-binding cassette B1) and, as a result, fail to retain rhodamine and similar dyes, such as MTG (16). Accordingly, we used a multicolor flow cytometric approach previously described in our laboratory, which incorporates MTG to provide a more conclusive differentiation between naive cells (CD19IgD’CD24’CD38’CD27’CD10’ MTG”), and late (T3) transitional cells (CD19IgD’CD24’CD38’CD27’CD10’ MTG”) than can be obtained with more commonly markers such as IgD, CD24, CD38, and CD10 (15). Our data indicate that IgMlo cells do not represent late transitional cells, but have a mature naive phenotype with expression patterns for other developmental Ags, CD21 and CD23, similar to other naive cells irrespective of their slgM level. Using this phenotype and based on the signaling results, we applied a stringent definition of naive cells to sort purify the 20% of naive cells with the lowest slgM levels (IgMlo) and analyzed these cells for their surface phenotype and cellular properties. As shown in Fig. 2A and 2B, this approach allowed for a consistently clear separation of the subset of interest from the majority of naive B cells, which express ~3-fold higher levels of slgM with relatively little difference in IgD expression (IgMhi).

**Functional properties of naive IgMlo B cells**

Given that BCR downregulation could either be secondary to acute antigenic stimulation or chronic engagement by exposure to self-Ags, we examined the expression of conventional activation markers on freshly isolated PBMCs. As shown in Fig. 3A, IgMlo cells lacked expression of CD69, CD80, CD86, and CD95, suggesting that slgM downregulation in these cells is not the consequence of acute antigenic stimulation. In addition, the IgMlo population had significantly lower upregulation of the early activation marker CD69 after in vitro BCR stimulation for 18 h. In contrast, as has been reported for anergic anti-insulin transgenic B cells (17), BCR-stimulated IgMlo cells were able to upregulate CD80/CD86 to similar levels as the control IgMhi naive B cells (Fig. 3B).

Ultimately, B cell responses are determined by the balance between BCR and costimulatory signals and the engagement of inhibitory receptors capable of dampening B cell activation (18–20). Thus, we used flow cytometry to examine the expression levels of accessory molecules known to modulate BCR signaling including CD19, CD21, CD22, and CD32b. As shown in Fig. 3C, although IgMlo cells express CD19 and CD21 (Fig. 2B, 2D), they express significantly lower levels of the BCR costimulatory complex CD19–CD21 as compared with naive IgMhi cells. Interestingly, IgMlo cells also have elevated surface expression of CD22 but reduced surface expression of CD32b, both members of the Ig-like superfamily receptors carrying ITIM in their cytoplasmic tails that powerfully dampens positive BCR-induced signaling (21). Expression of CD22 has been shown to increase as immature/transitional cells enter the mature naive compartment and its expression declines with B cell activation (22). Consistently, we observed that IgMlo naive cells expressed significantly higher levels of CD22 than do transitional cells (Fig. 3E and data not shown). Interestingly, CD22 expression increased significantly in transitional cells but not in IgMlo cells when cultured with BAFF (Fig. 3E), the main B cell maturation factor for transitional B cells (15, 23).

To better understand the significance of these findings, we also examined the expression of these markers in patients with SLE, an autoimmune condition in which one would expect to detect decreased anergy and increased activation of autoreactive B cells. As shown in Fig. 3D and 3F, SLE IgMlo B cells have significantly lower expression of CD22 but significantly enhanced expression of the activation marker CD95, both findings consistent with an activated phenotype and/or lower activation threshold in SLE IgMlo naive cells.

**IgMlo B cells are hyporesponsive to TLR costimulation**

In HEL/anti-HEL double-transgenic mice, anergic B cells are refractory to either TLR9 or TLR4 stimulation, although their hyporesponsiveness can be at least partly overcome by the high concentration of the corresponding ligands (CpG DNA and LPS, respectively) (24). We have shown that even in the absence of BCR engagement, TLR9 stimulation, with appropriate cytokines, can also drive proliferation of human naive B cells, and similar

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results have been published for transitional cells (25, 26). Accordingly, we analyzed cell divisions using measurements of CFSE dilution after in vitro stimulation with anti-IgM, IL-2, and CpG (note that expression of TLR9, the receptor for CpG, on IgMlo cells is similar to that of IgMin cells; Supplemental Fig. 1A, 1B). As shown in Fig. 4, after 4 d in culture, the frequency of IgMlo cells that underwent at least one division was diminished by 40% as compared with IgMin cells (Fig. 4A, 4B). Moreover, IgMlo cells are...
IgMlo cells display decreased Ab-secreting ability and are enriched for autoreactivity

A critical downstream consequence of B cell stimulation is the ability to secrete Ab. Hence, we stimulated IgMlo cells under different conditions known to induce Ab secretion by human naive B cells (27). Fig. 5A and 5B show that IgMlo cells produce significantly less IgM Ab than IgMhi cells. Importantly, decreased Ab production can also be demonstrated under powerful IgM-independent stimulation conditions, CD40L plus IL-21 (Fig. 5A). This result, because CD40 expression is similar between IgMlo and IgMhi cells (Supplemental Fig. 1C, 1D), is consistent with the anti-IgD Ca2+ flux results and indicates a global hyporesponsiveness of IgMlo cells that cannot be attributed simply to lower levels of IgM, but rather is a consequence of an intrinsic refractoriness to stimulation. Diminished Ab production was confirmed by ELISPOT experiments using equal numbers of input cells from 4-d cultures stimulated with CpG DNA, F(ab′)2 anti-IgM, and IL-2. As shown in Fig. 5A, in keeping with their lower division rate (28), IgMlo cells were significantly impaired in terms of differentiation to Ab-secreting cells.

Importantly, although IgMlo cells were inefficient in generating total anti-IgM Ab-producing cells, they contained a high frequency of autoreactive-producing cells as indicated by a newly developed HEp-2 ELISPOT, a high-throughput method to elucidate at the single-cell level the global autoreactivity of large numbers of cultured cells (Fig. 5D). To confirm that autoreactive cells detected with this method were not derived from the expansion of BND, HEp-2 ELISPOT experiments were repeated after the exclusion of BND, as described in the figure legend, and we obtained the similar results (Fig. 5B). The autoreactivity was detected up to 20% of Ab-producing IgMlo cells, but in only 3% of IgMhi cells. Of note, the frequency of HEp-2 autoreactive cells observed in our experiments was consistent with the frequency of HEp-2 reactivity observed by others for unfractionated naive B cells in single-cell analysis using recombinant mAbs (7). Also of interest, IgMlo appeared to contain the lowest frequency of autoreactive cells even though they yielded the highest frequency of IgM-secreting cells. The overall magnitude of autoreactivity detected in the current study is consistent with the level detected by Merrel et al. (6) in their analysis of wild-type mouse anergic naive B cells showing decreased expression of sIgM reactive with purified Ags contained in the HEp-2 extracts.

Finally, to validate the frequency and type of autoreactivity observed with our newly developed HEp-2 ELISPOT assay, we generated mAbs from single cells sorted from both the IgMlo and IgMhi populations using recombinant technology (as described in the Materials and Methods). This approach provides important complementary information as it reflects the frequency of autoreactive cells without the bias of preferential proliferation of particular subsets that may affect the result outcomes of the ELISPOT assays. Reassuringly, commercial anti-nuclear Ab ELISA assay testing of recombinant mAbs generated from single IgMlo and IgMhi cells showed that 40% of cells obtained from the IgMlo population were autoreactive compared with 7% obtained from the IgMhi population (Supplemental Fig. 2A, 2B).
The data presented in this study are consistent with the presence of anergy in a significant fraction of human naive B cells as documented by low Ca2+ flux, attenuated proliferation, and substantially decreased ability to secrete Ab in response to both BCR cross-linking and BCR-independent stimulation (CD40L plus IL-21). These cells are characterized by low surface expression of IgM, absence of activation markers, increased autoreactivity, and abnormal expression of inhibitory receptors as compared with control naive B cells, which express higher levels of sIgM and respond more vigorously to BCR cross-linking. Of note, the hyporesponse observed to BCR stimulation was not due solely to the decreased levels of sIgM because similar attenuated responses were obtained in response to anti-IgD stimulation. Rather, our results indicate that, as was observed in mouse anergic B cells (29, 30), the level of sIgM determines the threshold of response to stimulation.

Interestingly, intracellular staining detects similar levels of cytoplasmic IgM in IgMlo and IgMin cells (Supplemental Fig. 3A, 3C), indicating that decreased sIgM levels are not due to decreased transcription (as also supported by the sustained level of sIgD). Instead, these observations are consistent with increased IgM internalization and/or defective transport to the cell surface reported by Bell et al. (31). These mechanisms have also been invoked to explain low sIgM levels in anergic transgenic B cells, a phenotype that can be reversed when the responsible self-Ag is removed from the system. Our results indicate that similarly, human IgMlo cells are able to upregulate sIgM after resting for 48 h in culture medium in the absence of stimulation (Supplemental Fig. 3C). These conditions would also preclude IgMlo cells from engaging self-Ags that might have been present in vivo, other than those present in the surface of naive B cells themselves or those released from dead or apoptotic cells. Given that we observed negligible levels of cell death/apoptosis in the 48-h culture (not shown), our results suggest that downregulation of sIgM may be secondary to chronic engagement of self-Ag and may be reversed by interrupting this interaction.

As we and others have established (7, 15), transitional cells share some of the properties assigned in this study to anergic naive B cells (including attenuated BCR responses, hypoproliferation to TLR9 stimulation, and increased autoreactivity), and the differentiation between these populations may be difficult because the surface phenotypic markers typically used (IgM, IgD, CD24, CD38, and CD10) are expressed as a continuum in transitional and naive populations. The mature nature of IgMlo cells, however, is supported by their competency to extrude rhodamine or MitoTracker, a property characteristic of mature naive B cells owing to their expression of the ATP-binding cassette B1 transporter (15, 16). IgMlo maturity is also supported by the higher expression of CD22 and by the differential upregulation of this marker in response to BAFF stimulation. Collectively, our results are consistent with the recent description of An1 anergic B cells in wild-type mice rather with a T3 transitional phenotype (6).
CD22 is a critical inhibitory receptor that becomes operational in mature follicular B cells and is reduced upon B cell activation (22). In this compartment, CD22 determined the threshold for BCR-stimulated activation and is critical for the enforcement of B cell tolerance. In keeping with this model, CD22 deficiency may result in autoimmunity (32, 33). Thus, our finding of increased CD22 levels in IgMlo cells strongly indicates that, also in keeping with signaling, proliferation, and Ab secretion studies, these cells have a higher activation threshold as compared with other naive cells with lesser degree of autoreactivity. IgMlo cells expressed

**FIGURE 4.** IgMlo cells have reduced proliferative capacity in response to in vitro stimulation. Naive cells from peripheral blood were sorted as described in Fig. 1, loaded with CFSE, and placed in culture with Cpg (2.5 μg/ml), F(ab')2 anti-IgM (2.5 μg/ml), or IL-2 (10 ng/ml). Cultured cells were collected on days 3-5 for proliferation and cell survival analysis. A, CFSE histogram shows the 4-d proliferation of IgMlo and IgMin cells. B, Frequency of cells having undergone at least one division. C, Frequency of dividing cells within each cell division. D, Division index (includes only cells that made at least one division) versus time. Reciprocal slope of regression line gives time to subsequent divisions. E, Time to subsequent divisions of IgMlo and IgMin cells. F, Graph shows the percentage of live cells within total culture cells. All data were collected from seven independent experiments, and the analyses were performed as described in the Materials and Methods. *p < 0.05, ***p < 0.005.

**FIGURE 5.** IgMlo cells have reduced total IgM production but increased frequency of autoreactive IgM Ab-producing cells. A, Plot displays amount of total IgM Ab production of IgMlo and IgMin cells upon stimulation with Cpg (2.5 μg/ml), F(ab')2 anti-IgM (2.5 μg/ml), IL-2 (10 ng/ml), or CD40L and IL-21 (50 ng/ml). B, Graph displays frequency of IgM-producing cells (left y-axis) and frequency of autoantibody-producing cell (right y-axis) among IgMlo (IgMlo cells that were excluded of BND during cell sorting), IgMin, and IgMhi cells (49) following stimulation with Cpg, F(ab')2 anti-IgM, and IL-2. Images show developed spots from ELISPOT assays for total IgM (C) and autoreactive IgM Abs (D). Equal numbers of viable cells from each population were assayed. Anti-IgM autoantibody-producing cells (D) upon stimulation with Cpg (2.5 μg/ml), F(ab')2 anti-IgM (2.5 μg/ml), and IL-2 (10 ng/ml). *p < 0.05, **p < 0.005.
lower levels of CD19 and CD21, lacked expression of activation markers, and displayed attenuated upregulation of some early activation markers in response to in vitro stimulation. Collectively, this phenotype suggests that in healthy subjects, IgMlo cells represent cells chronically stimulated in vivo, presumably by self-Ags, leading to decreased levels of IgM and costimulatory BCR molecules. Also of interest, IgMlo cells express lower levels of CD32b, suggesting that in these cells, low levels of BCR signaling fail to recruit this important inhibitory receptor for the feedback inhibition of B cells engaged in productive Ag-specific responses (34). These features also argue against the alternative possibility that slgM downregulation could reflect the recent BCR internalization in response to acute B cell activation. Instead, our data suggest that IgMlo cells from SLE patients may represent acutely activated cells as indicated by increased CD95 and decreased CD22 expression.

Although the relative decrease in CD19 and increase in CD22 should impose an enhanced threshold for activation of IgMlo cells, thereby contributing to enforcing tolerance in autoreactive B cells, the upregulation of CD80/86 observed in vitro indicates that the state of unresponsiveness in IgMlo cells is reversible; therefore, these cells represent a dangerous reservoir of potentially pathogenic autoreactive B cells. Although some transgenic models suggest that this danger can be minimized by the shortened lifespan of anergic B cells (35, 36), our data highlight the risk created by the persistence of these cells. Consistent with their lack of CD95 expression, IgMlo cells did not appear to be proapoptotic, and in the absence of stimulation, on average, 90% of the 18-h cultured cells survived, which is similar to the survival of control cells (data not shown).

Similar scenarios indicating the ability of anergic B cells to be activated by both T-dependent and independent stimulation are well described in different animal models (17, 24, 37–39). Activated, but not resting, naive B cells can upregulate costimulatory molecules and effectively serve as APCs that mediate cognate activation of T cells (40–42). B cell APC activity may critically contribute to the initiation of T cell-mediated autoimmunity (43), and therefore, failure to upregulate critical T cell costimulatory molecules such as CD80 and CD86 has tolerogenic effects beyond the censoring of the B cells themselves. However, the ability of anergic B cells to express these costimulatory molecules has been inconsistent in different autoreactive transgenic models (17, 44, 45). The unaltered upregulation of CD86 in vitro culture suggests that the APC activity of IgMlo cells could be intact, as shown in the anti-insulin T125tg B cells (17), and thus the pathway leading to anergy of IgMlo cells may be distinct from the pathway leading to becoming an APC.

This article expands the spectrum of anergic naive B cells identified in humans. Indeed, our initial report on this tolerance mechanism identified anergic behavior in autoreactive 9G4-pos cells that represent 5–10% of all human naive B cells in healthy subjects and that display markedly depressed Ca2+ responses upon BCR stimulation despite expressing intermediate levels of slgM (46). Although other autoreactivities may also be involved in the censoring of 9G4 B cells (S. Jenks, E. Palmer, C. Tipton, C. Richardson, and I. Sanz, manuscript in preparation), the main antigenic target of 9G4-pos B cells is represented by the blood group i Ag, which is also expressed by a CD45/B220 glycoform preferentially upregulated in naive B cells (47). More recently, another population of human anergic naive B cells has been reported. These cells, termed BND, lack expression of surface IgM, account for ~2.5% of all B cells, and are likely to represent the IgM-negative tail of the population studied in our work. Given that naive cells represent 50–80% of all peripheral blood B cells, BND would account for ~3–5% of all naive cells. Of interest, BND demonstrate significant autoreactivity against nuclear and cytoplasmic Ags in anti-nuclear Ab testing as well as polyreactivity against two or more Ags (ssDNA, dsDNA, insulin, and LPS) when tested by ELISA (9). A third type of human naive anergic B cell has also been reported during the preparation of this article. These cells, characterized by poor BCR responsiveness and autoreactivity similar to the one described above for BND, are distinguished by the downregulation of CD21 (CD21lo cells). Of note, these anergic, autoreactive CD21lo cells were identified in patients with common variable immunodeficiency and in a subset of rheumatoid arthritis patients who may be deficient in receptor-editing mechanisms. However, CD21lo anergic cells did not seem to represent a substantial fraction of naive B cells in healthy subjects (48). Thus, previously reported anergic populations represent relatively small fractions of all naive B cells and therefore could not account for the large fraction of autoreactive cells consistently identified in the healthy naive B cell compartment (9). Our data help fill this gap by identifying a larger subset of autoreactive naive B cells with an anergic phenotype that are consistently identified in healthy subjects. Accordingly, this work contributes to the growing body of evidence identifying anergy as mechanism of tolerance enforcement in mature naive cells that is present universally in healthy subjects. Although our preliminary results suggest that this mechanism may be defective in SLE patients, larger and more detailed studies will be required to assess the participation of defective anergy in this and other autoimmune diseases.

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Disclosures

The authors have no financial conflicts of interest.

References

Supplemental figure 1: TLR9 and CD40 expression on IgMlo cells. PBMC were stained with MTG, anti-CD19, CD27, IgD, IgM, CD40, and fixed and permeabilized followed by staining with TLR9. A-D, Frequencies of TLR9 (A), CD40 (C) and the MFI of TLR9-pos cells (B), CD40-pos cells (D) among IgMlo and IgMhi cells. Unswitched IgD-pos/CD27-pos (UnS) and Switched IgD-neg/CD27-pos (Sw) memory cells were used as positive controls for TLR9 expression (ns, not significant).
Supplemental figure 2: Autoreactive antibodies within the IgM<sup>lo</sup> and IgM<sup>in</sup> populations. A and B, Plot displays the ANA ELISA result obtained from the recombinant monoclonal antibodies made from single IgM<sup>lo</sup> (n=10) and IgM<sup>in</sup> (n=13) cells. The data are expressed as Units vs monoclonal antibody concentrations (as described in the material and methods). The Units were calculated according to the recommended manufacturer formula with 20 Units representing the recommended positive cut off point.
Supplementary figure 3: Intracellular and surface IgM expression of IgM<sub>lo</sub> cells. B cells from naïve compartments (IgM<sup>lo</sup> and IgM<sup>hi</sup>) were sort-purified as described. Cells were re-stained with anti-IgM conjugated to a different fluorochrome, to analyze for the surface (sIgM) (A), or total, surface and intracellular (s+inIgM) expression of IgM (B). C, Bar graph (mean and SD) summarizes the fold differences of sIgM expression on IgM<sup>hi</sup> cells, surface staining (left column), and total IgM expression (right column) compared with IgM<sup>lo</sup> cells. D, The overlap histogram shows the representative results of sIgM expression following cell sorting of IgM<sup>lo</sup> cells (Fresh 0h), and after being in culture media for the time indicated (donors, n=4).