CD40 ligand and MHC class II expression are essential for human peripheral B cell tolerance

Maxime Hervé,[1] Isabelle Isnardi,[1] Yen-shing Ng,[1] James B. Bussel,[2,3] Hans D. Ochs,[4] Charlotte Cunningham-Rundles,[5] and Eric Meffre[1,3]

[1]Laboratory of Biochemistry and Molecular Immunology, Hospital for Special Surgery, New York, NY 10021
[2]Department of Medicine and Pediatrics, [3]Weill Medical College of Cornell University, New York, NY 10021
[4]Department of Pediatrics, University of Washington School of Medicine, Seattle, WA 98195
[5]Department of Medicine and Pediatrics, Mount Sinai Medical Center, New York, NY 10029

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Hyper-IgM (HIGM) syndromes are primary immunodeficiencies characterized by defects of class switch recombination and somatic hypermutation. HIGM patients who carry mutations in the CD40-ligand (*CD40L*) gene expressed by CD4⁺ T cells suffer from recurrent infections and often develop autoimmune disorders. To investigate the impact of CD40L–CD40 interactions on human B cell tolerance, we tested by ELISA the reactivity of recombinant antibodies isolated from single B cells from three CD40L-deficient patients. Antibody characteristics and reactivity from CD40L-deficient new emigrant B cells were similar to those from healthy donors, suggesting that CD40L–CD40 interactions do not regulate central B cell tolerance. In contrast, mature naive B cells from CD40L-deficient patients expressed a high proportion of autoreactive antibodies, including antinuclear antibodies. Thus, CD40L–CD40 interactions are essential for peripheral B cell tolerance. In addition, a patient with the bare lymphocyte syndrome who could not express MHC class II molecules failed to counterselect autoreactive mature naive B cells, suggesting that peripheral B cell tolerance also depends on major histocompatibility complex (MHC) class II–T cell receptor (TCR) interactions. The decreased frequency of MHC class II–restricted CD4⁺ regulatory T cells in CD40L-deficient patients suggests that these T cells may mediate peripheral B cell tolerance through CD40L–CD40 and MHC class II–TCR interactions.

CORRESPONDENCE
Eric Meffre:
meffree@hss.edu

Abbreviations used: AID, activation-induced cytidine deaminase; ANA, antinuclear antibody; BAFF, B cell-activating factor; BCR, B cell receptor; BLS, bare lymphocyte syndrome; CDR, complementarity determining region; CSR, class switch recombination; ds, double-stranded; GC, germinal center; HD, healthy donor; HIGM, hyper-IgM; SHM, somatic hypermutation; SLE, systemic lupus erythematosus; ss, single-stranded; XLA, X-linked agammaglobulinemia.

Hyper-IgM (HIGM) syndromes are primary immunodeficiencies characterized by defects in class switch recombination (CSR), resulting in severely decreased numbers of circulating isotype-switched memory B cells. As a consequence, HIGM patients who display normal to elevated serum IgM levels combined with severely decreased serum levels of IgG, IgA, and IgE suffer from recurrent bacterial infections (1–3). The genetic basis of HIGM is diverse and is caused either by defects in the CD40L–CD40 pathway, which is essential for B cell activation, germinal center (GC) formation, and CSR induction, or by defects involving the enzymes required for CSR (3). An X-linked form of the disease results from mutations in the *CD40L* (or *CD154*) gene; it is the most prevalent form of HIGM, accounting for a majority of the cases (4, 5). CD40L-deficient patients lack GCs and fail to develop normal antibody responses (4, 6). A B-cell intrinsic defect caused by rare mutations in the CD40L receptor CD40 results in an autosomal form of the disease, which is clinically indistinguishable from HIGM caused by defective CD40L (7). Abnormal CD40 signaling in the absence of functional nuclear factor-κB essential modulator (NEMO or IKKγ) also induces a form of HIGM that is associated with anhydrotic ectodermal dysplasia, which is a key feature of this syndrome (8, 9).

Mutations in genes encoding enzymes required for CSR and somatic hypermutations (SHMs) also result in HIGM. Activation-induced cytidine deaminase (AID) is induced in GC B cells, and defects in *AID* gene expression and/or function cause an autosomal recessive form of HIGM that is characterized by the presence of enlarged GCs and the absence of CSR and SHMs in both mice and humans (10–12).

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Another CSR deficiency has been correlated to mutations in the *uracil-DNA glycosylase* (*UNG*) gene that encodes a DNA repair enzyme that acts downstream of AID (13). In addition, 20–30% of HIGM patients have no identified mutations in *CD40L*, *CD40*, *NEMO*, *AID*, and *UNG*, suggesting that mutations in other unknown genes result in additional forms of HIGM (5, 14).

Impaired immune functions leading to primary immunodeficiencies often correlate with paradoxical autoimmune complications. Aside from the susceptibility to opportunistic and bacterial infections, CD40L-deficient patients are also prone to develop autoimmune diseases such as thrombocytopenia, hemolytic anemia, and nephritis, suggesting that B cell tolerance is not properly established and/or maintained in these patients (1–3, 15). In line with this hypothesis, it has been reported that sera from CD40L-deficient patients contain natural antibodies biased toward specific self-antigens that were not found in the sera of healthy donors (HDs), thereby suggesting an abnormal B cell selection in these patients (16). Although the molecular mechanisms by which CD40L induces B cell proliferation and differentiation are well described, its potential functions in counterselecting human autoreactive B cells are poorly characterized. Transgenic mouse models have suggested that $CD4^+$ T cells may play an important role in the elimination of peripheral autoreactive B cells (17). In addition, CD40L–CD40 and MHC class II–T cell receptor interactions were required for the counterselection of transgenic autoreactive B cells (18).

In humans, most developing autoreactive B cells are removed at two discrete checkpoints during early B cell development (19). A central B cell tolerance checkpoint silences polyreactive and ANA-expressing B cells between the early immature and immature B cell stages in bone marrow, and a peripheral B cell tolerance checkpoint further counterselects self-reactive clones at the transition between new emigrant and mature naive B cells in the periphery (19). The regulation of central B cell tolerance involves B cell receptor (BCR) signaling pathways that regulate recombination activating gene expression and central tolerance mechanisms such as receptor editing, anergy, and deletion in immature B cells (20). Indeed, we previously reported that alterations in BCR signaling in X-linked agammaglobulinemia (XLA) patients result in a defective central B cell tolerance checkpoint, which is illustrated by a failure to counterselect polyreactive developing B cells in those patients (21). Little is known about the molecules and pathways that regulate human peripheral B cell tolerance checkpoints. To determine the impact of CD40L and MHC class II expression on human B cell tolerance checkpoints, we cloned and expressed in vitro recombinant antibodies from single B cells from three CD40L-deficient patients and a MHC class II–deficient (bare lymphocyte syndrome [BLS]) patient, and compared their reactivity to that of recombinant antibodies isolated from HDs. We report that defects in CD40L and MHC class II expression result in the accumulation of autoreactive clones in the mature naive B cell compartment of CD40L-deficient and BLS patients. Thus, CD40L–CD40 and MHC class II play an important role in regulating a peripheral B cell tolerance checkpoint in humans. The decreased numbers of regulatory T (T reg) cells and elevated serum BAFF levels in CD40L-deficient and BLS patients suggest that T reg cells and BAFF are also involved in the regulation of peripheral human B cell tolerance.

RESULTS

Central B cell tolerance is functional in patients with CD40L mutations

A central B cell tolerance checkpoint normally removes most developing B cells that express highly polyreactive antibodies in the bone marrow, and only a small fraction of clones with low levels of polyreactivity migrate to the periphery (19). A peripheral B cell tolerance checkpoint further counterselects autoreactive new emigrant B cells before they enter the mature naive B cell compartment (19). Constitutive expression of CD40 is detected on human B cell precursors in the bone marrow and is retained at the mature naive B cell stage, but a role for CD40L–CD40 interactions on human B cell selection is unknown. To determine if human B cell tolerance checkpoints are affected by the absence of CD40L expression, we tested the reactivity of antibodies from 98 single peripheral $CD19^+CD10^+IgM^+CD27^-$ new emigrant B cells and 92 $CD19^+CD10^-IgM^+CD27^-$ mature naive B cells from three CD40L-deficient patients and compared it to that of antibodies from a teenage HD (HD08) and previously reported adult healthy controls (Fig. 1) (19, 21, 22). We found that polyreactive B cells represented 4.3, 6.7, and 9.5% of new emigrant B cells from the three CD40L-deficient patients, respectively, and was similar to that of new emigrant B cells from HD08 (5.3%) and previously reported adult controls, suggesting that there were no defects in central B cell tolerance in these patients (Fig. 1, A and B). Mature naive B cells from HD08 also contained a low proportion (6.7%) of polyreactive clones, similar to their new emigrant B cell counterpart (Fig. 1 A). In contrast, CD40L-deficient mature naive B cells displayed a higher frequency of polyreactive antibodies, with 13, 13, and 26.1% in patients 1, 2, and 3, respectively (Fig. 1 A). When the frequency of polyreactive clones from CD40L-deficient patients was compared with that of the five HDs of various ages analyzed so far, we found that it was increased in the mature naive B cell compartment, and that differences reached statistical significance ($P = 0.0262$; Fig. 1 B). In addition, all CD40L-deficient patients showed a higher proportion of polyreactive B cells in their mature naive B cells than in their new emigrant B cell counterpart from which they arose (Fig. 1 C).

Hence, polyreactive B cells are properly counterselected in the bone marrow of CD40L-deficient patients, revealing a functional central B cell tolerance checkpoint in the absence of CD40L expression. On the other hand, the peripheral B cell tolerance checkpoint seems to be defective in CD40L-deficient patients, resulting in an increase of circulating polyreactive B cells.

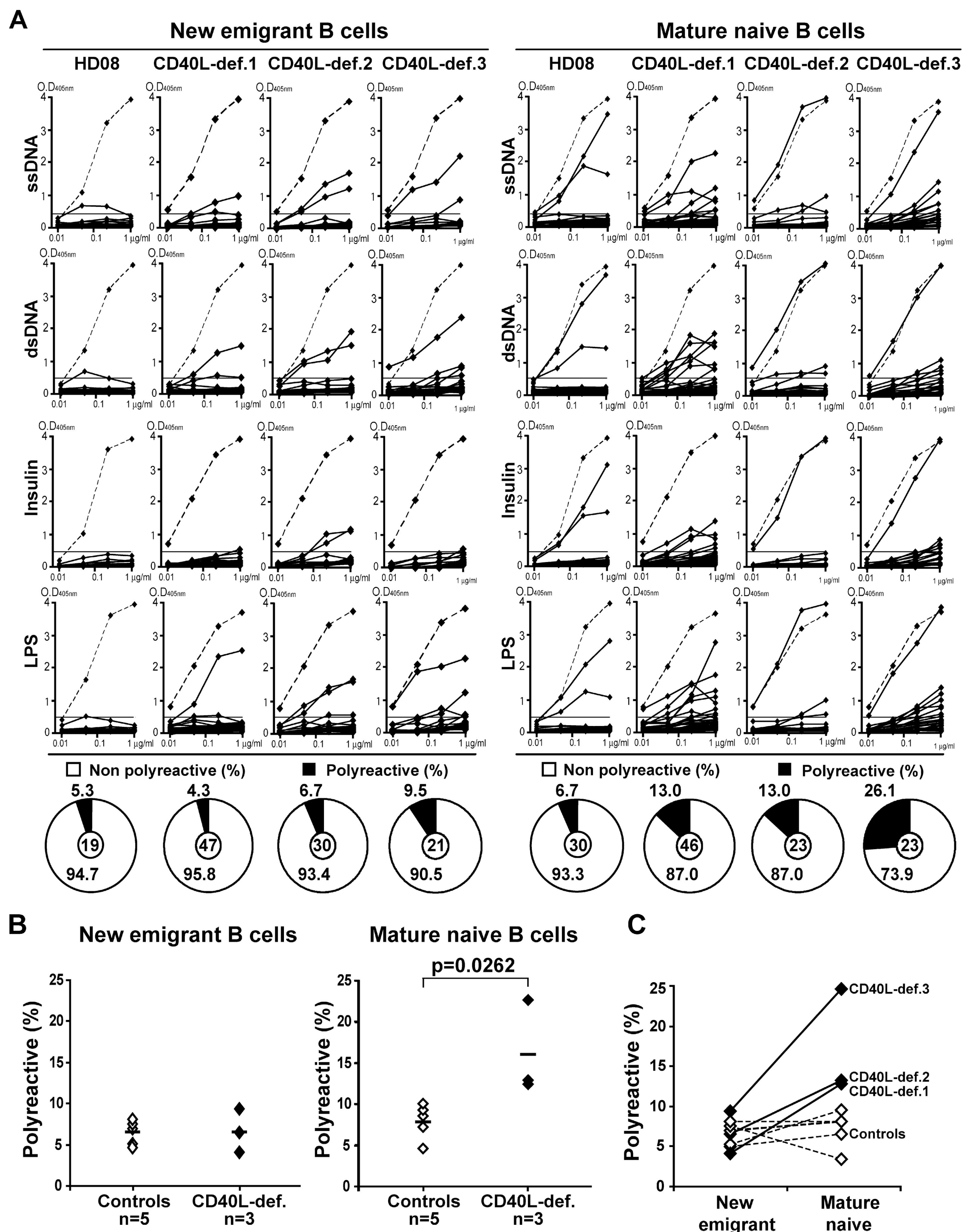

Figure 1. CD40L-deficient mature naive B cells express polyreactive antibodies. (A, top) Antibodies from new emigrant (left) and mature naive (right) B cells from a 14-yr-old HD (HD08) and three CD40L-deficient patients were tested in ELISA for reactivity with ssDNA, dsDNA, insulin, and LPS. Dashed lines show ED38-positive control (19, 54). Horizontal lines show cut-off OD_{405} for positive reactivity. (bottom) For each individual, the frequency of polyreactive (filled area) and nonpolyreactive (open area) clones is summarized in pie charts, with the number of antibodies tested indicated in the centers. (B) The frequency of polyreactive clones in the mature naive fraction of CD40L-deficient patients is higher than in healthy controls. The proportions of polyreactive B cells in new emigrant (left) and mature naive (right) B cells from five controls and three CD40L-deficient patients and (C) their evolution between these two B cell compartments are represented. Each diamond represents an individual, and the mean is shown with a bar. The controls shown include the two newly studied HD controls, HD08 and HD09, added to the previously reported controls (19, 21, 22). Statistically significant differences are indicated.

Peripheral mature naive B cells from CD40L-deficient patients express high levels of autoreactive antibodies

We further assessed the requirement of CD40L for central and peripheral B cell tolerance checkpoints by testing by ELISA the reactivity of recombinant antibodies from CD40L-deficient patients against HEp-2 cell lysates. We found that CD40L-deficient patients showed a frequency of HEp-2–reactive clones in new emigrant B cells that ranged from 36.2 to 47.6% and was similar to that of the teenage HD08 (31.6%) and of the previously reported healthy controls (29.2–46.2%; Fig. 2, A and B) (19, 21). Similar to adult healthy controls, the proportion of HEp-2–reactive antibodies decreased to 23.3% in the mature naive B cells from HD08 (Fig. 2) (19, 21, 22). In contrast, the frequency of mature naive B cells that expressed HEp-2–reactive antibodies was significantly increased in all three CD40L-deficient patients compared with controls ($P = 0.0001$; Fig. 2, B and C). Correlating with this increase in HEp-2–reactive mature naive B cells in CD40L-deficient patients, we found that these cells expressed heavy chain complementarity determining region (CDR) 3s with an increased frequency of positively charged residues, which favor autoreactivity (Fig. S1 and Tables S1–S12, available at http://www.jem.org/cgi/content/full/jem.20062287/DC1). Thus, the increase in HEp-2–reactive mature naive B cells in CD40L-deficient patients further demonstrates a defective peripheral B cell tolerance checkpoint when CD40L expression is deficient.

CD40L expression is essential to the counterselection of ANA B cells

Central B cell tolerance is responsible for the removal of most developing B cells expressing ANAs in HDs (19). In CD40L-deficient patients, the central B cell tolerance checkpoint appears to be normal, but a majority of peripheral mature naive B cells express HEp-2–reactive antibodies that may recognize nuclear antigens. To determine if HEp-2–reactive B cells from HIGM patients express ANAs, we performed indirect immunofluorescence assays on fixed HEp-2 cells (Fig. 3). We found that antibodies from a very small and similar proportion of new emigrant B cells from controls and CD40L-deficient patients expressed ANAs, further demonstrating that central B cell tolerance checkpoint was functional in the absence of CD40L expression (Fig. 3, A and B). The frequency of nuclear-reactive B cells remained very low in the mature naive B cell compartment of healthy controls (Fig. 3, A and B). In contrast, antibodies from CD40L-deficient mature naive B cells showed

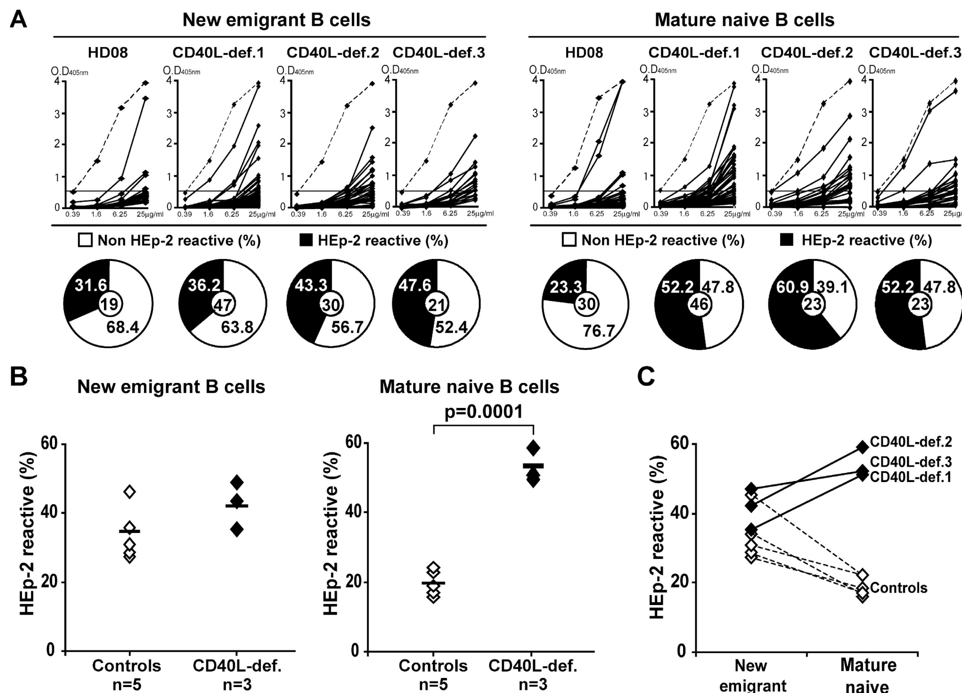

Figure 2. CD40L-deficient mature naive B cells express HEp-2–reactive antibodies. (A, top) Antibodies from new emigrant (left) and mature naive (right) B cells from a young HD, HD08, and three CD40L-deficient patients were tested in ELISA for anti–HEp-2 cell reactivity. Dashed lines show ED38-positive control (19, 54). Horizontal lines show cut-off OD_{405} for positive reactivity. (bottom) For each individual, the frequency of HEp-2–reactive (filled area) and non–HEp-2-reactive (open area) clones is summarized in pie charts, with the number of antibodies tested indicated in the centers. (B) The frequency of HEp-2–reactive clones in the mature naive fraction of CD40L-deficient patients is higher than in controls. The proportions of HEp-2–reactive B cells in new emigrant (left) and mature naive (right) B cells from five controls and three CD40L-deficient patients, and (C) their evolution between these two B cell compartments are represented. Each diamond represents an individual, and the mean is shown with a bar. The controls shown include the two newly studied HD controls, HD08 and HD09, added to the previously reported controls (19, 21, 22). Statistically significant differences are indicated.

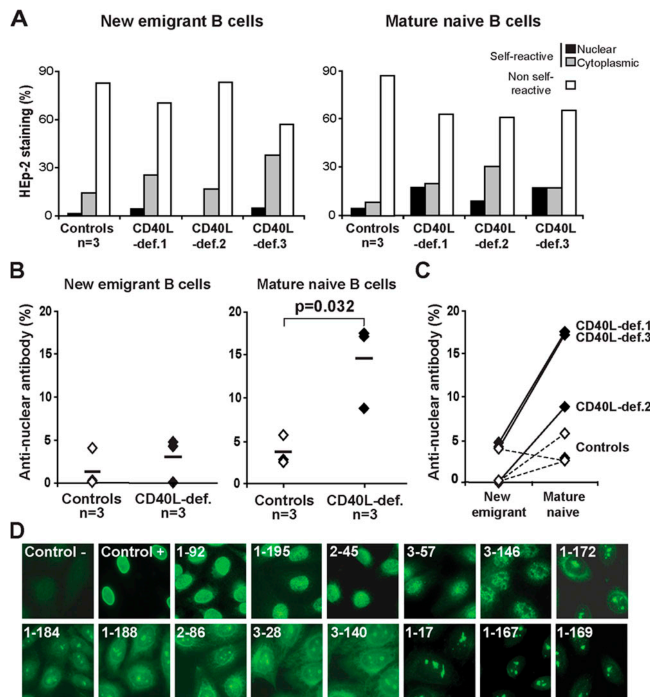

Figure 3. Mature naive B cells from CD40L-deficient patients express ANAs. (A) The frequency of self-reactive antibodies with nuclear (black bars) and cytoplasmic (gray bars) HEp-2 staining patterns, and the proportion of nonreactive antibodies (open bars) in new emigrant (right) and mature naive (left) B cells are shown for three controls and three CD40L-deficient patients. The data from three healthy controls (JH, HD08, and HD09) were pooled. (B) The frequency of ANA clones in the mature naive B cell compartment of CD40L-deficient patients is significantly higher than in healthy controls. The proportions of ANA-expressing B cells in new emigrant (left) and mature naive (right) B cells from controls and three CD40L-deficient patients and (C) their evolution between these two B cell compartments are represented. Each diamond represents an individual, and the mean is shown with a bar. The controls shown include the two newly studied HD controls, HD08 and HD09, added to the previously reported control (21). Statistically significant differences are indicated. (D) Antibodies from CD40L-deficient mature naive B cells show diverse antinuclear staining including homogeneous (1–92, 1–195, 2–45), speckled (3–57 and 3–146), nucleolar and cytoplasmic (1–172, 1–184, 1–188, 2–86, 3–28, and 3–140), and nucleolar only (1–17, 1–167, and 1–169).

a high frequency of ANA-reactive clones that represented, on average, 14.5% of this compartment and was significantly increased compared with healthy controls ($P = 0.032$; Fig. 3, B and C). In addition, all CD40L-deficient patients showed a higher proportion of ANA-expressing B cells in their mature naive B cell compartment than in the new emigrant B cell counterpart from which they originated (Fig. 3 C). ANA-expressing B cells were found to display the highest amount of positively charged residues in their heavy chain CDR3s compared with nonreactive or autoreactive non-ANA clones (Fig. S1 and Tables S1–S6). Autoreactive antibodies expressed by CD40L-deficient mature naive B cells showed diverse antinuclear staining patterns (Fig. 3 D). Some autoantibodies (1–92, 1–195, and 2–45) presented a homogeneous nuclear staining, whereas other recombinant antibodies (3–57 and 3–146) gave a speckled nuclear staining pattern that may correspond to antibodies directed against nuclear proteins (23). Most ANA clones in CD40L-deficient patients presented a nucleolar staining pattern that was associated (1–172, 1–184, 1–188, 2–86, 3–28, and 3–140) or not associated (1–17, 1–167, and 1–169) with the cytoplasmic reactivity that is often characteristic of anti-RNA polymerase complex antibodies (Fig. 3 D) (23). However, none of these autoantibodies recognized the condensed chromatin material in mitotic cells, suggesting that anti-DNA and chromatin-reactive clones are not recruited in the mature naive B cell pool of CD40L-deficient patients (unpublished data). To confirm this observation, we tested the antibody reactivity by indirect immunofluorescence on *Crithidia luciliae*. The kinetoplast of *C. luciliae* is an organelle composed of double-stranded (ds) DNA, and antibody recognition of the kinetoplast is the most specific assay to identify antinative dsDNA routinely used for the detection of these autoantibodies in patients with systemic lupus erythematosus (SLE). We found that there were no B cells that recognize the kinetoplast of *C. luciliae* in either new emigrant or mature naive B cells from CD40L-deficient patients (unpublished data). We conclude that ANA B cells are properly counterselected in the bone marrow of CD40L-deficient patients. However, some ANA B cells accumulate in the mature naive B cell compartment in the absence of functional CD40L, which is most likely expressed by $CD4^+$ T cells (24).

Peripheral B cell tolerance checkpoint requires MHC class II expression

The requirement of CD40L expression to counterselect human autoreactive B cells was reminiscent of a transgenic mouse model that demonstrated a role for $CD4^+$ T cells in this process (17, 18). To characterize a potential role for cognate B–T cell interactions, we analyzed B cell tolerance checkpoints in a BLS patient. BLS patients suffer from a rare primary immunodeficiency disorder characterized by defective expression of MHC class II molecules (25, 26). A BLS patient provided a unique opportunity to analyze the role of MHC class II molecules in the establishment of B cell tolerance. Peripheral blood B cells from the BLS patient almost completely lacked an IgM^-CD27^+ class-switched memory B cell population, whereas the frequency of IgM^+CD27^+ unswitched memory B cells was less affected, demonstrating the essential role of MHC class II molecules in the development of class-switched memory B cells in humans (Fig. 4 A). Similar to a majority of CD40L-deficient patients, the BLS patient also displayed an enlarged $CD10^+CD27^-$ new emigrant B cell population compared with the age-matched HD control (Fig. 4 A and Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20062287/DC1). Indeed, the expansion of immature transitional/new emigrant B cells is frequently associated with human immunodeficiencies (27). We also confirmed that BLS B cells failed to express HLA-DR MHC class II molecules, whereas HLA-A,B,C MHC class I molecule expression was unaffected (Fig. 4 A). To determine

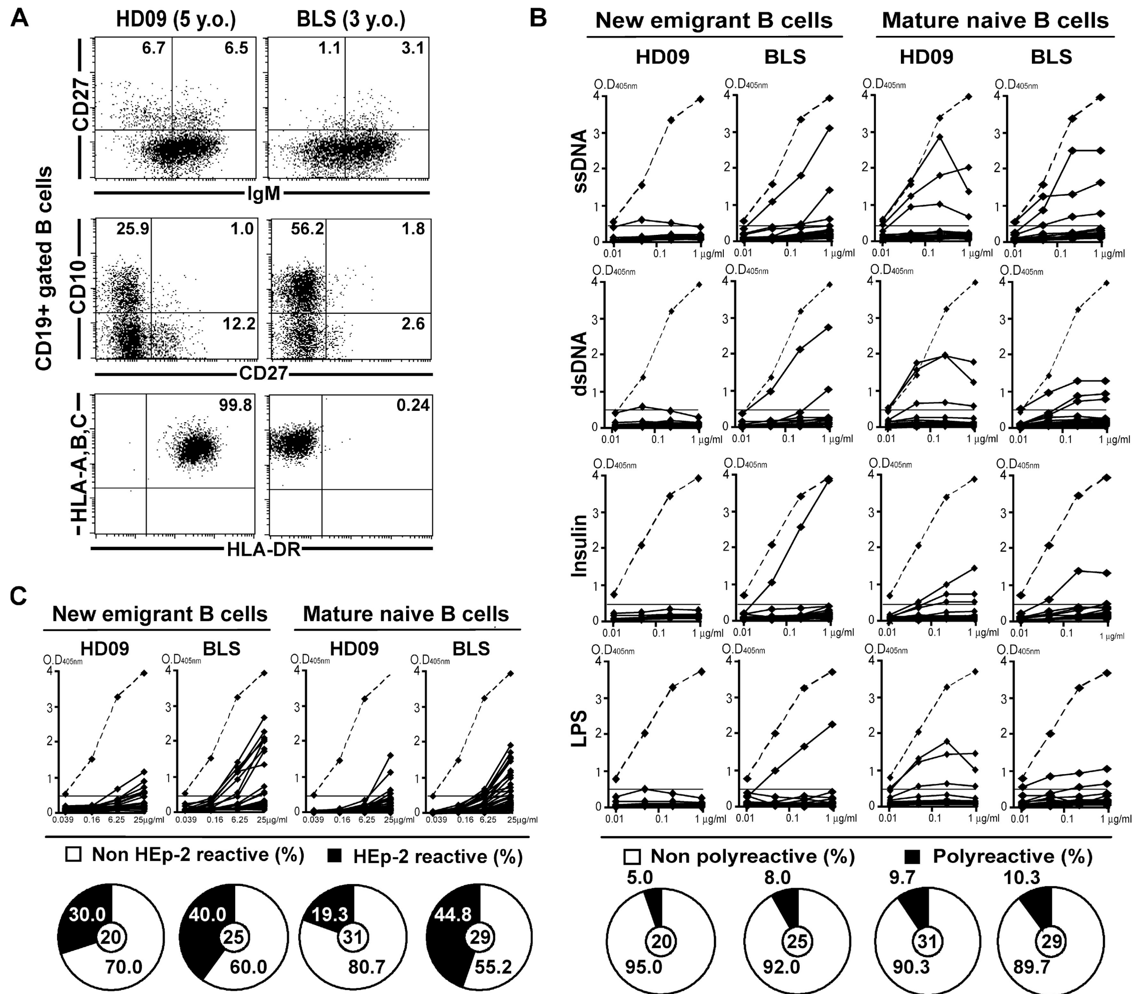

Figure 4. Mature naive B cells from a BLS patient express autoreactive antibodies. (A) A BLS patient showed decreased CD27$^+$ memory B cells. Dot plots show CD27/IgM (top), CD10/CD27 (middle), and HLA-A,B,C/HLA-DR (bottom) expression on CD19$^+$-gated B cells from a HD control HD09 (right) and a BLS patient. The BLS patient displayed decreased IgM$^-$CD27$^+$ class-switched and IgM$^+$CD27$^+$ memory B cell populations and an increased CD10$^+$CD27$^-$ new emigrant B cell population compared with HD controls. BLS B cells fail to express HLA-DR MHC class II molecules. (B) BLS B cells express polyreactive antibodies at a frequency similar to an age-matched control. Antibodies from new emigrant (left) and mature naive (right) B cells from a HD, HD09, and a BLS patient were tested in ELISA for reactivity with ssDNA, dsDNA, insulin, and LPS. Dashed lines show ED38-positive control (19, 54). Horizontal lines show cut-off OD_{405} for positive reactivity. For each individual, the frequency of polyreactive (filled area) and nonpolyreactive (open area) clones is summarized in pie charts, with the number of antibodies tested indicated in the centers. (C) BLS mature naive B cells express HEp-2–reactive antibodies. Antibodies from new emigrant (left) and mature naive (right) B cells from HD09 and a BLS patient were tested in ELISA for anti–HEp-2 cell reactivity. Dashed lines show ED38-positive control (19, 54). Horizontal lines show cut-off OD_{405} for positive reactivity. For each individual, the frequency of HEp-2–reactive (filled area) and non–HEp-2-reactive (open area) clones is summarized in pie charts, with the number of antibodies tested indicated in the centers.

the impact of MHC class II expression on the establishment of human B cell tolerance, we tested the reactivity of 25 and 29 antibodies cloned from single new emigrant B cells and mature naive B cells from the BLS patient, respectively. Because the BLS patient was a 3-yr-old child, we compared the reactivity of his antibodies to that of antibodies obtained from single B cells from a young, HD control (HD09; Fig. 4). The reactivity of antibodies expressed by MHC class II-deficient new emigrant B cells was similar to that of the young control in both polyreactivity and HEp-2 reactivity ELISA assays (Fig. 4, B and C). Thus, MHC class II expression does not appear to be required for the removal of developing autoreactive B cells during the central B cell tolerance checkpoint in the bone marrow. Similar to healthy adult donors, the frequency of HEp-2–reactive clones dropped from 30% in new emigrant B cells to 19.3% in the mature naive B cell compartment of the young HD09 control (Fig. 4 C). In contrast, the proportion of HEp-2–reactive mature naive B cells remained high (44.8%) in the BLS patient, and was similar to that of new emigrant B cells in this patient (Fig. 4 C). Contrary to CD40L-deficient patients who also showed defects in the peripheral B cell tolerance checkpoint, the frequency of polyreactive

and ANA-expressing B cells remained low in the mature naive B cell compartment of the BLS patient (Fig. 4 B and Tables S7 and S8). We conclude that MHC class II expression is dispensable for central B cell tolerance, whereas it is important for the additional removal of autoreactive B cells in the periphery at the transition between new emigrant and mature naive B cells.

Decreased frequency of regulatory T cells in CD40L-deficient patients

CD40L expressed by $CD4^+$ T cells and MHC class II requirement for peripheral B cell tolerance suggested that this checkpoint may depend on cognate B–T interactions. Two populations of T cells have been reported to express autoreactive TCRs that may be able to recognize self-antigens presented by autoreactive B cells: NKT cells that express TCRVα24 combined to TCRVβ11 and $CD4^+CD25^+CD127^{lo/-}$ regulatory T (T reg) cells that express the transcription factor Foxp3 (28–31). Additional HD controls and CD40L-deficient patients were enrolled to study NKT and T reg cell frequencies. The frequencies of NKT cells in CD40L-deficient and BLS patients were decreased compared with that of HDs, but differences failed to reach statistical significance (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20062287/DC1). In contrast, the frequency of T reg cells identified by the expression of CD25 either in the absence of or with low levels of IL-7 receptor/CD127 or in conjunction with Foxp3 was significantly decreased in all CD40L-deficient patients compared with HDs (7.3% in controls vs. 1.68% in CD40L-deficient patients; $P < 0.00001$; Fig. 5 A). The identification of T reg cells by intracellular detection of Foxp3 also revealed a significant decrease in the proportion of $CD25^+Foxp3^+$ T reg cells in CD40L-deficient patients in which these cells represent only 2.15% of their $CD4^+$ T cells compared with 6.52% in controls ($P < 0.00001$; Fig. 5 B). In contrast, the proportion of T reg cells in the BLS patients was similar to HDs (Fig. 5). However, $CD4^+$ T cells do not develop normally in the absence of proper class II transactivator/MHC class II molecule expression in BLS patients, and $CD4^+$ T cell numbers were decreased by four- to fivefold compared with HDs (unpublished data) (32). Thus, similar to CD40L-deficient patients, the absolute number of T reg cells in the BLS patient is also decreased by a factor of four to five. We conclude that T reg cells fail to develop or maintain in the absence of CD40L expression, and that decreased T reg cell numbers correlate with an altered peripheral B cell tolerance checkpoint in both CD40L-deficient and BLS patients.

Increased serum B cell–activating factor (BAFF) concentration in CD40L-deficient and BLS patients

BAFF is a critical B cell survival factor that controls the number of peripheral B cells (33). Transgenic mice overexpressing BAFF develop a SLE–like disease by rescuing developing autoreactive B cells from deletion and allowing their accumulation in the periphery (34–36). To determine whether the increased frequency of autoreactive B cells in the mature naive B cell compartment of CD40L-deficient and BLS patients results from increased BAFF levels, we measured by ELISA BAFF concentrations in the serum of these patients and compared them to that of HDs (Fig. 6). Serum BAFF levels in HDs were comparable among individuals, with an average concentration of 1,164 pg/ml. In contrast, serum BAFF levels were significantly increased by three- to fourfold in CD40L-deficient and BLS patients, in which it averaged 3,259 and 4,514 pg/ml, respectively (CD40L-deficient, $P < 0.00001$; Fig. 6). Thus, increased BAFF levels in CD40L-deficient and BLS patients correlate with a defective peripheral B cell tolerance checkpoint in these patients.

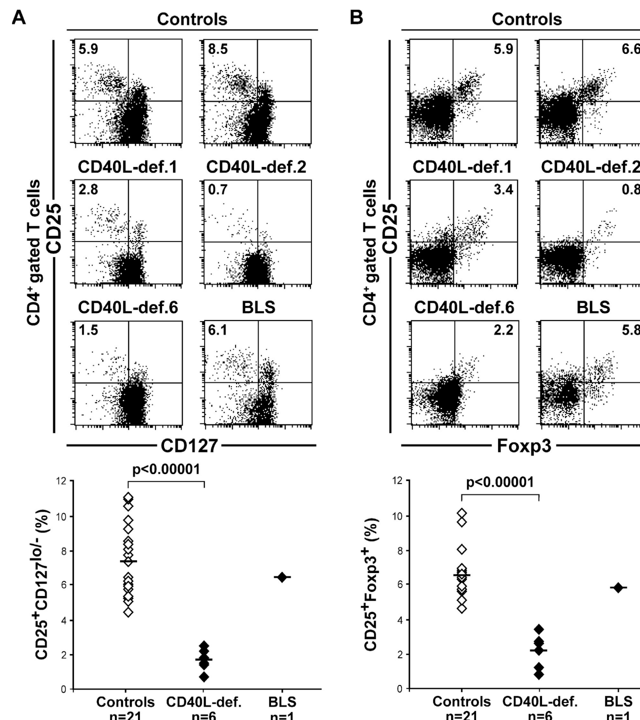

Figure 5. Low T reg cell frequency in CD40L-deficient patients. The frequency of T reg cells among peripheral $CD4^+$ T cells was assessed by analyzing the proportion of $CD25^+CD127^{lo/-}$ (A) and $CD25^+Foxp3^+$ (B) cells. Dot plots are shown for two healthy controls, three CD40L-deficient patients, and the BLS patient (top), and the data collected for controls (open diamonds) and patients (filled diamonds) are represented in the diagram. Each diamond represents an individual, and the mean is shown with a bar. The mean percentage of $CD25^+CD127^{lo/-}$ cells among $CD4^+$ T cells in HDs, 7.30 ± 1.6%; CD40L-deficient, 1.68 ± 0.5%; BLS, 6.4%; mean percentage of $CD25^+Foxp3^+$ cells among $CD4^+$ T cells in HDs, 6.52 ± 0.9%; CD40L-deficient, 2.15 ± 0.6%; BLS, 5.8%. Statistically significant differences are indicated.

DISCUSSION

B cell tolerance is established by silencing developing autoreactive B cells. Two major checkpoints are responsible for the removal of these clones. A central B cell tolerance checkpoint occurs in the bone marrow and counterselects most B cells expressing highly polyreactive and ANA-expressing B cells (19). The peripheral B cell tolerance checkpoint removes additional

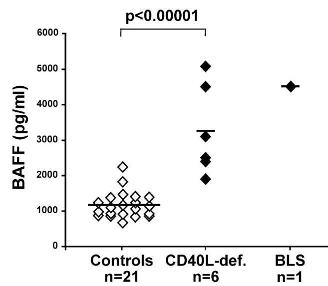

Figure 6. Elevated serum BAFF levels in CD40L-deficient and BLS patients. BAFF concentrations (pg/ml) in the serum of HD controls (open diamonds), six CD40L-deficient patients, and the BLS patient (filled diamonds) were measured by ELISA. Each diamond represents an individual, and the mean is shown with a bar: HD controls, 1,164 ± 364 pg/ml; CD40L-deficient, 3,259 ± 1,155 pg/ml; BLS, 4,514 pg/ml. Statistically significant differences are indicated.

autoreactive clones that may recognize self-antigens that were not expressed in the bone marrow environment. Little is known about the molecules and cell types involved in the regulation of these two checkpoints. We report that CD40L expression by T cells and MHC class II molecules are required for a functional peripheral B cell tolerance checkpoint in humans.

The frequency of autoreactive and polyreactive new emigrant B cells in CD40L-deficient and BLS patients was similar to that of HD controls, and therefore reveals that the central B cell tolerance checkpoint is functional in the absence of CD40L and MHC class II expression. Therefore, T cells expressing CD40L (24) and antigen presentation through MHC class II expression do not appear to play a role in counterselecting developing autoreactive immature B cells in the bone marrow. The regulation of central B cell tolerance seem to mainly involve sensing autoantigens binding to self-reactive BCRs on immature B cells (37, 38). Indeed, BCR triggering at this stage induces tolerance mechanisms, such as receptor editing, anergy, and, ultimately, deletion (20, 39). In humans, we previously reported that XLA patients, who carry mutations in the *BTK* gene, which encodes a cytoplasmic tyrosine kinase that plays an essential role in mediating BCR signaling, display a high proportion of autoreactive new emigrant B cells (21, 40, 41). The alteration of BCR signaling thresholds results in defective central B cell tolerance checkpoint and in the release of developing autoreactive B cells in the periphery.

The high proportion of autoreactive mature naive B cells in both CD40L-deficient and BLS patients revealed defects of the peripheral B cell tolerance checkpoints in the absence of either CD40L or MHC class II expression. Defects of the peripheral B cell tolerance checkpoint were also identified by the abnormal enrichment in CD40L-deficient patients of mature naive B cells that expressed antibodies with highly positively charged IgH CDR3s. These CDR3s were reminiscent of those expressed by bone marrow early immature B cells that frequently expressed ANAs, and they are often counterselected during early B cell development (19). Indeed, a majority of CD40L-deficient mature naive B cells were HEp-2–reactive and/or polyreactive, including ANAs, which were found to display the highest amount of positively charged residues in their CDR3s. The natural antibody repertoire of CD40L-deficient patients is biased toward specific autoantigens, and further demonstrates abnormal B cell tolerance mechanisms in the absence of CD40L expression (16). Thus, functional CD40L expression may maintain peripheral B cell tolerance by preventing the recruitment in the mature naive B cell compartment of autoreactive B cells, which may include ANA-expressing clones.

How may the absence of MHC class II or CD40L result in a failure to counterselect autoreactive clones in the mature naive B cell compartment? Although T–B cell interactions are severely compromised in the absence of either CD40L or MHC class II molecules and result in a failure to mount proper adaptive immune responses, it has been previously proposed that T–B cell interactions may also play an important role in the counterselection of autoreactive B cells in the periphery (17, 18). Using transgenic mouse models, $CD4^+$ T cells and the receptor/ligand pairs CD40L–CD40 and Fas–FasL have been shown to play an important role in removing tolerant hen egg lysozyme-binding B cells (17, 18). In this model, self-reactive B cells would present antigens via MHC class II molecules to $CD4^+$ T cells and trigger CD40L expression. In return, CD40L would cross-link CD40 on these autoreactive B cells and induce the expression of the death receptor Fas that would trigger B cell apoptosis (18). Hence, defects in Fas or FasL lead to the production of autoantibodies that resembled those found in SLE patients, both in mice and in humans (42–44). Our results agree with this model, and they further demonstrate an important role for T cells in counterselecting autoreactive B cells in humans.

What T cell subpopulation may be required for the establishment of peripheral B cell tolerance? Self-antigens captured by autoreactive BCRs may be presented to T cells through MHC class II molecules expressed on these autoreactive B cells. The putative T cells that may interact specifically with these autoreactive B cells should therefore express autoreactive TCRs. Two populations of T cells have been reported to express autoreactive TCRs: NKT cells and $CD4^+CD25^+$ T reg cells (for review see [29]). Because the peripheral B cell tolerance checkpoint required MHC class II expression, NKT cells are not likely to be involved in peripheral B cell tolerance because these cells that express a very restricted TCRVα24/TCRVβ11 repertoire recognize antigens presented by CD1 molecules (28). Indeed, we did not find any significant differences in the frequency of NKT cells between HDs and CD40L-deficient and BLS patients. In contrast, T reg cells display a diverse TCR repertoire restricted to MHC class II molecules, express CD40L and can inhibit B cell proliferation (24, 29). T reg cells can also suppress B cell responses and induce B cell death in vitro (45, 46). It has been reported that T reg cells do not develop normally in mice in the absence of CD40L–CD40 interactions (47, 48). In agreement with these observations, we found that CD40L-deficient patients displayed decreased

T reg cell numbers, suggesting that CD40L–CD40 interactions may also play an important role in the development or maintenance of these T cells in humans. Lack of T reg cells leads to severe autoimmune disorders in Foxp3-deficient mice and humans, revealing that T reg cells are required for the establishment and/or the maintenance of peripheral tolerance (49–51). Both CD40L-deficient and BLS patients displayed low numbers of T reg cells and showed defects in peripheral B cell tolerance, suggesting that T reg cells may play an important role in preventing the accumulation of new emigrant/transitional autoreactive B cells in the mature naive compartment of these patients.

BAFF is a serum cytokine that promotes transitional and mature naive B cell survival. BAFF-deficient mice display profoundly decreased numbers of peripheral B cells (33). In contrast, mice overexpressing BAFF develop autoimmune disorders similar to SLE and Sjogren syndrome, which are characterized by the production of autoreactive antibodies, including rheumatoid factor, anti-DNA, and other ANAs (34). Elevated BAFF concentration inhibits the counterselection of autoreactive new emigrant/transitional B cells that failed to be removed from the B cell population (35, 36). BAFF may also favor the proliferation of some clones by promoting the entry into the cell cycle (52). Hence, the elevated serum BAFF concentration in CD40L-deficient and BLS patients is likely to contribute to the accumulation of autoreactive mature naive B cells in the blood of these patients. Moreover, the increased frequency of B cells expressing ANAs in the mature naive compared with the new emigrant/transitional compartment of CD40L-deficient patients further suggest that elevated BAFF levels interfere with selection processes and result in the recruitment of autoreactive mature B cells in these patients.

In conclusion, our results suggest that decreased T reg cell numbers, in combination with elevated serum BAFF levels, may be responsible for the accumulation of autoreactive mature naive B cells in the blood of CD40L-deficient and BLS patients. Thus, proper T reg cell number and function and balanced serum BAFF concentration are two key players regulating the peripheral B cell tolerance checkpoint by CD40L-dependent mechanisms and through cognate B–T interactions.

MATERIALS AND METHODS

Patients. CD40L-deficient patients 1 and 4 are 8- and 2-yr-old brothers, respectively, who suffer from a single nucleotide deletion at position 580 (del580G), resulting in a frameshift and premature termination. CD40L-deficient patients 2 and 3 are 22- and 29-yr-old brothers, respectively, with a point mutation (G to C) in the splicing sequence of intron 4, which results in the deletion of exon 4 and in the absence of functional CD40L. CD40L-deficient patient 5 is an 11-yr-old boy with a point mutation in exon 5, resulting in a single amino acid substitution (L195P). The CD40L-deficient patient 6 is a 34-yr-old male patient who has two point mutations in exon 4 that result in the substitution of two adjacent amino acids (S128R and E129G) and prevent binding to CD40 (53). CD40L-deficient patient 7 is a 9-yr-old boy with a nonsense mutation (Y26X) in exon 1 that has been previously described (5). The BLS patient is a 3-yr-old boy who displays a defective class II transactivator gene, which is responsible for the defect of MHC class II molecular expression (complementation analysis done by J. Villard, University of Geneva Medical School, Geneva, Switzerland). At the age of 4 mo, he developed a *Pneumocystis carinii* pneumonia, which required mechanical ventilation, and he remained at the hospital for several weeks. At that time, an immunological evaluation revealed hypogammaglobulinemia with an IgG of 24 mg/dl, IgA of below 6, IgM of 19, and severe T cell deficiency with a reverse CD4 to CD8 ratio. The young controls (HD08 and HD09) were recruited during a visit to the General Pediatric Endocrine Clinic at Weill Cornell Medical Center. HD08 is an overweight 14-yr-old Hispanic girl who was referred to the Pediatric Endocrine Clinic at age 13 for the management of her weight and associated insulin resistance. She did not have any considerable past medical history. Her family history is negative for autoimmune-related disorders. Her physical examination was only remarkable for the presence of acanthosis nigricans in the skin of the axillary area, which is a manifestation of insulin resistance. Control HD09 is a 5-yr-old Hispanic girl who presented to the General Endocrine Clinic at the age of 2 with headache and central precocious puberty. The donor is otherwise a healthy girl without any significant medical history; her family history is negative for autoimmune disorders. Informed consent, as approved by the Institutional Review Board, was obtained from the parents or guardians of subjects <18 yr of age; in addition, consent was signed by children and adolescents >8 yr of age.

Single cell sorting and flow cytometry. Peripheral B cells were purified from the blood of CD40L-deficient and BLS patients and control donors by negative selection using the RosetteSep procedure (StemCell Technologies). Enriched B cells were stained with FITC anti–human CD27, PE anti–human CD10, anti–human IgM biotin, and allophycocyanin anti–human CD19 (Becton Dickinson). Biotinylated antibodies were revealed using streptavidin-PECy7 (Becton Dickinson). Single $CD19^+CD10^+IgM^+CD27^-$ new emigrant and $CD19^+CD10^-IgM^+CD27^-$ peripheral mature naive B cells from patients and control donors were sorted on a FACSVantage (Becton Dickinson) into 96-well PCR plates containing 4 μl of lysis solution (0.5× PBS containing 10 mM DTT, 8 U RNAsin [Promega], and 0.4 U 5′-3′ RNase Inhibitor [Eppendorf]) and immediately frozen on dry ice. All samples were stored at −70°C. For NKT and T reg cell analyses, peripheral mononuclear blood cells were stained either with FITC anti-TCRVα24, PE anti-TCRVβ11, (Immunotech), and PECy7 anti-CD3 or with FITC anti-TCRαβ, PE anti-CD25, PECy7 anti-CD4, and allophycocyanin anti-CD127 (Becton Dickinson). Intracellular Foxp3 stainings were performed according to the manufacturer's instructions (eBioscience).

cDNA, RT-PCR, antibody production, and purification. RNA from single cells was reverse-transcribed in the original 96-well plate in 12.5-μl reactions containing 100 U of Superscript II RT (Invitrogen) for 45 min at 37°C. RT-PCR reactions, primer sequences, cloning strategy, expression vectors, antibody expression, and purification were carried out as previously described (19, 54). Immunoglobulin sequences were analyzed by Ig Basic Local Alignment Search Tool comparison with GenBank. Heavy chain CDR3 was defined as the interval between the conserved cysteine at position 92 in the V_H framework 3 and the conserved tryptophan at position 103 in J_H segments.

ELISAs and IFAs. Antibody concentrations, reactivity against specific antigens, and indirect immunofluorescence were determined as previously described (19, 54). Highly polyreactive ED38 was used as positive control in HEp-2 reactivity and polyreactivity ELISAs (19, 54). Antibodies were considered polyreactive when they recognized at least two, and usually all of the four, analyzed antigens that include single-stranded (ss) DNA, dsDNA, insulin, and LPS. For indirect immunofluorescence on *C. luciliae*, fixed *C. luciliae*–coated slides (Antibodies, Inc.) were incubated at room temperature with purified recombinant antibodies at 50–150 μg/ml. FITC-conjugated goat anti–human IgG (Antibodies, Inc.) was used as a detection reagent. Serum BAFF concentration were determined by ELISA according to the manufacturer's instruction (R&D Systems).

Statistics. Differences between controls and patients were analyzed for statistical significance with unpaired Student's *t* tests, using SigmaPlot software (Systat). A P value of < 0.05 was considered significant.

Online supplemental material. Fig. S1 shows that mature naive B cells from CD40L-deficient patients are enriched in clones displaying highly positively charged heavy chain CDR3s. Fig. S2 shows that CD40L-deficient patients often display a high proportion of new emigrant/transitional B cells. Fig. S3 shows NKT cell frequency in control, CD40L-deficient, and BLS patients. Antibody repertoires and reactivity from new emigrant and peripheral mature naive B cells from HDs, CD40L-deficient, and BLS patients are presented in Tables S1–S12. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20062287/DC1.

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