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Ig Heavy Chain Promotes Mature B Cell Survival in the Absence of Light Chain

Pedro Geraldes,* Michelle Rebrowich,* Kai Herrmann,† Jamie Wong,‖ Hans-Martin Jäck,† Matthias Wabl,‖ and Marilia Cascalho2*‡‡

Survival of mature B cells is thought to depend on the BCR signaling (BCR) because ablation of either H chain (HC) expression or BCR signaling causes B cells to rapidly disappear. Whether a complete BCR is required for survival of mature B cells is not known. To address this question, we generated a mouse in which we can repress the expression of a transgenic Ig L chain (IgL) by doxycycline (IgL-repressible mouse). Repression of IgL abrogated expression. Surprisingly, however, IgL-negative B cells survived longer than 14 wk, expressed signal-competent HC on the cell’s surface, and active unfolded protein response factors. Like postgerminal center B cells, IgL-negative B cells were small lymphocytes, not dividing and expressed Bcl-6. Our results indicate that expression of unpaired HC, as it may occur as a consequence of Ag ligation, somatic hypermutation, or receptor editing, facilitates the survival of cells either by inducing receptor signaling or by inducing unfolded protein response and/or the expression of survival genes such as Bcl-6. The Journal of Immunology, 2007, 179: 1659–1668.

The development and survival of mature B cells is thought to require stable expression of surface Ig (1) and expression of functional Igαβ dimer (2). Lam et al. (1) and Kraus et al. (2) showed that repression of H chain (HC) and consequently of surface Ig expression causes death of mature B cells and inferred that survival depends on BCR signaling through the Igαβ dimer, much as the TCR is required for T cell survival (3). Whether instead the HC itself might promote survival independently of the BCR was not formally considered. We sought to test this question.

Expression of membrane-bound HC drives early B cell differentiation even in the absence of a complete surrogate light (SL) chain and conventional L chains. This idea is supported by the studies of Schuh et al. (4), who found that a transgenic μHC reaches the cell surface in the absence of the SL chain component A5 and conventional L chains induce IL-7-dependent cell growth and promote in vivo differentiation of pro-B cells. Similarly, Galler et al. (5) showed that μHC signals terminate the expression of terminal deoxynucleotidyltransferase and down-regulate the expression of the RAG 1 and 2 in the absence of SL and L chains.

B cells of camels, sharks, and ratfish produce HCs that cannot pair with L chains (LCs) (6). In these species (6) and when expressed in mice (7), unpaired HCs appear to drive B cell development and contribute to HC-only Abs which make up to 75% of the serum Ig (6). Synthesis of HC-only Abs may depend on some HC unique features including VH FR2 domain adaptations and lack of a CH1 domain. These features antagonize binding to L chains and possibly to the chaperone Ig HC-binding protein (BiP) that retains unpaired HCs in the ER (6), thus enabling trafficking from the ER to the cell surface in the absence of L chain. Synthesis of H chain-only Abs suggests that unpaired H chains may sustain B cell development and mature B cell survival.

In light of these properties of HC, we questioned whether murine HC expressed without L chain might sustain mature B cell survival. To test this concept, we generated a novel experimental system in mice: the IgL-repressible mouse. In the IgL-repressible mouse, expression of LC and surface BCR can be abrogated by feeding the mice doxycycline (DOX). Expression of HC remains unaffected in these mice. From the phenotype of the IgL-repressible mouse, we report here that, contrary to expectations, mature B cells survive repression of LC and that continued expression of HC alone drives long-term survival of B cells. We also report that HC is expressed on the surface of cells and can associate with Igαβ dimers to yield a functional complex that promotes survival. In contrast to studies by Corcos et al. (8, 9) showing that truncated H chains that lack the Vβ exon are expressed unpaired on the surface of B cells, B cells of the IgL-repressible mouse express the full-length protein. Although truncated H chains do not sustain survival of B cells (9), our studies indicate that expression of full-length HC does. These findings may explain how some cells of B lineage (e.g., plasma cells or neoplastic B cells) survive with little or no surface Ig.

Materials and Methods

Mice

The IgL-repressible mouse has monoclonal B and T cell compartments. Because the mice are on a RAG-1-negative background (RAG1−/−), no endogenous B or T cell Ag receptors are produced. Instead, T cells express the transgenic DO.11.10 βαTCR (10–12), while B cell Ig is encoded by a
combination of the knock-in pHc gene (V_{17.2.25}) (13) and a λLC transgene (14). The transgenic TCR is specific for an OVA peptide (OVA aa 323–339) and the combined transgenic knock-in BCR is specific for the human 4-hydroxy-3-nitrophenyl acetyl and its derivatives (13). Expression of λLC is regulated by the availability of DOX. In the absence of DOX, a transactivator binds the minimal promoter and drives λLC expression (see Fig. 1A).

The λLC gene was engineered using the tetracycline-inhibitable transactivator system (15). This system consists of a transactivator and a target λLC transgene. The transactivator or tTA (HSV VP16 fusion protein) is under the control of the mouse mammary tumor virus long terminal repeat promoter (MMTV-tTA) and is constitutively expressed. The MMTV-long terminal repeat targets the expression of tTA to lymphocytes and epithelial cells of the secretory organs (16, 17).

The transactivator (tTA) in the absence of tetracycline (or its derive DOX) acts upon the λLC transgene minimal promoter (Pmin) promoting transcription. DOX binds the tTA, preventing it from binding Pmin (16, 17) and effectively repressing λLC expression.

Tetracycline-responsive λI transgene

The repressible λ construct puts the λLC gene under the control of a tetracycline and transactivator-responsive promoter (TetO). The λ gene was obtained by the EcoRI digest of the C2 plasmid (a gift from Dr. F. Young, University of Rochester, Rochester, NY; Ref. 14). This fragment contains the λ endogenous promoter and the VJΑ rearrangement linked to Cα in a genomic configuration. The promoter region was subsequently excised by further digestion with SauAI, which cuts 15 bp upstream of the start codon ATG. The 5.8-kb fragment was then blunted and cloned at the Pvu1 site of the pBI-EGFP plasmid (catalog no. 6154-1; Clontech Laboratories). The final plasmid puts the λ gene under the control of a TetO regulatory element linked to the LCMY minimal promoter. Because the λ gene lacks an intronic enhancer and there is no 3′ enhancer in this construct, transcription depends on the binding of a transactivator to the TetO element.

Enhanced GFP expression, which in the pBI-EGFP plasmid is under the control of a TetO regulatory element, was lost upon breeding the founder mice.

Generation of the IgL-repressible mouse by breeding

The λ-repressible founders were mated to the MMTV-tTA mouse and to mice of the following genotype: RAG1−/−, V_{17.25} T/V_{17.25} T, and DO11 TCR, H-2/kl. The repressible Ig mice, obtained from the previous crosses, have the following phenotype: RAG1−/−, V_{17.25} T/V_{17.25} T, λ ind, tTA, and DO11-TCR, H-2/kl. The studies discussed here were performed with mice derived from two independent founders.

Animal care and DOX treatment

All mice were between 1 and 3 mo of age and were kept in a specific pathogen-free facility at the Mayo Clinic. All animal experiments were conducted in accordance with protocols approved by the Mayo Clinic Institutional Animal Care and Use Committee. IgL-repressible mice were fed a DOX diet (grain based) (200 mg/kg; $3888; Bio-Serv).

Genotyping

DNA was extracted from mouse tails according to standard protocols (13). Genotyping was done by PCR amplification of tail DNA with thermoprimer plus DNA polymerase (ABgene) and 12 pmol of each primer for 40 cycles using an Icycler (Bio-Rad) thermocycler. H chain knock-in (V_{17.2.25} D_{11.4}) forward-5′AAGTTCAAGTCGACAGCTGGTGC 3′; reverse-5′GGGAGCACATACTGCCGATTAAGTC 450 bp, melting temperature (Tm) 51°C; AILC forward-5′GGCT TTACATCGATGTTGATGAA 3′; reverse-5′GACCCATYCACTGAGCAGTAYSYYGTTC 3′, 500 bp, Tm 60°C; tetracycline transactivator (tTA) forward-5′ AGGAG ATGCTTATATCATGACTGCCG 3′; reverse-5′AGACCCGTATATCTGGTCTCTT 300 bp, Tm 52°C; IA-Ab forward-5′CATAGCCCAAATGTCGCTAATGTTT 300 bp, Tm 52°C; IA-A forward-5′CATACGGCCCAATGGTCGTCTCT 300 bp, Tm 52°C; ATGCTTACATCGATGTTGATGAA 3′; reverse-5′ AGTCCTTACATCGATGTTGATGAA 3′; reverse-5′ CATGGGATCATAGAGAGGCGAG TCTTGTGAC 3′, 200 bp, Tm 60°C; and IA-Ab forward-5′CATACGGCCCAATGGTCGTCTCT 300 bp, Tm 52°C; IA-A forward-5′CATACGGCCCAATGGTCGTCTCT 300 bp, Tm 52°C.

Cell lines and culture conditions

Ag8.8 cells were grown in complete RPMI (RPMI 1640 medium supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 5% FCS, 1 mM sodium pyruvate, and 2 mM l-glutamine. Ag8.8-Igκ transfectants were selected in complete RPMI supplemented with 1 mg/ml G418 (PA), whereas Ag8.8-Igκ expressing μHC was cultured in complete RPMI with 1 mg/ml G418, 1.25 μg/ml mycophenolic acid, 250 μg/ml xanthine, 100 μM hypoxanthine, and 16 μM thymidine. All cell lines were maintained at 37°C and 5% CO2 in a humidified incubator.

The Ag8.8-Igκ cell line was generated to analyze the cell surface transport of BCR complexes by transfection of Ag8.8, a subclone of the murine Ig-negative plasmacytoma cell line Ag8.653 (18). Murine Igκ, required for surface transport of BCR complexes (19), was amplified by PCR from cDNA of the cell line (18) in an appropriate forward primer (TTGATCCAGATGCGCAGGCTTAGA) and a BamHI restriction site and backward primer (TTGATCCAGATGCGCAGGCTTAGA) and cloned into the pBI-EGFP plasmid (purchased from BD Immunocytochemistry) with the same V_{17.25} D_{11.4} BamHI variable exon of the HC expressed in the repressible Ig mouse (21) were transfected in Ag8.8-H-Igκ by electroporation. Stable cell clones were established by limiting dilution and analyzed by flow cytometry.

Flow cytometry

Organ cell suspensions were prepared by pushing the organ through 0.70-μm mesh (spleens and lymph nodes) or by passing the tissue repeatedly through a 27-gauge needle (bone marrow). White blood cells were isolated using a Ficoll-Plaque (GE Healthcare) gradient and cells were counted with a Coulter counter (Beckman Coulter). Surface staining of splenocytes was done as described previously (13); for intracytoplasmic staining, splenocytes were fixed in PBS supplemented with 2% paraformaldehyde at 4°C for 1 h and permeabilized in 1 ml of 0.2% Tween 20 in PBS and in 1 ml of 0.1% NaNon, with 2% FCS. For membrane staining of cultured cells, 5 × 10^6 cells were incubated for 30 min on ice in PBS supplemented with 2% FCS and 0.1% NaNon, with the appropriate amount of Abs. For cytoplasmic staining of cultured cells, 5 × 10^5 cells were fixed in 4% paraformaldehyde in PBS at room temperature (RT) for 10 min, permeabilized with 0.1% Tween 20 at 37°C for 15 min, and stained at RT for 15 min with the appropriate amount of Abs. Data analyses were performed using a FACS Calibur (BD Biosciences) and CellQuest software (version 4.0.2).

Antibodies

Abs used were biotin-labeled, goat IgG directed against the murine IgM (H + L), purchased from Southern Biotechnology Associates and from BD Biosciences, allophycocyanin-labeled anti-mouse CD19 (13D), biotin-labeled anti-mouse Igκ, λ, and Χκ (R-26.46), R-PE-conjugated rat anti-mouse CD23 (FcεRII; B3B4) mAb, FITC-conjugated rat anti-mouse CD21/CD35 (CR2/CR1 and CD21a/CD21b) (706) mAb, FITC-conjugated rat anti-mouse CD24 (heat-stable Ag) (M169) mAb, biotin-conjugated mouse anti-mouse IgD (Igh-5b; 217-170) for B6 mice, and FITC-conjugated rat anti-mouse CD45R/B220 (RA3-6B2) mAb. Biotin-labeled Abs were detected with streptavidin-PE-Cy5 (BD Biosciences Pharmingen). Ki67 was detected with rat anti-mouse Ki67 (TEC-3; DakoCytomation) Ab in tissue sections.

Unconjugated affinity-purified goat Abs against mouse IgM (H + L) (Southern Biotechnology Associates) were labeled with the aCy5- labeling kit from Amersham Biosciences. The monoclonal mouse IgGl, κ Ab 24C2.5 against the intracellular tail of mouse Igκ was previously described (22).

TUNEL assay

The TUNEL assay was done with an apoptosis detection kit, ApopTag Red in Situ, according to the manufacturer’s instructions (Chemicon International).

Ca^{2+} influx

Ca^{2+} influx studies were performed by incubating splenocytes with 5 μM Indo-1 AM (Invitrogen Life Technologies) for 30 min at RT and then labeled with anti-CD19 and anti-B220 Abs for 30 min at 4°C. Cells were kept at 37°C for 2 min before adding unlabeled stimuli: polyclonal unlabeled goat anti-mouse IgM (H + L; 100 μg/ml), polyclonal unlabeled goat anti-mouse λ (λ-chain specific; 100 μg/ml), polyclonal unlabeled total goat anti-mouse IgG (100 μg/ml), all purchased from Southern Biotechnolce Associates. Ionomycin was obtained from Calbiochem and Merck. Data were collected and analyzed on a cytometer (LSR II; BD Biosciences) with FlowJo software (Tree Star). The results are shown in an indol1-violet: indol-blue ratio.
RT-PCR

RNA was extracted using a Qiagen RNeasy Kit (catalog no. 74104) according to the manufacturer’s instructions. The RNA yield was measured with the Nanodrop-no-1000 spectrophotometer (V3.0.1; Nanodrop Technologies). Reverse transcription was performed using an Invitrogen Life Technologies Thermoscript RT PCR System (catalog no. 1146-016) according to the manufacturer’s instructions. The cDNA was amplified using the following primers: Pax-5 forward CTACGGGCTTGGAGCTGAC and Pax-5 reverse TCTCGGCTTGACATAATGG (annealing 65°C, 439 bp); VpbrF forward GTCGTAATCTCCGAGCTGCG and VpbrR reverse CAGTCATGCTGGCAGTCTG (annealing 65°C, 600 bp); Aβ5 forward CCAAATCCGGTCTTAGGG (annealing 65°C, 205 bp); VpilR forward GGGGATATCCACACCAAACATC and VpiR reverse TCTTCATGGTGACATGCTGCAG (annealing 55°C, 500 bp); Irel1 forward AGAAGCTACTCTGTTGCCTGTTA and Irel1 reverse CTATCCTGGAAGAAGGTGGTTCTG (annealing 55°C, 150 bp); Edem1 forward ATCCTGCGGTACAGAGGCGG and Edem1 reverse GCTTCTCCGAGACCCTATGC (annealing 55°C, 150 bp); MrP1R forward GATCTAACATGACGGG and MrP1R reverse TCTACTGGTTGAGCTC (annealing 55°C, 174 bp); Xbp1s forward TTGCGGTCTTCACAGAAGT (annealing 53°C, 174 bp); Xbp1u forward GGGTCTAGTGGATGGTGTCC and Xbp1s reverse GAGACTTGTGGACACCCAGCC (annealing 58°C, 150 bp); BLIMP1 forward ACTTGGTTGAGCCAGACGGGAGG and BLIMP1 reverse CTGGTTGT GGCAGCATCATTGG (annealing 58°C, 150 bp); BcI6 forward TGCGTAATCCGACAGCTTGA and BcI8 reverse GGACATTGAAAATCGAGCGG (annealing 58°C, 150 bp); Aβ5 forward ACTTGTGGGAACAGGCAGCCAGGGGT and Aβ5 reverse ACTTGTGGGAACAGGCAGCCAGGGGT (annealing 58°C, 150 bp); and β-actin forward TCTCAAGCCACGTCGAAAAG and β-actin reverse TCTCTAGTGGTCAGAGCCGA (annealing 54°C, 600 bp).

Immunohistochemistry

The immunohistochemistry was performed essentially as described in João et al. (23). The primary Abs used were: unlabeled goat anti-mouse ALC and FITC-conjugated goat F(ab’)2, anti-mouse IgM (H + L) from Southern Biotechnology Associates, alkaline phosphatase was purchased from Cappel, rat anti-mouse CD19 (ID3) was purchased from BD Biosciences Pharmingen, and rat anti-mouse CD180 (RP10; RP/14) was purchased from Serotec. The secondary/tertiary Abs used were: rhodamine-conjugated donkey F(ab’)2 anti-rat IgG (H + L) from Jackson ImmunoResearch Laboratories, FITC-conjugated rabbit F(ab’)2, anti-goat IgG from ICN Pharmaceuticals/Cappel, and FITC-conjugated goat F(ab’)2, anti-rabbit IgG from ICN Pharmaceuticals/Cappel. Slides were examined on a Leica DMARD fluorescence microscope. Digital images were obtained using a high-resolution charge-coupled device digital camera (SPOT II; Diagnostic Instruments) mounted to the microscope and SPOT II software.

Immunoprecipitation and Western blot analysis of splenocytes

Spleens were harvested and cell suspensions were prepared by pushing homogenates through a 70-μm nylon mesh. BCR where depletion using Ficol-Paque (GE Healthcare). B cells were isolated using the MACS column (Miltenyi Biotec) and the B cell isolation kit (Miltenyi Biotec). Briefly, 0.25 million B cells where lysed using Beach buffer. Serial dilutions of lysates were incubated overnight at 4°C in 96-well flat-bottom microtiter plates (Nunc-Immuno 96 Micro well, Maxisorp; eBioscience) coated with 2 μg/ml unlabeled goat anti-mouse A (Southern Biotechnology Associates) and blocked with PBS supplemented with 0.1% gelatin and 0.1% Tween 20. ALC were revealed with HRP-conjugated goat anti-mouse A (Southern Biotechnology Associates) and developed with ABTS. Plates were read at 405 nm at 5, 10, and 20 min on a microplate reader (Power Wave X; Bio-Tek Instruments) and analyzed using the software KC4-Kinetica for Windows.

Results

The IgL-repressible mouse

The IgL-repressible mouse is bred onto a RAG1-negative background (RAG1−/−). B cells in this mouse express a monoclonal BCR consisting of a constitutively expressed knock-in μHC (VH417.2.25) (13) and of a DOX-repressible ALC transgene (14). Expression of the ALC transgene is driven by the binding of a tetracycline-controllable transcription factor (tTA) to the minimal Iε promoter (Pmin) (15–17). The activity of the tTA can be abolished by the addition of tetracycline or its derivative DOX (Fig. 1A). Because the tTA gene is under the control of the mouse mammary tumor virus long terminal repeat promoter (mouse mammary tumor virus promoter-driven-tTA (MMTV-tTA)), the expression of tTA is constitutive and targeted to lymphocytes and epithelial cells of the secretory organs (16, 17). The transgenic BCR is specific for the hapten 4-hydroxy-3-nitrophényl acetyl and its derivatives (13). T cells in the IgL-repressible mouse are also monoclonal and express the DO11.10 αβ TCR (11).
DOX treatment represses LC production

We first determined whether administration of DOX to the IgL-repressible mouse inhibits expression of ALC. ALC protein could not be detected by Western blot analysis of splenocytes isolated from mice that were fed DOX for 4 wk (Fig. 1B, lane 3). ELISA of homogenates (including cytoplasmic and membrane-bound protein) obtained from purified B cells confirmed the absence of ALC in DOX-treated IgL-repressible mice. ELISA limit of detection of ALC was 2 ng/ml. When all B cells expressed ALC (in the QM mice), we measured 64.6 ng/ml or 23 ng/million B cells. We detected only 5.5 ng ALC/ml in control B6 B cells, corresponding to 8.5% of the QM values, which is in line with the fact that only 5–10% of B cells in B6 mice express ALC.

Repressible Ig mouse B cells can only express ALC. In the absence of DOX, we detected 19.0 ng ALC/ml or 1.5 ng ALC/million B cells, indicating that B cells in the repressible Ig mouse produce 15-fold less ALC than QM B cells. The assay could not detect ALC above background in as many as 0.5 × 10^5 B cells obtained from a repressible Ig mouse following DOX treatment. This result indicated that DOX-treated repressible Ig B cells produced at least 32-fold less ALC as QM B cells and had at least 3-fold less ALC than non-DOX-treated repressible Ig B cells.

To determine the extent of transcription repression of the ALC gene in mice fed DOX, we performed RT-PCR with primers that were specific for cDNA. We failed to detect LC mRNA (Fig. 1C).

To determine whether repression of ALC protein expression effectively abrogated LC function as part of the BCR, we compared changes in the level of intracellular-free Ca^{2+} in B cells stimulated with anti-ALC Ab. Although B cells from IgL-repressible mice not treated with DOX responded to anti-ALC Ab by quickly increasing the intracellular Ca^{2+} (Fig. 1D, blue line), B cells from mice treated with DOX did not (Fig. 1D, red line). These results indicate that DOX treatment generated B cells functionally lacking LC, which we will refer to as “LC-negative B cells.”

**B cells survive and continue to express membrane-bound μHC upon LC repression**

Expression of surface BCR is thought to require the correct assembly of H and L chains. Hence, we questioned whether repression of LC affects surface μHC expression on splenic B cells isolated from DOX-treated mice. As expected from the analysis of ALC expression by Western Blot analysis, we detected surface ALC on all CD19-positive splenocytes from mice not fed DOX (Fig. 2A, first row), but not from mice fed DOX. Only ~5% of B6 splenocytes expressed ALC (Fig. 2A, first row, diagram on right). However, despite the complete repression of LC expression in mice fed DOX, we detected μHC in the cytoplasm as well as on the surface of B cells (Fig. 2A, second and third rows). Surface μHC was reduced by 10-fold in B cells that lack LC (Fig. 2A, second row), but cytoplasmic μHC was not.

The presence of cells with markers characteristic of B cells (CD180, CD19, and μHC) could be verified in the absence of LC in histological sections of spleen obtained from IgL-repressible mice fed DOX (Fig. 2B). These data show that LC-negative cells express CD180, CD19, and μHC, indicating that B cells survived (as indicated). Histograms represent ratio of fluorescence intensities of Indo-1-AM bound to Ca^{2+}/free Indo-1 AM over time (s). Arrows, Time the stimulus was added. Ca^{2+} influx curves obtained following LC cross-linking with goat anti-mouse ALC Ab are depicted in blue; curves obtained following addition or goat IgG control are depicted in red. LC-repressed B cells failed to generate Ca^{2+} influx following LC cross-linking (bottom diagram).
FIGURE 2. B cells survive LC repression and express μHC. A, Flow cytometric analysis of splenocytes from IgL-repressible mice fed or not fed DOX and B6 control, as indicated. *Left column.* The lymphocyte gate on the light scatter plot used in the analysis. The *three columns* to the right represent the fluorescence intensity plots of lymphocyte-gated events stained with labeled Abs, as indicated. Surface and cytoplasmic μHC were detected with a goat anti-mouse IgM (H + L), all other Abs were rat anti-mouse mAbs. Mature (MB) and immature (IB) B cells were identified in fluorescence intensity plots shown in row 4 obtained from lymphocyte and CD19-positive gated events. Marginal zone (MZ) and follicular (FO) B cells (row 5) were identified in fluorescence intensity plots shown in row 5 obtained from lymphocyte and B220-positive gated events. This figure shows that ALC-positive, surface CD19-positive B cells disappear in mice treated with DOX but CD19-positive, ALC-negative cells survive (upper row) expressing both surface (row 2) and cytoplasmic (row 3) μHC. Immature and marginal zone B cells are absent in DOX-treated IgL-repressible mice. Only ~5% of CD19-positive B cells in B6 mice are ALC positive. B, Frozen sections obtained from spleen of IgL-repressible mice fed (right) or not fed (left) DOX. Sections were costained with anti-μ (FITC-conjugated; row 2) and anti-CD180 Abs (rhodamine-conjugated; row 3). Sections shown in the row 4 were stained with an anti-CD19 Ab (FITC-conjugated) and in row 5 with an anti-IgM (H + L) Ab (FITC-conjugated), as indicated. All sections were also stained with 4',6-diamidino-2-phenylindole (DAPI) to identify nuclei as shown in row 1. Figure shows that ALC expression is abrogated by DOX and that surviving B cells that lack ALC express CD180, CD19, and μHC. C, RT-PCR analysis of Pax-5, VpreB, and λ5 mRNA in splenocytes obtained from IgL-repressible mice fed or not fed DOX, as indicated. Pax-5 expression marks B cell lineage, whereas the absence of both VpreB and λ5 expression rules out pre-B cells. PCR was done with 1/1, 1/5, and 1/25 cDNA dilutions. D, Analysis of μHC expression in Ag8.H clones by Western blot. Ag8.H cell lines were transfected with a construct expressing Igα and another construct expressing the μHC αVR17.2.25, the HC in the repressible IgL mouse that pairs with Igα. The mature B cell line NYC served as positive control for Igα and μHC in Ag8.H clones. Untransfected Ag8.H-Igα served as negative staining control (open histogram). *Left column.* Stable Ag8.H transfectants were cytoplasmic stained with 24C2.5 Abs against the intracellular tail of mouse Igα, FITC-conjugated Abs against mouse Fcγ served as secondary Abs (filled histogram). *Right column.* Stable Ag8.H transfectants producing μHC αVR17.2.25 μHC were cytoplasmic or membrane stained with Cy5-conjugated Abs against mouse μHC (gray histograms). Figure shows that μHC αVR17.2.25 is expressed on the surface. Results shown are representative of three independent experiments.

repression of LC. To determine which subsets of B cells survived repression of LC, we distinguished mature (CD19+CD24low IgDhigh), immature (CD19+CD24highIgDlow), marginal zone (B220+CD21highCD23low), or follicular B cells (B220+CD21low CD23high) by flow cytometry analysis of splenocytes obtained from IgL-repressible mice fed or not DOX. Fig. 2A, fourth row, shows that immature B cells and marginal zone B cells disappeared following LC repression. The surviving B cells included follicular B cells, the numbers of which decreased from 1.4 million to 0.3 million 4 wk after LC repression.

Pre-B cells that express HC do so in conjunction with surrogate LC (25). The surrogate LC is composed of λ5 and V pre-B proteins, and these along with HC form the pre-BCR that reaches the cell surface (25, 26). Because the pre-BCR is thought to sustain survival of pre-B cells (27), we asked whether B cells surviving repression of LC express a pre-BCR. Fig. 2C shows that mRNA for the surrogate L chain components VpreB and λ5 was absent in peripheral LC-positive as well as in LC-negative B cells. These results were corroborated by flow cytometry using Abs specific for pre-BCR components (data not shown) and indicate that surface LC-negative B cells were not pre-B cells. Because surviving B cells expressed CD19 and the Pax-5 transcription factor (Fig. 2C), and plasma cells do not (28), our results also indicate that LC-negative B cells were not terminally differentiated.

To confirm that the transgenic VH17.2.25 chain reaches the cell surface in the absence of IgL chains, we transfected SL and L anti-mouse mAbs. Mature (MB) and immature (IB) B cells were identified in fluorescence intensity plots shown in row 4 obtained from lymphocyte and CD19-positive gated events. Marginal zone (MZ) and follicular (FO) B cells (row 5) were identified in fluorescence intensity plots shown in row 5 obtained from lymphocyte and B220-positive gated events. This figure shows that ALC-positive, surface CD19-positive B cells disappear in mice treated with DOX but CD19-positive, ALC-negative cells survive (upper row) expressing both surface (row 2) and cytoplasmic (row 3) μHC. Immature and marginal zone B cells are absent in DOX-treated IgL-repressible mice. Only ~5% of CD19-positive B cells in B6 mice are ALC positive. B, Frozen sections obtained from spleen of IgL-repressible mice fed (right) or not fed (left) DOX. Sections were costained with anti-μ (FITC-conjugated; row 2) and anti-CD180 Abs (rhodamine-conjugated; row 3). Sections shown in the row 4 were stained with an anti-CD19 Ab (FITC-conjugated) and in row 5 with an anti-IgM (H + L) Ab (FITC-conjugated), as indicated. All sections were also stained with 4',6-diamidino-2-phenylindole (DAPI) to identify nuclei as shown in row 1. Figure shows that ALC expression is abrogated by DOX and that surviving B cells that lack ALC express CD180, CD19, and μHC. C, RT-PCR analysis of Pax-5, VpreB, and λ5 mRNA in splenocytes obtained from IgL-repressible mice fed or not fed DOX, as indicated. Pax-5 expression marks B cell lineage, whereas the absence of both VpreB and λ5 expression rules out pre-B cells. PCR was done with 1/1, 1/5, and 1/25 cDNA dilutions. D, Analysis of μHC expression in Ag8.H clones by Western blot. Ag8.H cell lines were transfected with a construct expressing Igα and another construct expressing the μHC αVR17.2.25, the HC in the repressible IgL mouse that pairs with Igα. The mature B cell line NYC served as positive control for H chain expression. E, Flow cytometry analysis of μHC αVR17.2.25 surface and cytoplasmic expression in Ag8.H clones. Intracellular and membrane expression of Igα, and μHC in Ag8.H clones. Untransfected Ag8.H-Igα served as negative staining control (open histogram). *Left column.* Stable Ag8.H transfectants were cytoplasmic stained with 24C2.5 Abs against the intracellular tail of mouse Igα, FITC-conjugated Abs against mouse Fcγ served as secondary Abs (filled histogram). *Right column.* Stable Ag8.H transfectants producing μHC αVR17.2.25 μHC were cytoplasmic or membrane stained with Cy5-conjugated Abs against mouse μHC (gray histograms). Figure shows that μHC αVR17.2.25 is expressed on the surface. Results shown are representative of three independent experiments. SSC, Side scatter.
chain-negative Ag8.653 first with a gene encoding Igα and then with a vector encoding the μH chain (V_{H}17.2.25-μHC) identical to the one expressed in the IgL-repressible mouse (Fig. 2D). Fig. 2E shows that the V_{H}17.2.25-μHC is transported to the surface of cells in the absence of LC.

Surface LC-negative B cells are long-lived

The survival of pre-B cells and the survival of mature B cells is thought to depend on expression of a surface receptor (1, 29). Lam and colleagues (1) and Kraus and colleagues (2) found that repression...
of HC abolishes the expression of a complete BCR and causes death of B cells. Whether B cells die because of the absence of surface BCR or to the absence of HC on its own was not determined. In contrast, repression of LC in the IgL-repressible mouse abolishes the assembly of a complete BCR but allows surface HC expression. To determine how long LC-negative B cells expressing neither complete BCR nor pre-BCR survive, we enumerated B cells in the spleen and in the peripheral blood of mice at different times following LC repression. The number of B cells in the spleen decreased 5- to 6-fold (from 2.4 million to 0.4 million) 4 wk after LC repression but it remained constant thereafter. The decrease in the number of B cells in the spleen was not observed in the peripheral blood. Fig. 3A shows that the number of CD19-positive B cells in the blood remained constant for up to 14 wk after the start of the DOX treatment. Long-lived B cells expressed bcl-6, blimp-1, and activation-induced cytidine deaminase (aid) and did not express c-myc mRNAs, consistent with a noncycling, post-germinal center phenotype (Fig. 3B). Expression of aid and blimp-1 suggests the possibility that these factors are needed to establish or maintain survival of B cells in a nonterminally differentiated state. Several factors could contribute to the maintenance of peripheral CD19-positive surface LC-negative cells: de novo production of B cells or proliferation in equilibrium with cell death and/or long life. To determine whether repression of LC completely eliminates de novo production of B cells in the bone marrow, IgL-repressible mice were treated with DOX for two generations (to exclude transfer of maternal B cells to the fetus), after which B cells in the periphery were sought. Flow cytometry analysis of splenocytes of IgL-repressible mice treated in this way revealed no B cells in the periphery (Fig. 3C, right panel). This result indicates that B cells are not produced de novo when production of A is repressed. It is possible that B cells surviving repression of LC are the progeny of rare B cells that proliferated to maintain the B cell compartment. To test this idea, we analyzed spleen sections costained with anti-Ki67 (a cell division marker). Fig. 3D shows that no μHC-positive cells express Ki67, and hence CD19-positive B cells were not proliferating to any great extent following repression of LC. Consistent with this concept, TUNEL analysis shows that splenocytes of mice treated with DOX for 4 wk were not undergoing apoptosis (as might be expected in rapidly proliferating populations of cells; Fig. 3E). These results show that repression of LC expression did not cause enhanced turnover of B cells and therefore the maintenance of surface LC-negative B cells must be due to long life.

FIGURE 4. Full-length μHC is expressed on the cell surface and signals. A, Western blot analysis of surface and cytoplasmic μHC in B cells isolated from spleens of IgL-repressible mice fed (right) or not fed (left) DOX or from B6 controls. Protein lysates obtained from isolated B cells were surface biotinylated and immunoprecipitated (with streptavidin beads). Biotinylated samples (surface) and nonbiotinylated samples (cytoplasm) were analyzed by 10% SDS-PAGE, blotted, and revealed with goat anti-mouse IgM (H + L) Ab. Figure shows that the full-length μHC is expressed on the surface of LC-negative B cells. Approximate molecular mass (m.w.) is noted on the right. B, Western blot analysis of IgG and IgM in B cells isolated from spleens of IgL-repressible mice fed or not fed DOX or from B6 controls. Samples were analyzed by 10% SDS-PAGE and blotted. Blotted proteins were revealed with a goat anti-mouse IgM (H + L) or goat anti-mouse IgG Abs as indicated. Western blot shows that the 50-kDa molecular mass bands detected by the anti-IgM (H + L) Ab are not IgG because they fail to be detected with an Ab directed against IgG. Approximate molecular mass (m.w.) is noted on the right. C, RT-PCR analysis of the length of the μHC mRNA obtained from splenocytes of IgL-repressible mice fed or not fed DOX, as indicated. Figure shows that the mRNA is full length, i.e., it does not contain deletions in the V or C exons. Below is shown a schematic representation of the RT-PCR primer sites respective to the μHC mRNA. In brief, 1/1, 1/5, and 1/25 dilutions of the cDNA were used. D, Flow cytometry analysis of Ca²⁺ influx in isolated B cells obtained from the spleen of QM or from IgL-repressible mice fed or not fed DOX for 4 wk. Histograms represent the ratio of violet:blue fluorescence intensities (Indo-bound to Ca²⁺/unbound Indo-1) over time. The cells were stimulated with either goat anti-mouse IgM (H + L) Ab (blue), with goat anti-mouse λ Ab (green), or with control Ab goat IgG (red) added at 2 min after the start (indicated by the arrow). Cross-linking of surface μHC but not ΔLC causes a modest Ca²⁺ influx by LC-negative B cells (lower panel). Results are representative of four independent experiments.
Our results indicate that surviving surface LC-negative B cells continue to produce \( \mu \)HC and suggest the possibility that \( \mu \)HC expression in the absence of LC functions as a receptor. Because unpaired full-length \( \mu \)HCS are thought to be retained in the ER (22), we asked whether \( \mu \)HC expressed on the surface was full length. Western blot analysis of cellular and surface \( \mu \)HC separated by SDS-PAGE shows that LC-negative B cells expressed predominantly the full-length \( \mu \)HC on the surface (Fig. 4A, lane 5). In addition to the full-length protein, splenocytes from repressible IgL mice also produced a lower molecular mass band, visible in lanes 3–6 of Fig. 4A, migrating with an approximate molecular mass of \( \sim 50 \) kDa. The 50-kDa molecular mass band could correspond to IgG or alternatively a truncated HC, as has been described in some B cell malignancies (31). Fig. 4B shows that the 50-kDa molecular mass bands are not IgG because they fail to be detected with an Ab directed against IgG.

To determine whether the 50-kDa band resulted from a truncated H chain, lacking CH1 or the V\( _{\text{H}} \) exon, we analyzed HC gene expression by RT-PCR. Fig. 4C shows that repressible IgL splenocytes express only the full-length RNA with no evidence of deletions in the variable or constant region exons (Fig. 4C), indicating that the 50-kDa molecular mass band apparent in lanes 3–6 of Fig. 4A, is likely a degradation product. The decrease in LC-repressed splenocytes in \( \mu \)HC mRNA, apparent in Fig. 4C, is attributable to the fact that LC-repressed mice have 5- to 6-fold fewer B cells compared with LC-expressing mice. Our results indicate the possibility that limited L chain availability renders the monoclonal V\( _{\chi} \)17.2.25 \( \mu \)HC more susceptible to proteolysis compared with the complete polyclonal \( \mu \)HC population in B6.

The surface expression of unpaired \( \mu \)HC suggested the possibility that HC alone delivers B cell survival signals. To answer this question, we compared changes in the level of intracellular-free \( \text{Ca}^{2+} \) in B cells stimulated with anti-IgM, whole Ab, or F(ab')\(_2\). Naive QM B cells and surface LC-positive B cells from repressible IgL mice responded to IgM cross-linking with a modest increase in the intracellular \( \text{Ca}^{2+} \) (Fig. 4D, upper and medium panels, blue line). Changes in the level of intracellular-free \( \text{Ca}^{2+} \) in QM or surface LC-positive B cells stimulated with anti-IgM had similar kinetics as those following stimulation with anti-IgG LC (Fig. 4D, green lines). In contrast, surface LC-negative B cells responded to IgM cross-linking with a modest increase in the intracellular \( \text{Ca}^{2+} \) originating a lower amplitude and somewhat retarded peak when compared with \( \chi \)LC-positive B cells or QM B cells (Fig. 4D, lower panel, blue line). The amplitude of the \( \text{Ca}^{2+} \) peak in response to IgM cross-linking on the surface LC-negative B cells was reduced relative to wild-type B cells, possibly due to decreased surface receptor density. Cross-linking IgL on surface LC-negative B cells originated no response (Fig. 4D, lower panel, green line). These results indicate that unpaired \( \mu \)HC generates signals.

**Unpaired HC in the cytoplasm triggers receptor-independent responses**

Because in the absence of LC, HC is retained in the ER by BiP (32), we investigated whether LC-negative B cells activated a stress response called the unfolded protein response (UPR). We tested activation of several UPR transducers. Activated inositol-requiring enzyme endoribonuclease (33) excises 26 bp from the X-box-binding protein 1 (XBP-1) mRNA to form XBP-1 spliced (s). Activation of activating transcription factor 6, another UPR transducer, induces the transcription of \( xbp-1 \) and ER chaperone genes; eukaryotic translation initiation factor a, subunit \( \alpha \) kinase (PERK) activation, transiently inhibits cap-dependent protein synthesis and induces C/EBP homologous protein (Chop). Fig. 5 shows that LC-negative B cells express the spliced (s) and unspliced (u) \( xbp-1 \) messages and have increased levels of BiP mRNAs consistent with inositol-requiring enzyme 1\( \alpha \) and activating transcription factor 6 activation. Chop expression is consistent with PERK activation. These results indicate activation of all three UPR transducers and suggest the possibility that the UPR may contribute to the ALC-negative B cells’ long life.

We also tested whether several UPR gene mRNAs were produced in SL and L chain-negative, IgM-positive Ag8.653 expressing or not the transgenic V\( _{\chi} \)17.2.25 chain. Our results (data not shown), indicate that \( ire1, xbp-1, \) spliced and unspliced, \( chop, \) and \( edem1 \) were equally expressed independently of the transgenic V\( _{\chi} \)17.2.25 chain. These results indicate that in transformed cells activation of the UPR occurs independently of H chain expression. These results do not contradict the possibility that accumulation of HC in the ER triggers the UPR in mature B cells independently of BCR stimulation.

**Discussion**

The property of memory that uniquely distinguishes adaptive immunity in host defense requires long-term survival of B cells after first exposure to Ag. How long-term survival is achieved is not completely understood but is thought to depend on surface expression of the Ag receptors. Thus, survival of T cells depends on engagement of the TCR with self-MHC plus self-peptide, assuring that only functional T cells live (3). In contrast to T cells, B cells recognize novel structures that are cleared from the organism. Thus, survival of mature B cells is thought to require a mature BCR (1, 2, 34), even though the necessity of a generic self-ligand has not yet been resolved.

As one possibility, BCR may promote B cell survival by signaling constitutively. This idea is supported by the work of Lam et al. (1) and Kraus et al. (2), who showed that ablation of HC or IgM signaling in mature B cells led to rapid cell death (in days). However, survival of B cells without BCR is not without precedent because BCR-less B cells expressing an EBV receptor instead of BCR survive in vitro and in vivo (35). Because EBV receptor-expressing B cells appear to activate tyrosine phosphorylation of BCR targets, Casola et al. (35) propose that BCR signaling is necessary for the survival of B cells.

In this study, we report that survival of mature B cells does not require the complete BCR but rather the mere production of unpaired HC suffices to assure B cell survival. In LC-negative B cells expressing BCR targets, Casola et al. (35) propose that BCR signaling is necessary for the survival of B cells.
cells, μHC can be expressed on the surface. Because surface μHC cross-linking induces a modest calcium influx, we concluded that μHC can signal. Signaling by unpaired surface μHC may be one mechanism promoting mature B cell survival. Our results showing long-term survival of B cells expressing HC unpaired with LC support the concept that a B cell autonomous mechanism governs B cell longevity in the absence of a complete BCR.

Functional Abs lacking L chains are produced by B cells in camels (36), nurse sharks, wobbegong sharks (37), and in ratfish (38), indicating that in these species B cells develop and persist in the absence of a conventional BCR. Moreover, expression of dromedary HC-only Abs in the mouse sustains B cell development (7, 39).

HC-only Abs in camels possess molecular adaptations, such as the loss of the CH1 domain, to avoid interaction with L chains and binding to BiP, thus escaping retention in the ER (6, 40). Truncated HC chains have also been associated with disease in mice and in humans causing myeloma (41) or H chain disease (8, 42, 43), respectively. H chain disease-associated μ proteins lacking the rearranged VDJ exon (ΔμHC) produce unpaired HC receptors that are signaling competent. Corcos et al. (8, 42) found that expression of ΔμHC promotes B cell differentiation in the bone marrow and in the periphery. However, mature B cells expressing ΔμHC are larger and have shorter half-lives than wild-type B cells (9, 44). This is despite the fact that ΔμHCs overcome BiP-mediated ER retention and are expressed on the surface (9). Because the HC produced by the IgL-repressible mouse is not truncated, our results indicate that when the availability of the L chain is limited, full-length H chains may escape ER trapping and form signaling competent receptors. Those may be important to promote survival of cells that lose LC expression due to somatic hypermutation or receptor editing.

That unpaired full-length μHC mediates some of the BCR functions was determined by Schuh et al. (4) and Galler et al. (5) who showed that wild-type full-length μHC is not expressed on the surface, promotes in vivo differentiation of pro-B cells (4), and induces IL-7-dependent growth (4) and signals, causing derepression of HC in the ER as described following cytokine and LPS treatment (7, 39). The authors have no financial conflict of interest.

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References


