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Essential Role for CAML in Follicular B Cell Survival and Homeostasis

Nicholas A. Zane,*1 Justin H. Gundelach,†,1 Lonn D. Lindquist,† and Richard J. Bram*‡

Calcium-modulating cyclophilin ligand (CAML) is a ubiquitously expressed protein that is important during thymopoiesis. However, whether it serves a function in mature lymphocytes is unknown. In this article, we show that CAML is essential for survival of peripheral follicular (Fo) B cells. Conditional deletion of CAML in CD19-Cre transgenic mice caused a significant reduction in Fo cell numbers and increased rates of homeostatic proliferation. CAML-deficient Fo cells showed increased cellular turnover and normal proliferative ability. Although CAML-deficient Fo cells responded to AgR stimulation and to B cell activating factor, they displayed decreased survival and increased apoptosis following stimulation with LPS and IL-4 in vitro. Failure to survive was not due to aberrant B cell development in the absence of CAML, because induced deletion of the gene in mature cells resulted in a similar phenotype. These data establish an essential and ongoing role for CAML in the long-term survival of mature B cells. The Journal of Immunology, 2012, 188: 3009–3018.

B lymphocytes play a critically important role in humoral immunity and may participate in regulation of cell-mediated immunity as well. In addition, malignancies of the B cell compartment make up a significant fraction of childhood and adult hematologic malignancies. It is important, therefore, to understand the molecular mechanisms that regulate the development and function of these cells.

B cell development is an orderly, stepwise process of maturation during which cells express a defined series of cell surface markers that enable their identification (1). Hardy, et al. (1) identified six developmental stages in the bone marrow, designating them fractions A through F, whereas Loder, et al. (2) further identified B cell developmental stages within the spleen. In this system, bone marrow fraction E cells become transitional type 1 (T1) cells in the periphery and further develop into transitional type 2 (T2) before becoming one of two different mature cell types, follicular (Fo) or marginal zone (MZ) (2).

B cells at the T2 and later stages rely heavily upon signaling from the ligand BAFF binding to and activating its highest-affinity cognate receptor, BAFF-R. Deletion in mice of either BAFF or BAFF-R substantially reduces the populations of mature splenic B cells (3–5).

Calcium-modulating cyclophilin ligand (CAML) was originally cloned in a two-hybrid search for cyclophilin B interacting proteins (6). CAML is a widely expressed intracellular protein that accumulates at internal cell membranes, particularly the endoplasmic reticulum (7). Although its mechanism of action is not yet deciphered, it is known to physically interact with several signaling molecules, including p56Lck (8), the B cell-specific TNFR family member transmembrane activator and CAML interactor (9), and the epidermal growth factor receptor, for which it is responsible for efficient receptor recycling after stimulation (10). CAML was shown to be targeted by several viral-encoded proteins in the context of which it was proposed to enhance cell survival (11, 12). Complete loss of CAML is embryologically lethal at an early stage in development (10), and deletion of CAML during thymopoiesis caused a block in T cell development at the CD4/CD8 single-positive stages (8).

To ascertain the role of CAML in the development and function of mature B lymphocytes, we have generated and examined B cell-specific knockout mice (designated bCAML−/−) and tamoxifen-inducible CAML knockout mice (designated eCAML−/−). CAML-deficient Fo B cells demonstrated a dramatic increase in cell death that was most prominent following LPS stimulation and was readily observed via in vivo adoptive transfer assays. These results implicate CAML as an essential mediator of B lymphocyte survival.

Materials and Methods

Reagents and Abs

Microbeads specific for murine Ags CD8a, CD4, CD19, and goat anti-rat IgG and allopurinolycocyanin-conjugated Abs specific for murine CD43 were from Miltenyi Biotec. Allophycocyanin-conjugated Abs specific for CD23 were from Southern Biotechnology Associates. FITC-conjugated Abs specific for CD23 were from eBioscience. Allophycocyanin-conjugated Abs specific for CD21 were from BioLegend. Tamoxifen, corn oil, and LPS from Escherichia coli O55:B5 were from Sigma-Aldrich. Unconjugated Abs specific for CD16/CD32 (to block FcR) and FITC-conjugated Abs specific for BP1, CD5, CD93, IgD, IgG1, BrdU, and isotype controls; PE-conjugated Abs against B220, CD21, CD69, IgA, MHC class II (MHCII), and TLR4; PerCP-Cy5.5-conjugated Abs against B220 and IgM; Cy5-conjugated annexin V; allophycocyanin-conjugated Abs against B220 and CD19; and propidium iodide (PI) staining solution were from BD Pharmingen. CAML-specific Abs were described previously (8).

Mice

Conditional B cell knockout mice, designated bCAML−/− (CD19-Cre, CAMLfloX/floX), were generated by crossing CD19-cre mice (13) with CAML floX/floX mice (8). Consistent with published results (13, 14), expression of Cre recombinase did not reduce B cell numbers in control Cre+CAMLfloX mice.
mice (data not shown). Tamoxifen-inducible CAML knockout mice, designated eCAML−/− (ESR-cre, CAMLΔ26) mice, were generated by crossing ESR-cre mice (15) to CAML flox/flox mice and sacrificed between 13 and 26 wk. NOD/SCID mice were acquired from The Jackson Laboratory and sacrificed between 20 and 32 wk. All mice were housed and bred at the Mayo Clinic under pathogen-free conditions. All animal studies approved by the Institutional Review Board of the Mayo Clinic.

Flow cytometric analysis

Lymphocytes from spleens, lymph nodes (LNs), and bone marrow were isolated, made into single-cell suspensions, and stained with fluorescent dye-conjugated Abs and CD16/CD32 (to prevent nonspecific Ab binding). Annexin V and PI staining was conducted as per the manufacturer’s instructions (BD Biosciences). Live lymphocytes were identified on the basis of forward and side scatter or, as indicated, by negativity for PI and annexin V.

Purification of mature murine B cells

B cells were cultured in RPMI 1640 medium with 10% FCS, 2-ME, penicillin and streptomycin, and l-glutamine for 30 min at 37 °C in tissue-culture-treated 6-well plates to allow for adherence of macrophages and dendritic cells. Nonadherent cells were bound to CD4 and CD8 microbeads (Miltenyi Biotec) to negatively select T cells. Transitional B cells were negatively selected on the basis of CD93 expression using rat anti-CD93 Abs and goat anti-rat IgG microbeads. Remaining mature B splenocytes were cultured and stimulated under conditions described previously.

BCR stimulation in culture

Total splenic B cell compartment was isolated, as previously described, and cultured at 1 million cells/ml without stimulation or with 10 μg/ml AfinityPure F(ab)2 fragment (Jackson ImmunoResearch Laboratories). Cells were stained at 0 and 12 h for expression of CD93, CD21, CD23, and CD69. Live cells were determined on the basis of forward and side scatter and PI exclusion. Fo cells were CD19+, CD23+, and CD93+. Live cells were determined on the basis of forward and side scatter and PI exclusion. Fo cells were CD19+, CD23+, and CD93+.

LPS and IL-4 stimulation and culture

Purified murine mature B cells, consisting of both MZ and Fo B cells, were cultured at 37 °C with 5% CO2 in sterile conditions and stimulated with LPS (15 μg/ml) and IL-4 (30 ng/ml) at 1 million cells/ml in 24-well plates. Fresh medium containing LPS (15 μg/ml) was added after 48 h. Cells were removed from culture and stained with CD23, annexin V, and PI at the indicated time points. Numbers of MZ and Fo cells were determined via FACS and plotted as the percentage of cells per milliliter present at each time point.

BAFF and IL-4 stimulation and culture

Purified mature splenocytes were cultured at 1 million/ml in 100-μl aliquots and stimulated with BAFF (1 μg/ml), IL-4 (50 ng/ml), or no stimulation. Samples were taken at 0-, 36-, 66-, and 96-h poststimulation in triplicate. Live cells were identified by negative staining for annexin V and PI; Fo cells identified via CD23+ staining, whereas MZ cells were CD23−.

CFSE staining and stimulation

Lymphocytes were stained in CFSE (5 μM) in PBS for 30 min at 37 °C and then washed three times in RPMI 1640 medium with 10% FCS. Cells were stimulated with LPS (15 μg/ml) and IL-4 (30 ng/ml) and cultured in media for the times described at an initial concentration of 1 million cells/ml. Cells were recovered and resuspended in fresh media containing LPS (15 μg/ml) after 24 h.

Tamoxifen and BrdU injections

Mice were injected i.p. with 1.5 mg sterile-filtered tamoxifen in corn oil (10 and 150 ml total per injection) once daily for 4 d prior to sacrifice. For BrdU injections, mice were injected i.p. with 1.0-mg sterile-filtered BrdU in 200 μl PBS once daily for 1–3 d, as indicated, prior to sacrifice.

BrdU and caspase-3 intracellular staining

BrdU and activated caspase-3 staining were performed as per the manufacturer’s instructions (BD Biosciences).

Inhibition of cellular division in vitro via paclitaxel

Purified mixtures of mature MZ/Fo were stimulated at 1 million/cells/ml with complete medium containing LPS (15 μg/ml), IL-4 (30 ng/ml), and paclitaxel (5 nM) at hour 0 and aliquoted into individual wells of a 96-well plate (10 μl each) to ensure equal cellular number. Samples were taken in triplicate at the times indicated.

Adoptive transfers of eCAML and eCAML CD19+ cells

We sacrificed three mice each of CAML+/−, CAML−/− (eCAML−/−, eCAML−/−, and eCAML−/−) and pooled the total splenocytes of each type. Total CD19+ splenocytes were isolated by positive selection using murine CD19 microbeads and resuspended at 50 million cells/ml in RPMI 1640 medium with nothing added. CD19+ cells from each mouse genotype were injected into five to six male NOD/SCID mice (ages, 20–32 wk) at 5.25–7 million cells per mouse. NOD/SCID mice were sacrificed after 3 wk, and the numbers of splenic CD4+ CD8− B220− CD19+ B cells were assessed. Total cell number per mouse was adjusted to assume 7 million cells initially transferred.

Statistical analysis

All p values are for Student t tests, one tailed with assumption of homoscedastic distribution, and all error bars are for SD, unless otherwise noted. Error bars labeled as SEM are for sample number (n) ≥ 10 and calculated as (SD/square root of n). A p value < 0.05 (<5% probability of null hypothesis) was considered statistically significant.

Results

bCAML−/− mice exhibit a 75% loss of mature splenic Fo B cells

We generated B cell-specific CAML knockout mice (referred to as bCAML−/−) by crossing the CD19-cre transgene (13) into CAML β/β animals (15). Expression of CAML was successfully ablated, as determined by Western blotting of lysates from CD19+ splenocytes from CAML-deleting (bCAML−/−) and littermate control (CAMLΔ26) mice (Fig. 1A). As was seen in previous models using CD19-Cre transgenic mice (13), gene deletion was not present in bone marrow B cell precursors, and the numbers of Hardy fractions A through E were not different from controls in bCAML−/− mice (data not shown).

Splenocyte cell development was examined, based on expression of cell surface proteins CD21, CD23, IgD, and IgM, which were used in all analyses to identify the appropriate populations. T1 and T2 cells were both CD93+IgMΔ and were distinguished by expression of CD21, CD23, and high IgD on T2, whereas T1 were CD21− CD23+ IgDΔ (16). MZ and Fo B cells were considered as IgMΔ IgDΔ CD93− CD21+. With CD23 expression used to delineate Fo from MZ cells (Fig. 1B) (2).

In CAMLΔ26 mice, CD19+ splenocyte populations were ~11% T1, 12% T2, 8% MZ, and 69% Fo (Fig. 1C), in agreement with published work (2, 17). In bCAML−/− mice, total splenocyte numbers were reduced by 37% (p < 0.003), and total B cell populations were reduced by 63% (p < 1.0 × 10−5). T1 cells were significantly reduced by 47% (p < 0.005) (Fig. 1C); however, T2 cells did not demonstrate a reduction in number in CAML-deficient mice. Similarly, mature MZ B cells exhibited only slightly decreased numbers in bCAML−/− mice, which was not statistically significant. The most salient feature of the bCAML−/− animals, however, was a 78% reduction in the number of mature Fo cells (p < 3 × 10−5) (Fig. 1B, 1C). Thus, CAML is essential for maintaining normal numbers of mature Fo cells but may not be required for MZ cell production or maintenance.

Peritoneal B cell populations are abnormal in bCAML−/− mice

To determine whether the loss of CAML similarly affected peritoneal B cells, we isolated and identified three populations (B1a, B1b, and B2) from the peritoneum, based on expression of IgM,
B220, CD5 (Ly-1), and CD19 (17, 18). Compared with littermate controls, \textit{bCAML\textsuperscript{−/−}} mice exhibited a reduction in the fetal liver-generated, self-renewing B220\textsuperscript{CD19\textsuperscript{+}} B1 cells (19) and mature circulating B220\textsuperscript{CD19\textsuperscript{lo}} Fo cells (B2 cells) (Fig. 1D). CAML-deficient B1 B cells were reduced by 71% (p < 0.0004) with the majority of cell loss coming from the CD3\textsuperscript{lo}CD4\textsuperscript{low} B1b fraction, which was reduced by 77% (p < 2.5 x 10\textsuperscript{-5}). The CD5\textsuperscript{+} B1a population was reduced by 42%; however, this did not reach statistical significance. The B2 cells were reduced by 60% (p < 0.025), indicating that mature B cells were globally reduced throughout the body. Taken together, these data indicate that loss of CAML causes a ~75% loss of peripheral B1 and B2 cells regardless of whether they originated in the fetal liver or the bone marrow.

**CAML-deficient B cells express an activated phenotype**

We next examined the activation state of normal and \textit{bCAML\textsuperscript{−/−}} B cells by analyzing surface expression of multiple proteins. By FACS analysis, there was no difference in the expression of B220, CD21, CD23, CD93, TLR4, IgA, IgD, IgG1, or IgM (Fig 1; data not shown) between CAML-deficient and control B cells. This suggested that the identification of B cell populations by flow cytometry was not impaired by loss of CAML. In contrast, MHCII expression was found to be reproducibly increased in the absence of CAML (Fig. 2), similarly to that reported for myb-deficient and inducible RAG-2 deleting mice (17, 20). Cell surface CD69 was marginally increased in Fo cells that lacked CAML, although MZ cells had normal CD69 levels. In addition, CAML-deficient Fo and MZ B cells had greater forward scatter than did cells from littermate controls (Fig. 2), consistent with an increased activation state in vivo. Taken together, these changes raise the possibility that deletion of CAML in B cells induced active homeostatic proliferation, likely resulting from the lymphopenic splenic environment (17, 20), thus suggesting that the homeostatic drive was intact in mutant mice.

**LN B cells fail to accumulate in \textit{bCAML\textsuperscript{−/−}} mice**

Reduced numbers of Fo cells might result from decreased production or accelerated loss because of impaired survival. Normally, circulating LN B cells are part of the long-lived mature B cell population (21), and they would be expected to be capable of accumulating and surviving in peripheral compartments. We hypothesized that if CAML loss caused a partial block in B cell development that allowed constant low-level production of (phenotypically normal) B cells, this might eventually lead to their accumulation up to normal levels within the LN over an extended period of time. In contrast, an effect of CAML on survival would be suggested by a failure of B cells to accumulate to normal levels, because the cells would proliferate but continually perish and be removed from circulation.

To explore this, we examined LN cells from mice with ages ranging from 5 to 76 wk. Inguinal LN cells were isolated and stained with B220 and CD3 Abs to determine the proportion of B and T cells, respectively (Fig. 3A). In both \textit{bCAML\textsuperscript{−/−}} and control mice, there was an increase in B cells in the LNs over time. However, at 5 wk of age, B cells were only 3.8% of cells within the LN of \textit{bCAML\textsuperscript{−/−}} mice compared with 18% in controls, representing a 79% reduction (p < 0.01), reminiscent of the reductions seen for splenic Fo cells. At all ages, LN B cells \textit{bCAML\textsuperscript{−/−}} mice never accumulated to proportions equivalent to controls and accounted for <12% of LN cells, whereas CAML\textsuperscript{fl/fl} LN B cells were always >18% of the cell population. These data suggest that loss of CAML in B cells might cause them to have a defect in survival, rather than simply to limit their production in the face of a normal life-span.

**CAML-deficient cells undergo higher turnover rates in spleen**

To specifically test whether CAML-deficient cells had reduced lifespan, we determined the rates of cell turnover in vivo. Mice were injected with BrdU at 24-h intervals to allow for identification of newly generated cells, and were euthanized 1–3 d later. Cells from the spleen were fixed and stained for BrdU, and quantified by FACS. At all time points, CAML-deficient B220\textsuperscript{+} cells had higher percentages of BrdU positivity, compared with cells from control littersmates (p < 0.035), whereas B220\textsuperscript{-} cells were not different between \textit{bCAML\textsuperscript{−/−}} and control mice (Fig. 3B). The 24-h BrdU incorporation time point is thought to be most reflective of homeostatic proliferation within the mature splenic B cell compartment, because transitional B cells are nondividing, and bone marrow precursors cannot reach the spleen within this time interval (17). At 2 d, the transitional B cells from the bone marrow were almost entirely BrdU\textsuperscript{−}, accounting for a large portion of the increase in BrdU\textsuperscript{+} B220\textsuperscript{+} cells (data not shown). Thus, we conclude that CAML-deficient B cells indeed have accelerated turnover, as a result of both enforced homeostatic proliferation and greater influx of bone marrow-generated cells, and in the face of dramatically reduced Fo numbers, this implies that they have a marked defect in survival.

Interestingly, although total numbers of peripheral MZ cells were not altered in mutant mice, the MZ cells, in addition to the Fo...
cells, displayed increased BrdU incorporation after a 2 d pulse of the nucleoside analog (Fig. 3C). Thus, MZ cells may have a partial reliance on CAML for maintenance of survival.

**Disrupted splenic architecture in bCAML<sup>−/−</sup> mice**

Histologic sections from CAML<sup>fl/fl</sup> and bCAML<sup>−/−</sup> spleens were fixed and stained with H&E to determine whether the loss of Fo cells had an effect on the architecture of the organ (Supplemental Fig. 1). CAML<sup>fl/fl</sup> spleens (left panels) had multiple well-defined darker-staining follicles (representative follicles indicated with arrows) with easily identifiable central arteries (22). By contrast, bCAML<sup>−/−</sup> (right panel) spleens displayed diffuse follicles with reduced cellularity and poorly visible margins. Central arteries were difficult to identify, and some arteries were surrounded by very few follicular cells. Overall, bCAML<sup>−/−</sup> mice showed a disrupted splenic architecture, because of low follicular cellularity and the lack of Fo cell accumulation within the spleen.

**Defective survival of CAML-deficient B cells in response to BAFF or LPS and IL-4 in vitro**

CD19<sup>+</sup> B cells purified from bCAML<sup>−/−</sup> and control mouse spleens were stimulated in vitro using IgM-specific Ab. Cells responded with increased cell size (determined by forward scatter) and by upregulation of CD69, regardless of mutant or wild-type status, indicating that CAML is not required for BCR activation (Fig. 4A). Next, purified B cells were stained with CFSE and tested in vitro for response to stimulation with LPS and IL-4. Cellular proliferation was determined based on CFSE dilution.

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**FIGURE 2.** bCAML<sup>−/−</sup> splenic B cells display increased size and MHCII expression. Representative histograms showing forward scatter and expression of cell surface CD69 and MHCII by bCAML<sup>−/−</sup> (black) and CAML<sup>fl/fl</sup> (gray) splenic F0 and MZ cells as indicated. Graphs show the average mean fluorescence intensities of CD69 and MHCII expression by CAML<sup>fl/fl</sup> (gray) and bCAML<sup>−/−</sup> (black) MZ and F0 cells as indicated. *p < 0.022, **p < 0.012.
over time. CAML-deficient cells proliferated as well as cells from littermate controls at all time points (Fig. 4B), in agreement with the robust incorporation of BrdU observed in vivo.

We next asked whether B cells from bCAML$^{−/−}$ mice had altered survival in vitro in response to the essential B cell survival factor, BAFF, which induces survival without proliferation (23). Purified Fo cells from bCAML$^{−/−}$ and control animals died rapidly in the absence of cytokines, as indicated by FACS analysis for annexin V and PI positivity. Control Fo cell survival in response to IL-4 was significantly better than that of CAML-deficient cells at all time points ($p < 0.01$) (Fig. 5A). Treatment with BAFF induced the greatest survival in both CAML$^{fl/fl}$ and bCAML$^{−/−}$ Fo cells, and both BAFF-stimulated groups showed significant survival compared with unstimulated cells ($p < 0.006$). Interestingly, bCAML$^{−/−}$ and control Fo cells displayed equal amounts of survival at 36 h after BAFF stimulation, indicating an equivalent response. However, at later time points, bCAML$^{−/−}$ cells underwent a significant reduction compared with controls ($p < 0.006$) (Fig. 5A).

To further explore the survival defect in bCAML$^{−/−}$ Fo cells, we cultured purified mature splenic B cells with LPS and IL-4 to provide a strong stimulus for survival, activation, and proliferation. Samples were harvested at various times and analyzed by FACS for annexin V and PI positivity. The number of surviving cells for the first 50–60 h of stimulation was the same regardless of CAML genotype, similar to that seen with BAFF stimulation. However, by 70–74 h, average bCAML$^{−/−}$ Fo cell numbers were...
CAMLfl/fl (solid line, open circles) and bCAML-/- (dashed line, open squares); IL-4 stimulated: CAMLfl/fl (solid line, closed diamonds) and bCAML-/- (dashed line, closed triangles). BAFF stimulated: CAMLfl/fl (solid line, closed circles) and bCAML-/- (dashed line, closed squares). Number of live cells at each time point was divided by initial number placed in culture. *p < 0.006. (B) In vitro number of splenic Fo cells from four CAMLfl/fl (black bar) and eight bCAML-/- (gray bar) mice, aged 15–22 wk, stimulated with LPS and IL-4 after 50–60 and 74–80 h in culture. Fo cell number is graphed as the number of cells at each time divided by the initial number cultured. Data representative of two separate experiments. *p < 0.0002.

FIGURE 5. bCAML-/- cells exhibit long-term survival defects. (A) Fo cells from 24-wk-old CAMLfl/fl (n = 3) and bCAML-/- (n = 5) mice were cultured for 96 h without stimulation or stimulated with 50 ng/ml IL-4 or 100 μg/ml BAFF. Unstimulated: CAMLfl/fl (solid line, open circles) and bCAML-/- (dashed line, open squares); IL-4 stimulated: CAMLfl/fl (solid line, closed diamonds) and bCAML-/- (dashed line, closed triangles). BAFF stimulated: CAMLfl/fl (solid line, closed circles) and bCAML-/- (dashed line, closed squares). Number of live cells at each time point was divided by initial number placed in culture. *p < 0.006. (B) In vitro number of splenic Fo cells from four CAMLfl/fl (black bar) and eight bCAML-/- (gray bar) mice, aged 15–22 wk, stimulated with LPS and IL-4 after 50–60 and 74–80 h in culture. Fo cell number is graphed as the number of cells at each time divided by the initial number cultured. Data representative of two separate experiments. *p < 0.0002.

Reduced by 80% compared with CAMLfl/fl cells (p < 0.0002) (Fig. 5B).

To determine whether cells were dying via apoptosis, we assessed the percentage of early apoptotic (annexin V+) and dead (annexin V+PI+) Fo cells that were present after 30 h in culture with LPS and IL-4. CAML-deficient Fo cells exhibited a 19% decrease in overall survival compared with controls (data not shown). Furthermore, CAMLfl/fl Fo cells were 6% early apoptotic and 9% dead, whereas bCAML-/- cells were 13% early apoptotic and 17% dead, a near-doubling of both populations (p < 0.0037 and p < 0.0081, respectively) (Fig. 6A). To verify that cells were activating the apoptotic response, we assayed for active intracellular caspase-3. Tested after 55 h of LPS stimulation, CAML-deficient B cells had higher levels of active caspase-3 (p < 0.0029) than control cells (11 versus 16%), confirming that loss of cells in these cultures occurred by apoptosis (Fig. 6B).

Death of CAML-deficient cells by apoptosis is independent of cellular division

To explore whether the delayed death of cells lacking CAML was related to a failure to survive during attempted cell division (24), we asked whether blocking division would also inhibit cytokine-induced apoptosis. First, cells were treated with various concentrations of the mitotic inhibitor paclitaxel to identify an optimal concentration that would suppress LPS and IL-4 induced proliferation yet have minimal toxicity on its own. (In these experiments, that concentration was determined to be 5nM [data not shown].) Isolated Fo cells were then stimulated with LPS and IL-4 in the presence of 5nM paclitaxel. We observed a modest reduction in survival after 30 and 50 h in culture, control Fo cells maintained numbers of surviving cells similar to those at 50 h, indicating strong continued survival in the absence of proliferation. In contrast, CAML-deficient Fo cells underwent a dramatic reduction in survival by 80 h of stimulation (p < 0.05). Taken together with the results of BAFF stimulation, these findings suggest that cell death resulting from loss of CAML does not require cell division.

Continuous requirement for CAML to maintain survival within mature B cells

Taken together, the results described above indicate that CAML has an essential role in blocking death of mature B cells. However,
because CAML may act during development, it was possible that the defect in B cells arising in \textit{bCAML}\textsuperscript{−/−} mice was due to aberrant development in the absence of the CAML gene during the transitional stages. To discern, therefore, whether CAML is specifically required after B cells have reached maturation, we used the inducible ESR-Cre transgene (15). These mice, designated \textit{eCAML}\textsuperscript{−/−}, were treated with tamoxifen to activate CAML gene excision. After 4 d of treatment, purified splenic MZ and Fo cells demonstrated efficient loss of CAML protein (Fig. 7A). At this early time point, \textit{eCAML}\textsuperscript{−/−} (CAML-deficient) Fo cells were only minimally reduced in number (to 73% of CAML\textsuperscript{+/+} control mice) (Fig. 7B). Next, cells were stimulated in vitro with LPS and IL-4 to evaluate effects on survival. We again found a dramatic loss of cell viability after 74–80 h of stimulation, with \textit{eCAML}\textsuperscript{−/−} cells being only minimally reduced in number (to 73% of CAML\textsuperscript{+/+} control mice) (Fig. 7B). Next, cells were stimulated in vitro with LPS and IL-4 to evaluate effects on survival. We again found a dramatic loss of cell viability after 74–80 h of stimulation, with \textit{eCAML}\textsuperscript{−/−} cells demonstrating an 80% reduction in number, compared with similarly treated control cells (Fig. 7C). We observed an intermediate effect in cells from \textit{eCAML}\textsuperscript{+/−} (heterozygote) mice, suggesting the possibility of a gene dosage effect. We conclude that CAML is required acutely in mature B cells to maintain their survival upon stimulation with LPS and IL-4.

Next, we revisited the question of BAFF signaling by examining the ability of BAFF to upregulate the cell surface proteins CD21 and MHCII, which are known to be directly induced by this cytokine (25, 26). Purified B cells from tamoxifen-treated \textit{eCAML}\textsuperscript{−/−} or littermate control mice were incubated with or without BAFF in vitro for 12 h and then stained and analyzed by FACS. BAFF treatment induced a robust upregulation of both cell surface proteins, indicating that BAFF signaling is not impaired by loss of CAML (Fig. 8), and cannot explain the failure of CAML-deficient B cells to survive.

\textit{bCAML} and \textit{eCAML CD19}\textsuperscript{+} cells show reduced survival after adoptive transfer

We next tested both \textit{bCAML}\textsuperscript{−/−} and \textit{eCAML}\textsuperscript{−/−} cells in an in vivo adoptive transfer experiment to analyze their survival in an unstimulated state, allowing for natural development within the spleen. Splenic CD19\textsuperscript{+} cells from \textit{CAML}\textsuperscript{+/+}, \textit{bCAML}\textsuperscript{−/−}, \textit{eCAML}\textsuperscript{+/+}, \textit{eCAML}\textsuperscript{−/−}, and \textit{eCAML}\textsuperscript{−/−} were transferred into NOD-SCID mice, which lack T, B, and NK cells and circulating complement. Three weeks after transfer, the number of B splenocytes (identified as CD4\textsuperscript{−}/CD22\textsuperscript{−}/CD12\textsuperscript{−}/CD19\textsuperscript{+}) was assessed via FACS. Recovered B splenocytes from \textit{bCAML}\textsuperscript{−/−} mice showed significantly reduced (\textit{p} = 0.01) numbers compared with \textit{CAML}\textsuperscript{+/+} cells (Fig. 9), indicating a loss of cellularity that cannot be attributable to abnormal division or failure to respond to extrinsic signals, such as LPS. Likewise, \textit{eCAML}\textsuperscript{−/−} mice showed an even greater reduction in number compared with \textit{CAML}\textsuperscript{+/+} controls (\textit{p} = 0.00005) (Fig. 9). Heterozygote \textit{eCAML}\textsuperscript{+/−} mice displayed no statistically significant differences from \textit{CAML}\textsuperscript{+/+} controls but were likewise more numerous (\textit{p} = 0.02) than CAML-deficient cells. Taken together, these data show that CAML-deficient cells from two separate models display a survival deficit that is not attributable to differences in development, stimulation, division, or environment.

Discussion

CAML is critical for the long-term survival of splenic Fo cells

We report in this paper a critically important role for CAML in mediating the long-term survival of Fo B cells. This effect is evidenced in vivo by dramatically reduced steady-state numbers of splenic and LN Fo cells, accompanied by more rapid BrdU incorporation, indicative of faster proliferation in \textit{bCAML}\textsuperscript{−/−} mice. In vitro, B cells lacking CAML initially responded normally to stimulation in number compared with \textit{CAML}\textsuperscript{+/+} controls but ultimately died in greater numbers by 90 h of culture. Their failure to survive long term following stimulation with LPS + IL-4 was even more dramatic.

Recently, we reported that CAML was necessary for proper cellular division in mouse embryonic fibroblasts by its effects on the regulation of the mitotic spindle and the spindle assembly checkpoint (24). However, this effect appears to be specific to mouse embryonic fibroblasts (MEFs), and we did not find any evidence that CAML participates in regulation of cell proliferation in B lymphocytes. We instead found that Fo cells fail to survive in vitro even when cellular division is ruled out as a complicating factor. This was accomplished via two methods, by stimulation with BAFF, which causes survival in the absence of division, or by the addition of the mitotic inhibitor paclitaxel. In both BAFF and paclitaxel-treated experiments, control Fo cells showed no significant losses from 60 to 90 h poststimulation, whereas CAML-deficient cells were reduced in number. Furthermore, cell proliferation in the absence of CAML was normal, both in vivo as well as in vitro. This stands in sharp contrast to MEFs, which immediately cease division upon loss of CAML.
Finally, we note the distinction between these two cell types and mouse embryonic stem cells, which appear to have both normal proliferation and survival characteristics regardless of CAML presence or absence (10).

**BCR signaling is intact in the absence of CAML**

Induced deletion or defects in the BCR have been shown to lead to losses of splenic cellularity that somewhat resemble the phenotype of bCAML \(^{-/-}\) mice (27). However, multiple observations indicate that the key role for CAML in B cell survival is not due to an effect on BCR processing or signaling. First, bCAML \(^{-/-}\) B cells had normal levels of IgM and IgD, unlike those from mice with inducible deletion of IgM. Second, responses of CAML-deficient cells to cross-linking of surface IgM were normal, including upregulation of CD69, increased forward scatter indicative of cell size, and Ca\(^{2+}\) influx (data not shown).

**The role of CAML in B cell survival is likely independent of BAFF**

The interaction of BAFF with its natural receptor, BAFF-R, is essential for the maintenance of the mature MZ and Fo cells (5). In our studies, we found that bCAML \(^{-/-}\) Fo cells responded to BAFF stimulation as well as controls, as indicated by normal upregulation of CD21 and MHCII and by their survival at 36- and 60-h time points, compared with unstimulated cells. It was only in long-term (>3 d) cultures that a deviation between CAML-deficient and control cells was observed. We suspect, therefore, that CAML is not essential for BAFF signaling. Consistent with this, we note that BAFF-deficient mice exhibit a much more severe splenic phenotype than bCAML \(^{-/-}\), because they almost completely lack T2, MZ, and Fo cells (5). A/WySnJ mice, which bear a disrupting mutation in BAFF-R (28), exhibit a ∼90% reduction in mature splenic B cells and a shortened B-2 B cell half-life.

### FIGURE 8

**BAFF increases expression of CD21/35 and MHCII despite loss of CAML.** Purified B cells from tamoxifen-treated eCAML \(^{-/-}\) or littermate control mice were incubated in vitro for 12 h with or without recombinant BAFF (100 ng/ml). Fo cells were analyzed for expression of CD21/35 and MHCII by FACS analysis. (A) Example FACS plots of control and eCAML \(^{-/-}\) stained for CD21/35 (top) and MHCII (bottom) (black, unstimulated; gray, BAFF treated). (B) Summary of mean fluorescence intensity increases specific for the indicated cell surface proteins (CAML\(^{+/+}\); \(n = 8\); eCAML\(^{-/-}\); \(n = 4\)).

### FIGURE 9

**Reduced survival of CAML-deficient B splenocytes following adoptive transfer.** (A) Total number of B splenocytes recovered from NOD-SCID mice after adoptive transfer of CD19\(^{+}\) splenocytes from CAML\(^{+/+}\) (black bars, \(n = 6\)) or bCAML \(^{-/-}\) (gray bars, \(n = 5\)). Cell number recovered is adjusted to account for the 7 million cells initially transferred. (B) Total number of B splenocytes recovered from NOD-SCID mice after adoptive transfer of CD19\(^{+}\) splenocytes from eCAML \(^{+/+}\) (black bars, \(n = 6\)), eCAML \(^{-/-}\) (striped bars, \(n = 6\)), and eCAML \(^{-/-}\) (gray bars, \(n = 5\)).
life (4). Interestingly, A/WySnJ mice show little to no effect upon the number and turnover rate of B-1 B cells. bCAML−/− mice also show a higher-than-control BrdU labeling rate and reduced B-2 B cell numbers but are differentiated by their large deleterious effect on B-1 B cell numbers. This further suggests that the loss of CAML imparts a survival defect that is more broadly applicable and qualitatively different from that seen in mice with either BAFF deficiency or BAFF-R disruption.

CAML expression is fundamental to mature cells

In these studies, we have also resolved an important issue regarding the role of CAML in cellular development about whether CAML is necessary solely in developmental processes or whether it contributes to the function of fully matured somatic cells. In studies by Tran et al. (10), the major role of CAML was found to be its involvement in recycling of epidermal growth factor receptor for epithelial cells and serving as a negative regulator of p56lck during thymocyte development (8). Likewise Liu et al. (24) found mitotic spindle defects in CAML-deleted MEFs. Whether mature cells, many of which are nondividing, require CAML for additional functions remained unresolved. By the use of the eCAML model, we were able to allow normal development of mature long-lived B cell populations in the presence of CAML and subsequently examine the peripheral cells following its induced deletion. If CAML would participate solely during development, one would expect eCAML−/− cells to survive and proliferate normally in response to LPS and IL-4. Instead, mature cell function was dependent on CAML expression, such that even heterozygotes displayed a significant survival disadvantage compared with control cells. Indeed, bCAML−/− Fo cells that developed in the absence of the protein and eCAML−/− Fo cells that deleted the protein acutely responded to LPS and IL-4 in a strikingly similar manner.

Splenic Fo cells in the absence of Ag stimulation and normal bone marrow production are mostly naïve, nondividing cells. In contrast, cells transferred into lymphopenic hosts would be subject to enhanced proliferation because of homeostatic influences. CAML was required for survival of Fo cells in both circumstances and thus serves a cell autonomous function in this role.

Two prior reports from other laboratories (11, 12) have shown that certain viral proteins are capable of binding to and targeting CAML to inhibit the apoptosis of infected cells. A novel protein from Kaposi’s sarcoma-associated herpesvirus named K7 was identified by Feng et al. (11), based on its expression during viral lytic replication and the presence of a hypothesized mitochondrial targeting sequence. K7-expressing cells showed a resistance to apoptosis upon treatment with thapsigargin, a proapoptotic agent that increases cytosolic calcium (29). Using a yeast two-hybrid screen and mutational analysis, the authors determined that amino acid residues 22–74 of K7 bound to the cytosolic domain of CAML and that this interaction was necessary for the anti-apoptotic effects (11).

More recently, Grant, et al. (12) discovered that the adenovirus E3-6.7 protein bore sequence homology to transmembrane activator and CAML interactor and that it bound to CAML as revealed by yeast two-hybrid and coimmunoprecipitation experiments. Interestingly, the homologous region of E3-6.7 formed a large portion of the CAML binding domain, with three conserved cysteines being required for the interaction (12). Similar to K7, E3-6.7 interaction with CAML imparted antiapoptotic properties to thapsigargin-treated cells.

In this study, we expand the role of CAML beyond the phenotype shown in viral-induced inhibition of apoptosis. Rather than simply serving a role in the response to proapoptotic stimuli, CAML appears to be fundamental in its ability to prevent apoptotic death on an ongoing basis in mature, uninfected B cells. Thus, we hypothesize that these two viral proteins may not be subverting CAML to serve an aberrant role but instead activating its natural anti-apoptotic function to provide increased survival to infected cells. Although the mechanism by which CAML suppresses apoptosis is not yet known, this work clarifies its role as an important mediator of long-term survival in peripheral B cells and provides new insight into how CAML serves as a potential target that regulates the immune system.

Disclosures

The authors have no financial conflicts of interest.

References